THE UNIVERSITY OF MANITOBA

A COUPLING FACTOR FOR PHOTOSYNTHETIC PHOSPHORYLATION IN PEA AND BEAN PLASTIDS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

WINNIPEG, MANITOBA

October, 1971

ABSTRACT

A coupling factor for photophosphorylation and its latent adenosine triphosphatase activity were studied in pea and bean plastids.

Buffered sucrose-ethylenediaminetetracetic acid extracts of pea chloroplasts stimulated photophosphorylation in partially deficient chloroplast residues indicating that they probably contained a coupling factor for photophosphorylation. Optimal removal of the coupling factor from the chloroplasts was achieved with extraction media containing 0.5 to 1 mM ethylenediaminetetracetic acid. Trypsin treatment of pea chloroplasts caused activation of a Ca^{2+}-dependent adenosine triphosphatase suggesting that pea plastids contained a latent adenosine triphosphatase similar to that of the spinach chloroplast coupling factor.

Ethylenediaminetetracetic acid extracts of bean etioplasts and chloroplasts contained a latent adenosine triphosphatase with similar properties. The enzyme was activated by dithiothreitol, its activity was higher in the presence of Ca^{2+} than in the presence of Mg^{2+} ions and it was inactivated upon cold treatment. The etioplast and chloroplast enzymes were inhibited by Dio-9 and had identical R_f values upon disc gel electrophoresis. Optimum extraction of the enzyme from both types of plastids was accomplished with 1 mM ethylenediaminetetracetic acid. Photophosphorylation capacity could be partially restored to depleted chloroplast preparations by addition of NaCl-bovine serum albumin-ethylenediaminetetracetic acid extracts of etioplasts or chloroplasts indicating that such extracts contained a coupling factor for photophosphorylation. The results suggested that

the adenosine triphosphatase from bean etioplasts and chloroplasts represented a modified coupling factor for photophosphorylation.

The specific activity of the adenosine triphosphatase in ethylenediaminetetracetic acid extracts of bean plastids increased upon greening of etiolated plants. This light-induced increase was inhibited by both chloramphenicol and cycloheximide, specific inhibitors of chloroplastic and cytoplasmic protein synthesis. There was no accumulation of adenosine triphosphatase in postribosomal supernatants isolated from chloramphenicol or cycloheximide treated bean leaves. The results indicated that both the chloroplast and the cytoplasmic ribosomal systems were required for the formation of the bean chloroplast adenosine triphosphatase.

ACKNOWLEDGEMENTS

The author would like to express his sincere thanks to:

Dr. R. D. Hill for his interest, guidance and many helpful suggestions during the course of the present work and preparation of this manuscript.

Dr. W. Bushuk for the use of the disc gel electrophoresis equipment and of the analytical ultracentrifuge.

Mr. R. Zillman for operation of the ultracentrifuge and calculation of the sedimentation coefficients.

The financial support from The University of Manitoba Graduate Research Fund is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

The following non-standard abbreviations are used in the present work:

ATP-ase .....adenosine triphosphatase

BSA .....bovine serum albumin

CAP .....chloramphenicol

CF_1 .....coupling factor 1

CHI .....cycloheximide

DTT .....dithiothreitol

EDTA .....ethylenediaminetetracetic acid

NBE .....10 mM NaCl, 0.2% BSA, 1 mM EDTA at pH 8

PMS .....phenazine methosulfate

RuDP .....ribulose diphosphate

STB .....0.4M sucrose, 40 mM TES-NaOH, 0.2% BSA at pH 8

STN .....0.4M sucrose, 20 mM Tris-HCl, 10 mM NaCl at pH 7.9

TES .....tris (hydroxymethyl)methylaminoethane sulfonic acid

Tris .....tris (hydroxymethyl) aminomethane

INTRODUCTION

Photosynthesis is one of the most important biological phenomena on Earth as it is the major process by which the energy of solar radiation is converted to chemical energy necessary for supporting the life of both autotrophic and heterotrophic organisms.

In higher plants the energy conversion takes place in specialized organelles - chloroplasts. Light is absorbed by chloroplast pigments where it causes electron excitation. The energy of the excited electrons is used to drive a series of redox reactions along a chain of electron carriers of increasing redox potential. This process is accompanied by ATP synthesis. The mode by which ATP formation is coupled to electron flow is yet unknown but at least two protein components called coupling factors are required.

As a large part of the solar energy is converted to ATP, an understanding of the function of the coupling factor in light-dependent ATP synthesis (photophosphorylation) is of prime importance.

There have been two basic approaches used in the study of the role of the coupling factor in photophosphorylation. One consists of resolution of the system responsible for photophosphorylation into individual components, elucidation of their functions and interactions with the ultimate goal to reconstitute the original system from its parts. The other makes use of the comparison of a complete system with one that is deficient in certain components and functions. The absence of one component from a photophosphorylation system usually affects either partial reactions within the system or the overall

system itself. Studies of these effects often give insight into the role of the missing component in the system. The deficient system in plants can be a mutant or an etiolated plant. The latter has the advantage that it can evolve into the complete system upon illumination.

The purpose of the present work was to study the role of one coupling factor in photophosphorylation using etiolated plants as the deficient system and following their evolution into the complete one upon greening. Specifically, answers to the following questions were sought:

1) Is the coupling factor present in etioplasts?
2) Are its properties similar to the chloroplast coupling factor?
3) Does the amount of coupling factor increase upon greening?
4) Is the coupling factor formed on the chloroplast or on the cytoplasmic ribosomal system?

LITERATURE REVIEW

1. Photophosphorylation

As mentioned in the introduction, light causes electron flow in chloroplasts resulting in O_2 evolution and NADP reduction. ATP formation is coupled to this light-induced electron transport.

Oxygen evolution upon illumination of isolated chloroplasts supplied with an artificial electron acceptor was first observed by Hill in 1937 (1). However, photophosphorylation was not discovered until 1954 when Arnon, Allen and Whatley observed light-dependent ATP formation from ADP and orthophosphate by chloroplast preparations (2).

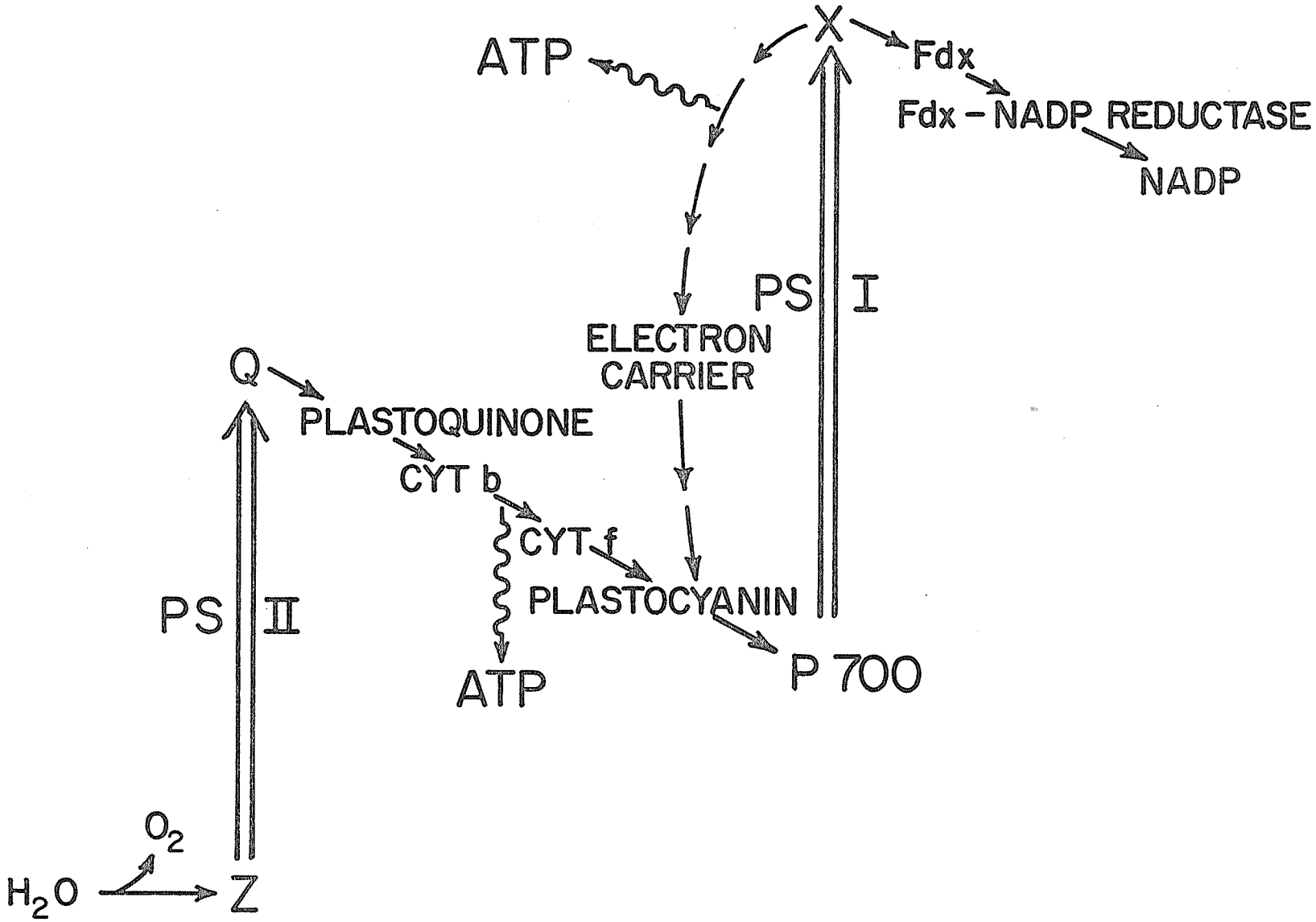
Since their discovery, both light-driven electron transport and photophosphorylation have been intensively studied. As a result, many individual reactions involved in these processes are known today but the overall picture is still far from complete.

Emerson *et al.* (3) observed that the quantum yield of photosynthesis (molecules of oxygen evolved per quantum of light absorbed) decreased in far red light which was still readily absorbed by chlorophyll. Simultaneous illumination by light of a shorter wavelength caused an increase in oxygen evolution which was greater than the sum obtained by separate illumination with the two types of light. The enhancement suggested that more than one light reaction might be involved in photosynthesis. This evidence together with spectroscopic observations on the redox states of some of the electron transport

carriers in chloroplasts led Hill and Bendall (4) to the formulation of a hypothetical chain of redox reactions driven by two separate light reactions. A recent version (5) of their scheme that became widely accepted is shown in Fig. 1. The short wavelength Photosystem II yields a strong oxidant and a weak reductant upon light absorption. The oxidant excepts electrons from H_2O yielding oxygen and the reductant is oxidized by a chain of electron carriers of increasing redox potential. The long wavelength Photosystem I oxidizes the last member of the redox chain upon illumination and produces a strong reductant which is used for reduction of NADP. It can be seen from Fig. 1 that operation of both Photosystems is required to obtain electron flow from H_2O to NADP. This "noncyclic" electron flow may be accompanied by ATP formation that is termed "noncyclic photophosphorylation". Noncyclic photophosphorylation was discovered by Arnon et al. (6, 7) who have shown that reduction of exogenous NADP or ferricyanide was accompanied by ATP formation and that there was a stichiometric relationship between the number of moles of ATP formed and the number of electrons transported.

Another type of light-dependent ATP formation is the "cyclic photophosphorylation" discovered by Arnon's group (8, 9). This type of phosphorylation requires addition of catalytic amounts of exogenous electron carriers such as phenazine methosulfate (PMS). As this type of light-dependent ATP formation is accompanied by no net change in oxidation and reduction, it is assumed to be coupled to a cyclic flow of electrons sensitized by Photosystem I.

Although chloroplasts isolated from spinach (10) or swiss chard


X
ATP
Fdx
Fdx – NADP REDUCTASE
NADP
PS I
ELECTRON CARRIER
Q
PLASTOQUINONE
CYT b
CYT f
PLASTOCYANIN
P 700
ATP
PS II
O_2
H_2O
Z

(11) catalyzed high rates of cyclic photophosphorylation, plastids isolated from maize (12) or beans (13) by the same isolation techniques had low rates of ATP formation. Miflin and Hageman (12) found that maize leaf extracts inhibited photophosphorylation by spinach chloroplasts and that active phosphorylating maize chloroplasts could be prepared by including glutathione and polyethylene glycol in the homogenization medium. The latter probably prevented the inhibition by tannins (14). Margulies (13) has shown that photophosphorylation activity of bean chloroplasts depended upon growing conditions as well as the mode of grinding of leaves. While homogenization with a high speed blendor gave good chloroplast preparations, plastids prepared by grinding with mortar and pestle had low rates of photophosphorylation. The bean leaf homogenates prepared in this way inhibited phosphorylation by spinach chloroplasts. McCarty and Jagendorf (15) have established that linolenic acid was released due to hydrolysis of galactolipids when bean plastids were isolated below pH 8. Free linolenate caused 50% inhibition of cyclic photophosphorylation at a concentration of 10^{-6}M. Friedlander and Neumann (16) have shown that serum albumin stimulated photophosphorylation by chloroplasts. Maximal stimulation was obtained when serum albumin was present in both the homogenization and the incubation medium. The effect was thought to be due to binding of unsaturated fatty acids and phenols to serum albumin. The beneficial effect of bovine serum albumin on photophosphorylation rates by bean plastids was also observed by Gyldenholm and Whatley (17) as well as by Howes and Stern (18) who have also shown stimulation of photophosphorylation

by glutathione, cysteine and mercaptoethanol. They suggested that bovine serum albumin might behave as a sulfhydryl reagent in addition to its effect on binding of fatty acids.

To obtain more information on the mechanism of photophosphorylation, attempts were made to study partial reactions of the process. Shen and Shen (19) as well as Hind and Jagendorf (20) were able to divide photophosphorylation into a light and a dark reaction. They observed that chloroplasts illuminated in the presence of an electron carrier such as pyocyanine can form ATP from ADP and phosphate added in the subsequent dark period. They inferred the formation of a nonphosphorylated high energy intermediate X_E during the light stage which could be utilized for the ATP formation in the following dark step. They demonstrated that in the darkness and in the absence of substrates X_E underwent rapid decay (20).

The search for the nature of the high-energy intermediate X_E led to the investigation of pH gradients, the formation of which was proposed to be the driving force for ATP synthesis according to Mitchell's chemi-osmotic hypothesis (21). It follows from this hypothesis that the light-induced electron transport should be accompanied by formation of a pH gradient across the thylakoid membrane. Formation of such pH gradients was actually found to take place as a pH rise was observed in unbuffered chloroplast suspensions upon illumination (22). The pH rise had similar pH and cofactor requirements as X_E. The pH rise of the external medium upon illumination was found to be accompanied by acidification on the inner side of the membrane as measured by membrane-bound bromothymol blue (23).

Hind and Jagendorf (24) and Jagendorf and Uribe (25) have shown that the pH gradient may serve as driving force for ATP synthesis. They created an artificial pH gradient across the thylakoid membrane by first exposing the chloroplasts to an acidic medium and then transferring them into a basic medium containing ADP, phosphate and Mg^{2+}. Small amounts of ATP were formed under these conditions. Use of succinate in the acid stage increased the yield of ATP manyfold when compared to HCl. It was suggested that the succinic acid enters the chloroplasts in the undissociated form at the acidic stage and serves inside as a reservoir of protons upon transfer to the basic medium, thus increasing the amount of ATP formed. Although it was shown by these experiments that the pH gradient may serve as the driving force for ATP synthesis, it is still an open question whether it is identical with X_E or whether its formation is a process parallel to ATP synthesis, both being dependent on the X_E formation. The latter situation which may be visualized by the following scheme

$$h\nu \longrightarrow e^- \text{flow} \longrightarrow X_E \overset{\text{pH}}{\rightleftharpoons} \text{ATP}$$

seems more probable in view of the results obtained by Nelson et al. (26) who observed high rates of ATP formation but no light-induced pH rise in subchloroplast particles incubated with digitonin.

Partial reversal of photophosphorylation is represented by light-induced ATP-ase activity in chloroplasts. Although chloroplasts have low ATP-ase activity in the dark (27), ATP hydrolysis can be induced by illumination of the plastids under certain conditions. Two types of ATP-ase activity have been observed. Avron (28) has described a

light-dependent Ca^{2+}-requiring ATP-ase. The enzyme was inhibited by Mg^{2+} and inactivated immediately after switching off the light. Its activity was small compared to the rate of photophosphorylation. Another light-activated ATP-ase has been described by Petrack and Lipmann (29). They observed that chloroplasts catalyzed ATP hydrolysis in the presence of light, Mg^{2+} and a sulfhydryl compound. The ATP-ase required light for its activation but retained its activity in the subsequent darkness if ATP was present (30, 31, 32). The rate of ATP hydrolysis was comparable with rates of photophosphorylation. Recently Kaplan et al. (33, 34) were able to show that the same type of ATP-ase activity could be induced using an acid-base transition for the activation stage instead of light.

2. Coupling factors

Attempts to resolve the system responsible for photophosphorylation into individual components led to the isolation of coupling factors from chloroplasts. A coupling factor can be defined as a substance which can be isolated from chloroplasts and which stimulates photophosphorylation without affecting the rate of electron transport (35).

A coupling factor from chloroplasts was first described by Avron in 1963 (36). He observed that EDTA caused uncoupling of photophosphorylation from electron flow. It inhibited ATP formation and stimulated photoreduction. The photophosphorylation activity of chloroplasts pretreated with EDTA could be partially restored when the EDTA extract was re-added in the presence of Mg^{2+} ions. The

activity of the high molecular weight factor responsible for the restoration was rapidly lost upon storage of the extract.

In 1965 Vambutas and Racker (37) reported stimulation of photophosphorylation by a latent, Ca^{2+} ion dependent ATP-ase from spinach chloroplasts. This enzyme was isolated from acetone extracted chloroplasts and purified by ammonium sulphate and protamine fractionation. The Ca^{2+}-dependent ATP-ase activity of this protein (coupling factor) was activated by trypsin. However, the coupling factor activity of the enzyme was lost upon this treatment. The authors proposed that the coupling factor catalyzed the last transphosphorylation step of the ATP synthesis in which ADP reacted with a phosphorylated high energy intermediate $X\sim P$ to form ATP: $X\sim P + ADP \rightleftharpoons X + ATP$.

In 1966 McCarty and Racker (35) produced further evidence for the participation of the coupling factor designated CF_1 in photophosphorylation, in the light-induced pH rise and in ATP synthesis which accompanies an acid-base transition of chloroplasts in the dark. Using EDTA for chloroplast extraction as described by Avron (36) and varying its concentration, they were able to correlate the release of the latent ATP-ase to the loss of phosphorylating capacity by the chloroplast residue. They observed maximal release of the latent ATP-ase with 0.5 mM EDTA and showed that such extracts contained a coupling factor identical to the coupling factor they isolated from acetone-extracted chloroplasts (37). The enzyme was cold labile and dissociated into subunits upon cold treatment. An antiserum to the coupling factor inhibited photophosphorylation as well as the Ca^{2+}-

dependent ATP-ase. ATP synthesis upon acid-base transition was also inhibited by the antiserum, indicating that the coupling factor participated in this process. On the other hand, the light-induced pH rise was not affected by the antiserum, indicating that the coupling factor did not participate in this process. It was observed, however, that EDTA treatment of chloroplasts abolished the pH rise (22) which could be reconstituted upon readdition of the EDTA extract (35). It seemed, therefore, that the physical presence of the coupling factor was required for the light-induced pH rise rather than its enzymatic activity and that the coupling factor had actually two functions, a catalytic one and a structural one.

In 1968 McCarty and Racker (38) demonstrated that the ATP-ase activity of the soluble coupling factor can be unmasked not only by trypsin or heat (37) but also by the use of a strong reducing agent such as dithiothreitol (DTT). Whereas exposure of chloroplasts to trypsin or heat caused detachment of CF_1 from the chloroplasts and abolished photophosphorylation, dithiothreitol treatment left CF_1 bound to the membrane and did not impair phosphorylation. The membrane bound enzyme was Mg^{2+}-dependent for photophosphorylation and ATP-ase activity, whereas the solubilized enzyme was Ca^{2+}-dependent. The membrane bound CF_1 had also higher sensitivity to inhibitors than the soluble enzyme. This phenomenon of the conferral of properties to a soluble protein upon its combination with a membrane has been called allotopy (39).

Bennun and Racker (40) purified the coupling factor to homogeneity and labeled it by ^3H acetic anhydride. Binding studies

with the tritium-labeled CF_1 have shown that trypsin or heat treatment of CF_1 abolished its capacity to bind to the chloroplast membrane. Mg^{2+} or Ca^{2+} ions were required for CF_1 binding to the chloroplast membrane. Combination of CF_1 with the membrane resulted in an increase in the cold stability of the enzyme.

Livne and Racker (41) observed that polynucleotides and lipids extracted from chloroplasts protected CF_1 against heat inactivation. Moreover, lipids also protected CF_1 against cold inactivation, suggesting that the cold-stability conferred upon CF_1 by its combination with the chloroplast membrane might be due to a lipid component in the membrane. Besides the lipid component they have also found a protein component capable of interacting with CF_1 (42, 43). The protein could be solubilized from chloroplasts with 0.4 M ammonium hydroxide. It could bind CF_1 and inhibited its DTT-activated ATP-ase. The protein was heat-labile and sensitive to trypsin. It stimulated photophosphorylation in chloroplasts that have been exposed to trypsin while having no effect on their rate of electron transport. It behaved therefore as a coupling factor and was designated CF_2.

Lynn and Straub (44) isolated from spinach chloroplasts a coupling factor required for photophosphorylation and proton uptake. The purified enzyme had no trypsin-activated ATP-ase. Removal of cations converted the protein into an enzyme with properties identical to CF_1. They concluded therefore that their coupling factor was probably identical with CF_1 but contained bound Mg^{2+} or other divalent cations.

CF_1 of spinach chloroplasts was also studied by Howell and

Moudrianakis (45) as well as Karu and Moudrianakis (46). These workers have isolated the enzyme from EDTA extracts of plastids and purified it by ammonium sulfate precipitation and density gradient centrifugation. The purified enzyme had properties similar to Racker's CF_1 and restored photophosphorylation when added in the dark stage of the two-stage photophosphorylation experiment. The enzyme showed subunit structure upon high-resolution electron microscopy and had a sedimentation coefficient of 12.7 upon centrifugation.

The localization of CF_1 on the outer surface of the thylakoid membrane of chloroplasts was confirmed by electron microscopy (47, 48, 49).

Recent studies on CF_1 by Farron (50) have shown that the coupling factor and the ATP-ase produced by mild heating of the coupling factor in the presence of ATP are identical with regard to electrophoretic mobility on polyacrylamide gel, sedimentation in the analytical ultracentrifuge and amino acid composition. The molecular weight of both proteins was 325,000 ($S^{o}_{20,w}$ = 13.8 S). Further studies on the mechanism of CF_1 conversion to the ATP-ase by Farron and Racker (51) have shown that this conversion is accompanied by the appearance of titratable SH groups. Although the coupling factor activity of the protein decreased upon heating while the ATP-ase activity increased, the two processes were found to be independent of each other, as their sensitivity to heat treatment as well as to thiol reagents was different. It was concluded that the conversion from a latent into a manifest ATP-ase represented a conformational change which proceeded possibly via disulfide interchange.

Studies by Roy and Moudrianakis (52) have shown that the coupling factor could strongly bind ADP in vitro at two sites. The bound ADP underwent a transphosphorylation to give AMP and ATP which remained bound to the enzyme. Chloroplasts supplied with AMP, Pi, pyocyanine and light were able to phosphorylate AMP. The resulting ADP remained bound to the coupling factor upon isolation. They suggested that the coupling factor-bound ADP was derived through a light-dependent phosphorylation of AMP and that it might serve as the last stable high-energy intermediate in photophosphorylation.

Although most of the work on coupling factor and the ATP-ase derived from it was done on spinach chloroplasts, the enzyme was also found in plastids from other sources. Ranalletti et al. (53) have observed a coupling factor in bean chloroplasts with properties identical to the spinach chloroplast coupling factor 1. On disc gel electrophoresis both enzymes had identical electrophoretic mobility.

Lockshin et al. (54) have recently found a coupling factor in maize chloroplasts as well as in maize etioplasts which are the plastids of dark grown plants. The enzyme from NaCl-EDTA extracts of both types of plastids could restore photophosphorylation in depleted chloroplast membranes. The properties of the enzyme such as trypsin, dithiothreitol and Ca^{2+}-activation of its ATP-ase were similar to the spinach CF_1. Electron microscopy of the etioplast and chloroplast membranes has shown the presence of 90 Å particles. The removal and reassociation of the particles with membranes was correlated with the ability to carry out photophosphorylation.

A coupling factor was also isolated from Euglena chloroplasts

(55). Unlike spinach CF_1, the Ca^{2+}-dependent ATP-ase activity of this enzyme was not masked. Lee et al. (56) have found a coupling factor for photophosphorylation in Anabaena variabilis. This coupling factor had no overt ATP-ase activity. A coupling factor for bacterial photophosphorylation was obtained from membranes of the photosynthetic bacterium Rhodopseudomonas capsulata by sonication in the presence of 1 mM EDTA (57). The enzyme was cold labile and had low ATP-ase activity. Another coupling factor for bacterial photophosphorylation was extracted from Chromatium strain D bacteria by Hochman and Carmeli (58).

3. Chloroplast development during greening

Higher plants germinated in complete darkness produce pale seedlings which contain no chlorophyll. Such etiolated plants can transform readily into normal green plants upon a few days of illumination. This change in appearance is accompanied by profound changes at the cellular level.

(a) Ultrastructural and biochemical changes during greening. Dark grown plants contain plastids with a structure different from that of chloroplasts. These plastids, called etioplasts, can develop into normal chloroplasts upon illumination of etiolated plants. The etioplast to chloroplast transformation was followed by means of electron microscopy by von Wettstein (59, 60), Gunning and Jagoe (61) as well as other workers (62, 63). These authors observed that the membraneous structure present in etioplasts, called the prolamellar body, had lost its crystalline character within a few minutes of

illumination. Upon further illumination, double membrane sheets (primary lamellae) were formed and extended out of the prolamellar body throughout the plastid. After several hours of illumination the membranes doubled in some places and started to form grana. Gyldenholm (64) has shown that the conversion of an etioplast to a chloroplast was completed within 45 hrs of illumination of dark grown plants.

The structural changes are accompanied and probably caused by chemical changes occurring in the plastids upon greening. Etiolated leaves contain protochlorophyll which can be photoreduced to chlorophyll a. This process which occurs very rapidly is followed in greening corn seedlings by a lag period and afterwards by synthesis of large amounts of chlorophyll a (65). Chlorophyll b synthesis is slower. Etiolated bean leaves were found to contain 0.2 µg protochlorophyll per leaf. During 45 hrs of illumination the chlorophyll content increased to 110 µg per leaf after an initial lag period of 3 to 5 hours (64). Carotenoids (66) and plastid lipids (67) are also formed in large quantities upon illumination of etiolated plants.

The greening process is accompanied by protein synthesis. De Deken-Grenson (68) as well as Mego and Jagendorf (69) have found a large increase in protein content of plastids upon greening. This increase is probably related to the synthesis of a number of proteins required for the various metabolic activities of chloroplasts.

Since etioplasts are lacking chlorophyll, they are not able to carry out electron transport, light-dependent ATP synthesis and CO_2 fixation. The inability to carry out these functions, however, is

not only due to the absence of chlorophyll but also due to the lack of other components in etioplasts as became apparent from studies of the above mentioned light-dependent reactions in greening plants.

Smith et al. (70) were following the development of the Hill reaction which represents part of the electron transport chain using dichlorophenol-indophenol as electron acceptor. They were able to detect the Hill reaction in plastids isolated from etiolated barley plants which were illuminated for at least 7 hours. The activity of the chloroplasts increased with prolonged illumination. Anderson and Boardman (71) followed development of the Hill reaction in plastids from greening bean plants using ferricyanide as electron acceptor. They observed a rapid increase in activity after 6 hours of illumination. Photoreduction of NADP, on the other hand, was absent after 8 hours and after 16 hours of illumination it was only about 30% of that of fully green chloroplasts.

Studies on the individual electron transport carriers have shown that ferredoxin which is absent in etiolated bean leaves and ferredoxin-NADP reductase, the activity of which is low in dark grown bean plants, increase considerably upon light-induced greening (72). Smillie et al. (73) have observed increases in ferredoxin-NADP reductase, cytochrome-552 and cytochrome-561 upon illumination of dark grown Euglena gracilis cells. Plastoquinone was also found to increase in greening Euglena (74).

The onset of photophosphorylation in chloroplasts isolated from greening bean leaves was studied by Gyldenholm and Whatley (17). They have found no light-dependent ATP formation in etioplasts. Cyclic

photophosphorylation with PMS as cofactor could be detected after 10 hours of illumination, non-cyclic photophosphorylation with ferricyanide as electron acceptor after 15 hours of illumination and non-cyclic photophosphorylation with NADP as electron acceptor after 20 hours of illumination. All the activities increased considerably with further greening. Electron microscopy of the developing chloroplasts demonstrated transformation of the prolamellar body into primary thylakoids and later grana formation. The authors have suggested a correlation between the attainment of a minimum structural complexity with the onset of photophosphorylation.

The inability of etioplasts to catalyze photophosphorylation could not only be due to the absence of light-driven electron transport in these plastids but also due to lack of the coupling factor required for coupling ATP formation to the electron transport chain. It has been shown recently (54), however, that the coupling factor is present in maize etioplasts. Basing the activity on a per mg of leaf tissue or a per mg of membrane protein as well as from the results of electron microscopic observations, the authors concluded that most if not all of the coupling factor present in the chloroplast was already present in its etioplast precursor. The finding that the enzyme had properties identical to the chloroplast enzyme and that it could restore photophosphorylation in green plastid membranes suggested that the coupling factor was not the limiting component causing absence of phosphorylation in etioplasts. Since etioplasts were found to contain the coupling factor there was a possibility that they might form ATP upon acid-base transition which does not require the light-driven electron

flow. It was found by Forger and Bogorad (75), however, that etioplasts did not form ATP upon acid-base transition and that they could not respond to osmotic changes in the environment. They suggested that the lack of osmotic responsiveness in etioplasts was the cause for their failure to catalyze acid-base phosphorylation.

Changes in activities of the enzymes of the CO_2-fixation cycle upon greening were examined in detail by Bradbeer (76). Following enzymic activity in leaf homogenates prepared from greening bean plants, he has observed light-induced increase in activity of most of the CO_2-fixation pathway enzymes.

(b) Effect of protein synthesis inhibitors on the transformation of etioplasts to chloroplasts. Chloramphenicol is known to inhibit protein synthesis by the 70S ribosomes of bacteria where it binds to the 50S subunit and probably interferes with the attachment of m-RNA or aminoacyl t-RNA to the ribosome (77, 78). Chloramphenicol was also found to inhibit protein synthesis by the 70S ribosomes of plant mitochondria (79) and by the 70S ribosomes of chloroplasts (80). In all these cases, the inhibition of protein synthesis at the ribosomal level is stereospecific. D-threo chloramphenicol causes inhibition while the L-threo isomer does not. However, both isomers inhibit ion uptake (81) and oxidative phosphorylation (82) in higher plants. This indicates that there could also be indirect inhibition of protein synthesis by inhibiting the energy supply. This effect would be caused by both isomers. Ellis (80) has suggested that the effect of both isomers on protein synthesis should be studied. If only D-threo chloramphenicol will cause inhibition of protein synthesis, then the

effect of the inhibitor is at the ribosomal level. If both isomers are inhibitory, the effect is an indirect one. In contrast to the inhibition of 70S ribosomes of chloroplasts, the 80S ribosomes of the higher plant cytoplasm are not inhibited by chloramphenicol (80).

Cycloheximide, on the other hand, is known to inhibit protein synthesis on the 80S cytoplasmic ribosomes from animal (83) as well as plant (84) sources. It does not inhibit protein synthesis by the 70S chloroplast ribosomes (80). In reticulocyte ribosomes, cycloheximide was found to inhibit protein synthesis by affecting binding, movement and release of t-RNA from the ribosomes (85). In addition to inhibiting protein synthesis at the ribosomal level, cycloheximide was also inhibitory to ion uptake by nongreen plant tissues, but leaf tissues were unaffected by the inhibitor (86). Kirk (87) has found no change in respiration or motility of Euglena gracilis cells at cycloheximide concentrations which inhibited chlorophyll synthesis. He concluded that cycloheximide can be used as a selective inhibitor of protein synthesis on 80S ribosomes of green cells.

Margulies (88) has observed 60% inhibition of chlorophyll synthesis and a 90% inhibition of the development of Hill reaction and CO_2 fixation ability upon greening of etiolated bean plants treated with chloramphenicol (4 mg CAP/ml). In further studies (89) he has shown that chloramphenicol partially inhibited formation of leaf and plastid protein upon greening. The increase in ribulose diphosphate carboxylase upon greening was also inhibited, while the NADP-linked glyceraldehyde-3-P dehydrogenase was not affected. Plastids from chloramphenicol treated plants had low rates of cyclic photophos-

phorylation and Hill reaction when compared to controls.

The effect of chloramphenicol on the ultrastructural changes of pea plastids upon greening was studied by Srivastava et al. (90). They have observed no effect on formation and structure of the prolamellar body or on conversion of the prolamellar body into thylakoids. They found, however, a strong effect on the thylakoid aggregation into grana.

The effects of D-threo and L-threo chloramphenicol on the light-induced formation of enzymes of the CO_2 fixation pathway in greening bean plants was studied by Ireland and Bradbeer (91). The increase in activity of ribulose diphosphate carboxylase and phosphoribulokinase was inhibited by D-threo but not by L-threo chloramphenicol indicating that these enzymes were formed on the plastid ribosomes. Several other enzymes of the pathway were not inhibited by chloramphenicol and were concluded to be formed by cytoplasmic ribosomes.

Criddle et al. (92) have measured incorporation of radioactive amino acids into the two subunits of the ribulose diphosphate carboxylase in the presence of chloramphenicol and cycloheximide. They observed that chloramphenicol specifically inhibited synthesis of the larger subunit while formation of the smaller subunit was inhibited by cycloheximide.

Smillie et al. (73) have observed that chloramphenicol inhibited formation of ribulose diphosphate carboxylase, NADP-linked glyceraldehyde-3-P dehydrogenase, ferredoxin - NADP reductase, cytochrome-552 and cytochrome-561 upon greening of Euglena gracilis cells. Formation of the latter three was also inhibited by cycloheximide, whereas

synthesis of the first two was not inhibited by this antibiotic. They concluded that the two soluble Calvin cycle enzymes were formed by chloroplast ribosomes, that the three enzymes of the electron transport system were also made by chloroplast ribosomes and that the inhibition by cycloheximide might have been indirect by blocking synthesis of structural elements essential for formation of the electron transport enzymes and their incorporation into the plastid membranes.

Hoober et al. (93) have observed that greening of the y-1 mutant of Chlamydomonas reinhardi in the presence of chloramphenicol caused drastic reduction of photoreductive activities while chlorophyll and plastid membranes were formed at the normal rate. However, the membranes in chloramphenicol treated cells rarely fused to form grana. In the presence of cycloheximide the chlorophyll synthesis and membrane formation was slowed down, but photoreduction and grana formation was the same as in controls when compared on the chlorophyll basis. The deficient membranes formed in the presence of chloramphenicol gained full activity upon transfer to a medium containing cycloheximide. The results indicated that products of both the cytoplasmic and the chloroplastic ribosomal systems were required for the formation of a normal active plastid membrane.

Eytan and Ohad (94) who also studied greening of the Chlamydomonas reinhardi y-1 mutant observed formation of a protein which they designated L protein upon greening. The formation of L protein was inhibited by cycloheximide. Preincubation of greening cells with chloramphenicol led to accumulation of L protein which protected the

greening system against complete inhibition of chlorophyll formation upon transfer to a cycloheximide containing medium. On the basis of their data, the authors suggested that proteins of cytoplasmic and of chloroplast origin were required for the formation of functional photosynthetic membranes and that the synthesis of these two types of proteins was probably regulated by a feedback mechanism.

Armstrong et al. (95) have followed the cellular origin of several plastid components using a number of antibiotics inhibiting specific transcriptional and translational steps of protein synthesis in Chlamydomonas reinhardi. They have found that chloroplast DNA had information for synthesis of cytochromes 553 and 563. Both chloroplast and cytoplasmic ribosomes were required for the synthesis of the two cytochromes. Only cytoplasmic ribosomes were required for synthesis of chlorophyll, ferredoxin, ferredoxin-NADP reductase and phosphoribulokinase. Both chloroplast and cytoplasmic ribosomes were required for the formation of ribulose diphosphate carboxylase.

MATERIALS AND METHODS

I. Experiments with pea plants

Growing of pea plants. Seedlings of Pisum sativum var. Laxton's Superb were soaked in distilled water for 24 hours and subsequently grown for two weeks on vermiculite in the greenhouse.

Chloroplast isolation. Chloroplasts were usually isolated according to Nobel (96) by cutting 10 g of plants into small pieces, placing them into a bag made out of a fine nylon cloth (15 XX Flour Silk, Strong-Scott Ltd., Winnipeg) and grinding them in a chilled mortar containing 10 ml of Nobel's isolation medium (0.2M sucrose in 20 mM TES-NaOH pH 7.9) for about 20 to 30 seconds. Afterwards the homogenate was squeezed out of the bag and centrifuged at 4° for 1 minute at about 1000 g to collect the plastids. The supernatant was decanted. The pellet was resuspended in a small amount of the homogenization medium and kept on ice. In some experiments an isolation medium containing 0.4 M sucrose, 20 mM Tris-HCl and 10 mM NaCl at pH 7.9 (STN medium) was used (The composition of the medium is similar to that used by Jagendorf and Avron (10)). The isolation procedure was the same as above except that the centrifugation was performed at 2000 g for 5 minutes.

Photophosphorylation assay. Light-dependent ATP formation was measured by incubating chloroplasts containing 30-100 µg chlorophyll in an incubation medium with 105 µmoles NaCl, 30 µmoles Tris-HCl, 60 mµ moles phenazine methosulfate, 15 µmoles $MgCl_2$, 9 µmoles ADP and 9 µmoles sodium phosphate containing ^{32}Pi in a volume of 3 ml at pH 7.9

(96) for 1 minute at 20° and 100,000 lux of incandescent light. The illumination was followed by deproteination with 0.2 ml of 60% trichloroacetic acid. The amount of ATP formed was measured by ^{32}Pi incorporation into ATP after centrifuging down the denatured protein.

^{32}Pi incorporation into ATP was measured by the method of Nielsen and Lehninger (97). A 2 ml aliquot of the supernatant was mixed with 2 ml of molybdate reagent (3% ammonium molybdate in 2N H_2SO_4). After 5 minutes, 4 ml of water-saturated isobutanol-benzene (1:1) mixture was added and mixed on a Vortex mixer at maximum speed for 10 seconds. After separation of the phases the organic layer containing the phosphomolybdate complex was removed and the aqueous layer was extracted two more times with isobutanol-benzene to remove all inorganic phosphate. A 2 ml aliquot of the aqueous layer containing the ^{32}P-labeled ATP was counted in a liquid scintillation counter after dilution with 10 ml of H_2O or in a Geiger-Muller counter after drying the sample on a planchet.

The light-dependent label incorporation into ATP was obtained by subtracting the incorporation obtained in a dark incubation from that obtained upon illumination. All steps of the assay except the incubation were carried out on ice.

Chlorophyll and protein determination. Chlorophyll was measured spectrophotometrically in 80% acetone extracts of chloroplasts using the equation of Mackinney (98). Protein was measured according to Lowry et al. (99).

Restoration of photophosphorylation by EDTA extracts of chloroplasts. The experimental procedure was essentially that of Avron (36)

and of McCarty and Racker (35). In the experiment 0.3 ml aliquots of a chloroplast suspension in Nobel's medium (90 µg of chlorophyll) were resuspended in 3 ml of 0 to 10 mM EDTA in 0.2 M sucrose and 10 mM TES-NaOH pH 7.9. After 10 minutes at room temperature the suspensions were centrifuged for 20 minutes at 27,000 g at 20°. The resulting supernatants were stored at room temperature while the pellets were resuspended in 0.3 ml of Nobel's medium and stored on ice. Photophosphorylation of the chloroplast residue was measured as described above using 0.1 ml of the resuspended plastid residue (30 µg of chlorophyll). To measure the stimulation of ATP formation by the EDTA extracts, 1 ml aliquots of these extracts were mixed with 0.1 ml of the corresponding chloroplast residues and all the other components of the reaction mixture except phenazine methosulfate (PMS) were added. After 10 minutes on ice, PMS was added in the dark and the light incubation was carried out as described above.

Trypsin activation of the Ca^{2+}-dependent ATP-ase in chloroplasts. Trypsin activation of the ATP-ase was performed as described by Vambutas and Racker (37). Chloroplasts containing 117 µg of chlorophyll were incubated with 10 µmoles Tris-HCl, 1 µmole EDTA, 0.5 µmole ATP and 150 µg trypsin in a volume of 0.4 ml at pH 8. After a 0 to 30 minute incubation at room temperature, 450 µg of soybean trypsin inhibitor were added in a volume of 0.1 ml. ATP-ase activity was assayed by incubating 0.1 ml of the activated enzyme with 50 µmoles Tris-HCl, 5 µmoles $CaCl_2$ and 5 µmoles ATP in a final volume of 1 ml at pH 8. After 20 minutes of incubation at 37° the reaction was stopped by addition of 0.2 ml of 10% trichloroacetic acid and the

released inorganic phosphate was measured as the unreduced phosphomolybdate complex according to Mozerski et al. (100). Controls for each sample were performed by adding trichloroacetic acid at zero time of the ATP-ase assay.

Light-dependent pH rise. The light-dependent pH rise of an unbuffered chloroplast suspension was measured essentially according to Neumann and Jagendorf (22). However, $MgCl_2$ was included in the incubation mixture as it is known to increase the pH rise by rendering the plastid membranes less soluble to protons (101). Chloroplasts isolated in the STN medium were washed twice with a solution of 0.4M sucrose, 10 mM NaCl and 1 mM $MgCl_2$ to lower their buffering capacity. To measure the light-induced pH rise, chloroplasts containing 1.4 mg of chlorophyll were incubated with 350 µmoles NaCl, 10 µmoles $MgCl_2$ and 0.24 µmoles PMS where indicated in a volume of 10 ml. The pH rise during illumination with 100,000 lux of incandescent light at 10^{o} was followed with a pH meter (Radiometer 26 Copenhagen) using a combined glass-calomel electrode. The buffering capacity of the incubation mixture was measured by following the pH change upon addition of 0.1 to 0.2 ml of 10^{-3} M HCl in the dark.

Acid-base phosphorylation. The experiment was performed according to Jagendorf and Uribe (25). Plastids were isolated in STN and washed once with 10 mM NaCl. Chloroplasts containing 375 µg of chlorophyll or etioplasts containing 760 µg of protein were incubated with 10 mM succinate pH 3.8 in a volume of 1.5 ml (acid stage). After a 1 minute incubation at room temperature the solution was transferred

into a base bath containing 1.5 ml of 3 mM sodium phosphate containing ^{32}Pi, 3 mM ADP, 5 mM $MgCl_2$, 0.1 M Tris-HCl at pH 8.3 and an amount of NaOH that just neutralized the succinate. To deproteinate the incubation mixture, 0.2 ml of 60% trichloroacetic acid was added 1 minute after the transfer. All stages of the reaction were performed in the dark. The amount of ATP formed was measured as described in the section "Photophosphorylation assay". Control experiments in which the pH of the "acid stage" was 8.3 were performed and the obtained values of ATP formation under these condition (representing actually polyphosphate contamination of the ^{32}Pi) were subtracted from the values obtained upon pH 3.8 to pH 8.3 transition to obtain corrected values of ATP formation due to acid-base transition.

II. Experiments with bean plants.

Growing of bean plants. Seeds of Phaseolus vulgaris var. Kinghorn Special were rinsed with a dilute hypochlorite solution (Javex diluted with H_2O in a 1:5 ratio) and grown for 2-3 weeks on soil either in the greenhouse to produce normal green plants or in a dark growth chamber to produce etiolated plants.

Plastid isolation. Plastids were isolated by homogenizing the primary bean leaves with "STB medium" consisting of 0.4 M sucrose, 40 mM TES-NaOH and 0.2% bovine serum albumin (BSA) (Calbiochem) at pH 8. A Vir-tis 45 homogenizer was used and operated either at top speed for 20 seconds when using the 250 ml homogenizing flask or at a reduced speed (setting 10 on the rheostat) for 1 minute when using

the 30 ml homogenizing flask. After filtering the homogenate through a layer of fine nylon cloth (15XX Flour Silk, Strong-Scott Ltd., Winnipeg) to remove unhomogenized material and most cell debris, it was centrifuged 5 minutes at 2000 g. The plastid pellet obtained was resuspended in a small amount of homogenizing medium or directly extracted with H_2O or EDTA as indicated in each experiment. During the isolation the temperature was kept below 4°.

Photophosphorylation assay. Photophosphorylation was measured in an incubation mixture similar to that of Gyldenholm and Whatley (17) containing 300 µmoles TES-NaOH, 15 µmoles $MgCl_2$, 9 µmoles sodium phosphate containing ^{32}Pi, 6 µmoles ADP, 3 mg BSA, 90 m µmoles PMS and chloroplasts containing 20-50 µg chlorophyll in a volume of 3 ml at pH 8. The reaction mixture was incubated for 1-2 min at 100,000 lux and 20°, deproteinated with 0.2 ml 60% trichloroacetic acid and the amount of ATP formed was measured as described above for pea chloroplasts.

Restoration of photophosphorylation by plastid extracts. Chloroplasts containing 200-400 µg chlorophyll were resuspended in 10-20 ml of extraction medium containing 10 mM NaCl, 0.2% BSA and 1 mM EDTA at pH 8 (NBE solution). After 10 minutes on ice, the suspension was centrifuged for 15 minutes at 35,000 g and 0°. The supernatant was withdrawn, the chloroplast sediment resuspended in 1 ml of a medium containing 10 mM NaCl and 0.2% BSA pH 8 and stored on ice. A portion of this chloroplast suspension (0.1 ml) was preincubated on ice with 0.3 ml of 50 mM $MgCl_2$ and 1.2 ml of either NBE or NBE plastid extract. After 10 minutes, 1.4 ml of an incubation mixture at pH 8 and containing

300 µmoles TES-NaOH, 9 µmoles sodium phosphate containing ^{32}Pi, 3 mg BSA, 6 µmoles ADP and 90 mµmoles PMS was added. The test tubes were incubated for 5 minutes at a light intensity of 100,000 lux and either 5° or 20° as indicated in Results. Deproteination and estimation of the amount of ATP formed were performed as described above in the section on Photophosphorylation. Various extracts of both etioplasts and chloroplasts were used in place of the NBE extract in this type of experiment. The types of extracts used in the particular experiments will be specified in Results.

Light and DTT-activation of the Mg^{2+}-dependent ATP-ase in plastids. The assay was similar to that of McCarty and Racker (38). Chloroplasts containing 4-20 µg of chlorophyll were activated in an incubation mixture containing 100 µmoles TES-NaOH, 5 µmoles $MgCl_2$, 30 mµmoles PMS and 10 µmoles DTT in a total volume of 0.9 ml at pH 8. After a 5 minute activation in dark or light (100,000 lux) at 20°, 0.1 ml of 50 mM ATP was added. The mixture was incubated at 37° in the dark for 20 minutes and the reaction was terminated by adding 0.1 ml of 30% trichloroacetic acid. The released phosphate was measured as the unreduced phosphomolydate complex (100) in an 0.7 ml aliquot after oxidation of DTT by 5 µl of 30% H_2O_2.

The same type of experiment was performed with etioplasts containing 213 µg of protein. The conditions of incubation were identical to those for chloroplasts except that the incubation time was 30 minutes.

DTT-activated, Ca^{2+}-dependent ATP-ase in plastid extracts. The activity was assayed according to McCarty and Racker (38). Chloroplasts containing 0.5 to 2 mg of chlorophyll were resuspended in 30 ml of 1 mM EDTA, pH 8. After 10 minutes at room temperature the suspension was centrifuged 20 minutes at 35,000 g and 20°. To activate the ATP-ase activity of the enzyme, 3.5 ml of the supernatant were incubated with 250 µmoles TES-NaOH and 250 µmoles DTT in a total volume of 5 ml at pH 8. After 2 hours at room temperature, 0.1-0.4 ml aliquots were transferred to an incubation mixture containing 50 µmoles TES-NaOH, 5 µmoles $CaCl_2$ and 5 µmoles ATP in a final volume of 1 ml at pH 8. After a 10-20 minute incubation at 37°, the reaction was stopped by addition of 0.1 ml of 30% trichloroacetic acid and released inorganic phosphate was measured in the whole volume after DTT oxidation with H_2O_2 as described in the previous section. Controls containing no enzyme were performed.

Etioplast extracts were prepared by resuspending etioplasts containing 2-5 mg of protein in 10 ml of 1 mM EDTA, pH 8. The extracts were obtained, activated and incubated as described above for chloroplast extracts. Except if otherwise stated, the ATP-ase activity is expressed as µmoles Pi released per mg soluble protein in the extract per hour.

Disc gel electrophoresis, localization of the ATP-ase band. Disc gel electrophoresis was performed according to Davis (102) using a 3% spacer gel pH 6.7 and a 7% running gel pH 8.9 (Tris-HCl buffer). Tris-glycine buffer pH 8.3 was used as reservoir buffer. Plastid extracts for electrophoresis were prepared by extracting chloroplasts

or etioplasts as described in the previous section using H_2O, 1 mM EDTA pH 8, or both. The obtained extracts were concentrated 5 to 10 fold by blowing air over dialysis tubing containing the extracts. After sucrose addition to increase their density, 50-100 µl of these solutions were applied on the top of the gels. The electrophoresis was performed for about 30 minutes at 2 mA per tube. After the front marker (bromophenol blue) entered the running gel, the current was increased to 4 mA per tube and the electrophoresis was continued until the marker was close to the bottom of the tubes. The gels so obtained were stained for protein with Amidoblack or for ATP-ase activity by submerging the gels into a solution at pH 8 containing 100 mM TES-NaOH, 5 mM ATP and 50 mM $CaCl_2$.

Restoration of photophosphorylation by extracts of disc gel electrophoresis zones containing ATP-ase activity. Chloroplasts containing 18 mg of chlorophyll were extracted with 250 ml of 1 mM EDTA pH 8 as described above. The obtained extract was concentrated 50-fold by blowing air over dialysis tubing containing the extract and 0.1 ml of the resulting solution containing 396 µg of protein was applied to each of 24 gels used for disc gel electrophoresis under conditions described in the previous section. After electrophoresis the gels were stained for ATP-ase activity for 30 minutes as described above. After the ATP-ase bands became visible, the zones containing ATP-ase activity were excised and homogenized with 6 ml of H_2O using a ground glass pestle fitting a standard size test tube. The homogenate was dialyzed overnight at room temperature against 1.5 l of a solution at pH 8 containing 5 mM TES-NaOH and 1 mM EDTA. The dialyzed

gel homogenate was centrifuged 10 minutes at 2000 g to remove the gel material. The resulting supernatant was tested for coupling factor activity by combining 5 to 200 µl of this solution containing 410 µg of protein per ml with 0.1 ml of a depleted chloroplast residue containing 29 µg of chlorophyll as described for the NBE extracts and residues in the section on "Restoration of photophosphorylation by plastid extracts". The incubation of the combined system was performed at 5°.

Ribulose diphosphate carboxylase activity. Ribulose diphosphate carboxylase activity in the plastid extracts was assayed according to Bradbeer (76). The incubation mixture contained 50 µmoles TES-NaOH, 1 µmole DTT, 5 µmoles $MgCl_2$, 30 µmoles $NaH^{14}CO_3$ (5.8×10^6 dpm), 0.5 µmole ribulose diphosphate and 0.1 ml of plastid extract in a total volume of 0.5 ml at pH 8. After 10-20 minutes of incubation at 25°, the reaction was stopped by adding 50 µl of 30% trichloroacetic acid. The radioactive CO_2 was removed by bubbling air through the solutions for 1 minute. Aliquots (0.2 ml) of these solutions were counted in a liquid scintillation counter. Controls containing no enzyme were performed.

Inhibition of the light-induced increase in ATP-ase activity by chloramphenicol and cycloheximide. Parts of 14 days old etiolated bean plants cut about 1 cm below the cotyledons were greened for 48 hours at room temperature and 3000 lux while being partly submerged in a solution containing either chloramphenicol or cycloheximide as described by Margulies (88). This experimental design was used for

following inhibition of the light-induced increase in ATP-ase activity as a function of chloramphenicol (CAP) or cycloheximide (CHI) concentration.

In all other experiments, the excised etiolated plants were transferred to test tubes containing 20 ml of the antibiotic solution or H_2O and greened at 10,000 lux and 21° in a growth chamber.

ATP-ase activity was measured after DTT activation of plastid extracts obtained by extracting plastids isolated from ten leaves with 4 ml of 1 mM EDTA at pH 8.

D-threo chloramphenicol and cycloheximide used in these experiments were purchased from Sigma. L-threo chloramphenicol was a generous gift from Dr. H. E. Machamer, Parke-Davis & Co.

The antibiotic Dio-9 used for inhibition of photophosphorylation and ATP-ase activity was a gift from Royal Netherlands Fermentation Industries, Ltd.

RESULTS AND DISCUSSION

I. Experiments with pea plants

Photophosphorylation and its partial reactions have been studied, for the most part, in spinach chloroplasts. However, studies of the development of photophosphorylation in greening tissue are hampered by the difficulties encountered in growing spinach in the dark. Thus for this study on a coupling factor for photophosphorylation in etiolated and greening plants, peas (Pisum sativum) were chosen as experimental material.

1. Optimal conditions for photophosphorylation

Pea chloroplasts isolated and incubated according to Nobel (96) as described in the Methods (section I) catalyzed cyclic photophosphorylation at rates of 120-700 µmoles ATP formed/mg chlorophyll/hr. These rates were quite variable and considerably lower than the 2,300 µmoles ATP/mg chlorophyll/hr reported by Nobel (96). It was therefore decided to check the effects of various isolation and storage media, of different exogenous electron carriers, and of variation of the amount of chloroplasts in the incubation mixture on the rate of photophosphorylation.

Effect of the isolation and storage medium

Use of an isolation medium containing 0.4M sucrose, 20 mM Tris-HCl and 10 mM NaCl at pH 7.9 (STN) did not improve the rate of ATP formation when compared to the isolation of chloroplasts in 0.2M sucrose and 20 mM TES-NaOH pH 7.9 (Nobel's medium (96)). The photophosphorylation rate was 125 µmoles ATP formed/mg chlorophyll/hr for the

plastids isolated in STN compared to 143 μmoles ATP/mg chlorophyll/hr for plastids isolated in Nobel's medium. The latter medium was therefore used in further photophosphorylation experiments.

The stability of the chloroplast suspension after isolation was measured in an approximately isotonic (0.2M sucrose in 20 mM TES-NaOH pH 7.9) and in a hypotonic (10 mM NaCl) medium. It can be seen from Table 1 that the retention of photophosphorylation ability of the plastid suspension was higher in the isotonic than in the hypotonic medium. The greater loss of activity after 2 hours of storage in the hypotonic medium could be due to loss of chloroplast components required for photophosphorylation or due to structural damage of the thylakoid membranes.

Photophosphorylation with PMS and pyocyanine

The two exogenous electron carriers known to enable high rates of cyclic photophosphorylation by isolated plastids are phenazine methosulfate (PMS) and pyocyanine (5). To determine which of the two electron carriers will catalyze higher rates of ATP formation in pea chloroplasts, the plastids were incubated with 20 μM PMS (96) or with 50 μM pyocyanine (35) under conditions described in Methods. PMS was found to be superior to pyocyanine as it catalyzed formation of 125 μmoles ATP/mg chlorophyll/hr compared to 45 μmoles ATP formed/mg chlorophyll/hr with pyocyanine.

Optimal chloroplast concentration

The optimal chloroplast concentration for cyclic photophosphorylation was determined by varying the amount of chloroplasts in the incubation mixture over a tenfold range of concentration. It can

Table 1. Effect of the storage medium composition on photophosphorylation stability of the pea chloroplast preparation. Chloroplast suspensions were stored on ice for the time indicated in the table. Cyclic photophosphorylation was measured under conditions described in Methods.

Time elapsed from plastid isolation (min)	ATP formation (μmoles/mg chlorophyll/hr)	
	Nobel's medium[1]	10 mM NaCl
15	369	349
30	330	355
60	259	252
120	184	79

[1] 0.2M sucrose in 20 mM TES-NaOH pH 7.9

be seen from Table 2 that the highest specific activity for ATP formation was observed when incubation mixtures containing about 50 μg of chlorophyll per tube were used. The optimum is not very sharp, however, and good rates of photophosphorylation can be obtained over a wide range of chloroplast concentrations under the present assay conditions.

Mitochondrial contamination of chloroplasts

Chloroplasts isolated by differential centrifugation are known to be contaminated by mitochondria (e.g. 37). To ascertain whether oxidative phosphorylation by mitochondrial contamination in the chloroplast preparation contributed to ATP formation the photophosphorylation assay was performed in the presence of 2.5 μg of oligomycin per 3 ml of incubation mixture (103). Oligomycin is known to inhibit oxidative phosphorylation in mitochondria but it has no effect on photophosphorylation in chloroplasts (104). The photophosphorylation rate of the pea chloroplast preparation was found to be 540 μmoles ATP formed/mg chlorophyll/hr in the presence or absence of oligomycin indicating that mitochondrial contamination did not contribute to ATP formation under the incubation conditions described in Methods.

2. Coupling factor in pea chloroplasts

No reports on the presence of a coupling factor in pea chloroplasts could be found in the literature. Assuming that pea plastids might contain a coupling factor with properties similar to the spinach chloroplast coupling factor (36, 37, 38), the two types of assays available to establish its presence in pea chloroplasts were:

Table 2. Optimal chlorophyll concentration for ATP synthesis in pea chloroplasts.

Chloroplasts were isolated and resuspended in Nobel's medium as described in Methods to give a chloroplast suspension containing 52 µg chlorophyll/100 µl. The photophosphorylation assay was performed with 25 to 300 µl of this suspension.

Chloroplast concentration (µg chlorophyll/test tube)	ATP formation (µmoles/mg chlorophyll/hr)
13	554
52	688
104	539
156	480

a) Extraction of the coupling factor from the thylakoid membranes. The residual membranes should have low rates of photophosphorylation which should be stimulated upon readdition of the extract (35, 36, 37).
b) Measurement of the ATP-ase activity of the coupling factor after induction by trypsin, heat (37) or dithiothreitol (38).

Restoration of photophosphorylation by EDTA extracts

The initial attempts to extract the coupling factor from pea chloroplasts with 1 mM EDTA and follow restoration of photophosphorylation after readdition of the extract to the chloroplast residue were unsuccessful. The plastid membranes completely lost their capacity for light-dependent ATP formation upon exposure to 1 mM EDTA and this activity was not restored upon readdition of the EDTA extract. In further experiments this was found to be true for extraction with 0-4 mM EDTA. It was concluded that the exposure of the pea plastids to the hypotonic solution caused irreversible loss of their photophosphorylation capacity. It was hoped that an increase in osmolarity of the extraction medium might prevent this irreversible loss of activity. The EDTA solutions used for coupling factor extraction were therefore supplemented with 0.2M sucrose and 10 mM TES-NaOH pH 7.9. It can be seen from the results in Table 3 that extraction of pea chloroplasts with such solutions gave chloroplast residues capable of catalyzing photophosphorylation and that their activity was stimulated upon readdition of the extracts. Optimal extraction of coupling factor and the highest restoration due to readdition of the extracts was obtained with solutions containing 0.5 and 1 mM EDTA. This EDTA concentration is also known to be most effective with spinach

Table 3. Optimal EDTA concentration for coupling factor extraction from pea plastids.

Chloroplasts were extracted and reconstituted with EDTA-sucrose-TES solutions of varying EDTA concentration and light-dependent ATP formation was measured.

EDTA (mM)	ATP formation (μmoles/mg chlorophyll/hr)		
	Chloroplast residue	Chloroplast residue + extract	Difference
0	282	344	62
0.5	169	268	99
1	172	257	85
2	178	210	32
5	220	240	20
10	94	49	--

chloroplasts (35). The residual photophosphorylation activity in pea chloroplast membranes extracted with sucrose-TES-EDTA solutions was high when compared to spinach chloroplast residues obtained by EDTA extraction (35). The higher osmolarity of the extraction medium used in the present work may have to a large extent prevented the rupture of the chloroplast envelope membranes and consequently reduced the exposure of the thylakoid membranes to the extraction medium. This may have resulted in a decrease in the coupling factor extraction. The low activity in the case of 10 mM EDTA may have been due to irreversible removal of essential components required for photophosphorylation.

Trypsin-activated ATP-ase

Vambutas and Racker (37) have shown that trypsin treatment of spinach chloroplasts caused activation of a Ca^{2+}-dependent ATP-ase. They were able to purify this ATP-ase in its latent form and demonstrated that the latter acted as a coupling factor for photophosphorylation.

An attempt was made to show the presence of a trypsin activated ATP-ase in pea chloroplasts. It is evident from the results in Table 4 that pea plastids do contain such an ATP-ase as there was a fourfold activation of ATP-ase activity after a 10 minute trypsin treatment. Longer incubation with trypsin resulted in a decrease in activity, probably due to digestion of the ATP-ase enzyme.

The presence of a trypsin-activated, Ca^{2+}-dependent ATP-ase in pea chloroplasts together with the evidence on restoration of photophosphorylation by EDTA containing extracts indicate that pea chloro-

Table 4. Activation of pea chloroplast ATP-ase activity by trypsin. Chloroplasts were treated with trypsin for the time indicated in the table and the Ca^{2+}-dependent ATP-ase activity was measured after addition of trypsin inhibitor.

Time of trypsin activation (min)	ATP-ase activity (μmoles Pi released/mg chlorophyll/hr)
0	15.3
10	60.0
20	46.5
30	43.8

plasts probably contain a coupling factor similar to the spinach chloroplast enzyme.

3. Partial reactions of photophosphorylation

Light-induced pH rise

Since a light-induced pH rise of an unbuffered chloroplast suspension has been shown to be related to photophosphorylation, it was thought that following the ability of plastids isolated from greening plants to carry out the light-induced pH rise might provide some information as to the minimum requirements necessary for photophosphorylation.

It can be seen from Table 5 that pea chloroplasts isolated from green plants are capable of catalyzing the light-induced pH rise. As in spinach chloroplasts (22), addition of an exogenous electron carrier (PMS) caused a 50% increase in the extent of the pH rise and a 2.5 fold increase in its rate as measured by the pH change during the initial 15 seconds of illumination.

The attempt to follow the ability of plastids isolated from greening plants to catalyze the light-induced pH rise was unsuccessful as difficulties with the greening of etiolated pea plants were encountered. Etiolated pea plants did not turn green synchronously upon illumination. Rather, the greening started at the top of the plants where it was associated with growth and proceeded slowly to the lower parts. Isolation of plastids from such plants would result in a mixture of etiolated, partially green and fully green plastids. Experiments with such preparations could not provide reliable information.

Table 5. Measurement of a light-induced pH rise in pea chloroplasts. The light induced pH rise of the chloroplast suspension was measured as described in Methods in the presence or absence of PMS. Buffering capacity of the incubation mixture in the dark was 0.05 pH unit per 0.1 μmole H^+.

Addition	ΔpH[1]	Extent of H^+ uptake (μmoles H^+/mg chlorophyll)	ΔpH/initial 15 sec	Rate of H^+ uptake[2] (μmoles H^+/mg chlorophyll/min)
Chloroplasts	0.20	0.29	0.11	0.63
Chloroplasts + 0.24 μmoles PMS	0.31	0.44	0.27	1.54

[1] after equilibrium was reached (in less than 1 min)

[2] calculated from ΔpH per initial 15 sec

ATP formation by acid-base transition

ATP formation due to an artificially created pH gradient can proceed in darkness and does not require light-driven electron transport (25). Etioplasts do not contain chlorophyll and cannot therefore support light-driven electron transport. It was interesting to investigate, however, whether they could form ATP under conditions where electron transport was not required for ATP formation as in an acid-base transition. The experiment was performed according to Jagendorf and Uribe (25) under conditions described in the Methods. Pea chloroplasts were found to catalyze formation of 200 mμmoles ATP per mg chlorophyll upon the transition from a medium of pH 3.8 containing succinate to a medium of pH 8.3 containing ADP, Pi and $MgCl_2$. This yield is comparable to that obtained in spinach chloroplasts (25). Pea etioplasts incubated under identical conditions did not form any ATP indicating a defective energy transfer sequence in the prolamellar body membranes of these etioplasts.

A similar finding was recently made by Forger and Bogorad (75) who have shown that maize etioplasts did not form ATP upon acid-base transition. They have suggested that this may be due to the lack of osmotic responsiveness which they found in etioplasts.

Although it would have been interesting to follow the development of the acid-base phosphorylation activity in greening plants, this was not possible due to the nonsynchronous greening of etiolated pea plants exposed to light, as mentioned in more detail in the section on the light-induced pH rise. Since the objective of the present study was to follow coupling factor activity during greening of etiolated plants,

it was concluded that pea plants were not suitable experimental material for these studies. It was therefore decided to continue the work on etiolated bean plants, the primary leaves of which were reported to turn green synchronously upon illumination (17).

II. Experiments with bean plants

1. Assays

Photophosphorylation

Initial attempts to demonstrate cyclic photophosphorylation in bean chloroplasts by using the isolation and incubation media used for the experiments with pea chloroplasts (Section I) were unsuccessful. It was found, in agreement with other workers (16, 17, 18), that bovine serum albumin (BSA) was required for optimal rates of photophosphorylation.

Figure 2 demonstrates that the presence of 0.2% BSA in the homogenizing medium is essential for obtaining good rates of photophosphorylation. Variation of the BSA concentration in the incubation medium has a less pronounced effect on the ATP formation. Optimum rates are obtained, however, when the medium contains 0.1% BSA. The beneficial effect of BSA on light dependent ATP formation was suggested to be due to its ability to bind unsaturated fatty acids and phenols (16) as well as due to its effect as a sulfhydryl reagent (18).

With optimal BSA concentration in both the homogenizing and incubation medium, and incubation conditions described in Methods, the rate of photophosphorylation was linear for at least 5 minutes (Fig. 3). Bean etioplasts incubated under identical conditions showed no light-dependent ATP formation. This could be expected as the etioplasts contain no chlorophyll and, for this reason at least, are not capable of the light-driven electron transport necessary for photophosphorylation.

Figure 2

Effect of BSA on photophosphorylation in bean chloroplasts.

Curve A: no BSA in the homogenizing medium.

Curve B: 0.2% BSA in the homogenizing medium.

Conditions for incubation are described in Methods.

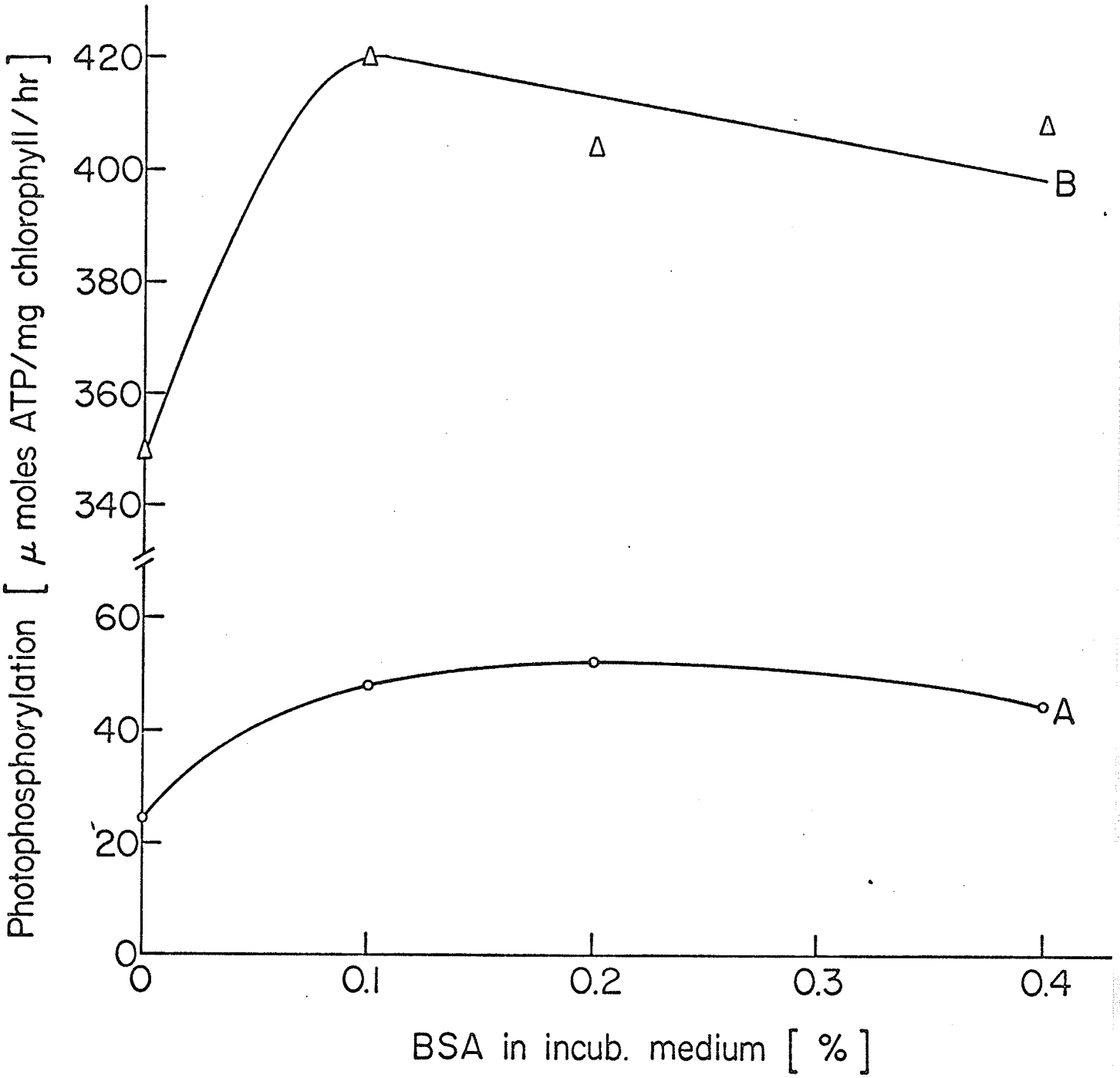
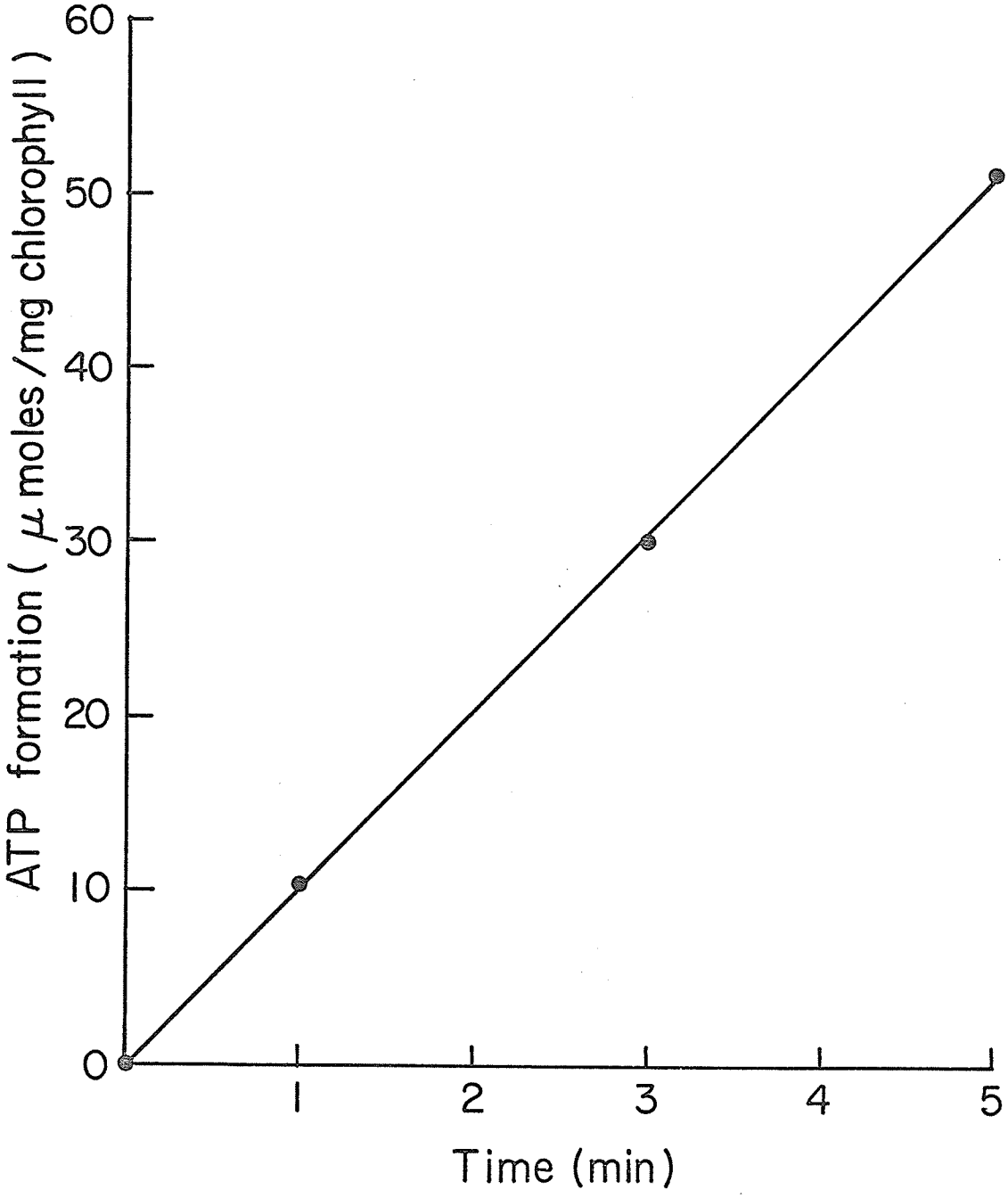

Photophosphorylation [μ moles ATP/mg chlorophyll/hr]
420
400
380
360
340
60
40
20
0
0
0.1
0.2
0.3
0.4
BSA in incub. medium [%]
B
A

Figure 3

Time course for photophosphorylation in bean chloroplasts. Incubation conditions are described in Methods. Chloroplasts contained 27 μg of chlorophyll.


ATP formation (μ moles / mg chlorophyll)
60
50
40
30
20
10
0
1
2
3
4
5
Time (min)

The isolation and incubation conditions used in these experiments could also be used for measuring light-dependent ATP synthesis in spinach chloroplasts. Photophosphorylation rates of 805 μmoles ATP formed/mg chlorophyll/hr were obtained with chloroplasts isolated from fresh field-grown spinach and rates of 525 μmoles ATP/mg chlorophyll/hr were measured in chloroplasts isolated from commercially available spinach.

Restoration of photophosphorylation

Spinach chloroplasts are known to release a coupling factor upon extraction with EDTA (35, 36). The membranous residue after EDTA extraction has low rates of photophosphorylation and is stimulated upon readdition of the EDTA extract.

Table 6 demonstrates, however, that bean chloroplasts exposed to 1 mM EDTA, pH 8, completely lost their capacity for light-dependent ATP formation and this activity was not restored after readdition of the EDTA extract to the residue. Inclusion of 0.2% BSA in the extraction medium to remove unsaturated fatty acids did not help to retain any photophosphorylation.

Use of a hypertonic isolation medium (STB), supplemented with 1 mM EDTA to extract the coupling factor, resulted in good retention of photophosphorylation but no extraction of the coupling factor. However, with a medium containing 10 mM NaCl, 0.2% BSA and 1 mM EDTA at pH 8 (NBE), it was possible to retain low rates of photophosphorylation in the chloroplast residue that could be stimulated upon readdition of the NBE extract (Table 6).

Similar observations were recently made by Lockshin et al. (54),

Table 6. Effect of the coupling factor extraction media on the ability to reconstitute photophosphorylation in bean plastids.

The chloroplast extraction and reconstitution was performed as described in Methods except that media of the composition listed in this table were used. All extraction media were adjusted to pH 8. The reconstituted plastids were incubated at 20°.

Extraction medium	ATP formation (μmoles/mg chlorophyll/hr)	
	Chloroplast residue	Chloroplast residue + extract
1 mM EDTA	0	0
1 mM EDTA 0.2% BSA	0	0
0.4M sucrose 40 mM TES-NaOH 0.2% BSA 1 mM EDTA	521	523
10 mM NaCl 0.2% BSA 1 mM EDTA	12	40

who have found that maize chloroplasts irreversibly lost their photophosphorylation activity upon extraction with 0.15 mM EDTA alone. As with bean plastids, inclusion of 10 mM NaCl in the extraction medium prevented the irreversible loss of activity in maize chloroplasts and enabled preparation of chloroplast residues whose photophosphorylation capacity was stimulated upon readdition of the NaCl-EDTA extract.

Although restoration of photophosphorylation with the NBE extracts was usually satisfactory upon illumination of the reconstituted system at 20°, occasionally low restoration rates were encountered. In these cases the ATP-ase activity of the NBE extract was found to be high even before DTT stimulation. This suggested that the poor restoration of photophosphorylation might be due to interference by the ATP-ase. Howell and Moudrianakis (45) have found that lowering the temperature of the photophosphorylation assay from 22° to 4° resulted in a greater decrease in ATP-ase activity relative to photophosphorylation. Using this technique, a greater stimulation of photophosphorylation in deficient chloroplast residues upon readdition of the extracts was obtained and the levels of restoration were more consistent. As shown in Table 7, restoration was proportional to the amount of NBE extract added at 5°. Addition of 1.2 ml of the NBE extract resulted in a nearly 5-fold stimulation of phosphorylation compared to the chloroplast residue and the rate reached one half of that of the original chloroplast preparation. Because of the more consistent results and greater stimulation, incubation at 5° was used in most reconstitution experiments.

Table 7. Restoration of photophosphorylation in bean chloroplast residues at 5°. The NBE extraction and reconstitution was performed as described in Methods. The volume of the NBE extract used was adjusted to 1.2 ml with NBE solution before adding the other components. The chloroplast residue contained 41 µg chlorophyll per tube of incubation. Photophosphorylation rate of the chloroplast preparation before NBE extraction was 20.2 µmoles ATP/mg chlorophyll/hr at 5°.

Amount of NBE extract added to chloroplast residue (ml)	ATP formation (µmoles/mg chlorophyll/hr)
0	2.3
0.2	3.8
0.4	4.7
0.8	8.5
1.2	10.8

DTT-activated, Ca^{++}-dependent ATP-ase in plastid extracts

EDTA extracts of bean etioplasts and chloroplasts were found to contain DTT-activated, Ca^{++}-dependent ATP-ase activity similar to that described in spinach chloroplasts (38). The time course of the ATP-ase activation with 50 mM DTT (Table 8) indicated that the activity of the enzyme increased most during the first hour of preincubation with DTT. After 2 hrs the increase in activity was not significant and, therefore, 2 hrs of activation were used in all experiments of this type.

In chloroplasts the ATP-ase activity after DTT treatment was usually about 40 µmoles Pi released/mg soluble protein/hr, while the activity of the extract before DTT treatment varied from 4 to 20 µmoles Pi/mg protein/hr. The variable activity of the ATP-ase in the plastid extracts before DTT treatment might have been caused by an activation of the enzyme before its extraction from the chloroplasts. Light or BSA were suspected to cause this activation of the ATP-ase. However, light-activation can probably be ruled out since the variable activation of the ATP-ase was also obtained with etioplasts which contain no chlorophyll. Furthermore, the extraction of the ATP-ase from chloroplasts isolated either in the dark or light had no effect on its activity. The activation by BSA can also be excluded as isolation of chloroplasts in a medium without BSA did not effect the ATP-ase activity of the EDTA extract. The reason for the variable DTT activation remains, therefore, unknown.

Staining of disc electrophoresis gels for ATP-ase activity

As EDTA extracts of plastids contain a number of proteins, a staining procedure specific for ATP-ase activity was required to

Table 8. Time course of bean plastid ATP-ase activation with 50 mM DTT. An EDTA extract of etioplasts was used as the enzyme source. The activation and incubation were performed as described in Methods except that the time of activation with DTT was varied as indicated in this table.

Activation time (min)	ATP-ase activity (μmoles Pi released /mg protein/hr)
0	2.0
10	3.4
30	3.7
60	4.5
120	6.1
180	6.7

distinguish the ATP-ase band on the disc electrophoresis gels from those of other proteins. Karu and Moudrianakis (46) were able to demonstrate ATP-ase activity derived from spinach chloroplasts in gels by using $Pb(NO_3)_2$ in the incubation medium and converting the lead phosphate formed to lead sulfide.

However, in the present investigation it was found that the ATP-ase activity of the EDTA extract of bean chloroplasts was inhibited 50% by 1 mM Pb^{2+}. This finding, together with the observation that Pb^{2+} ions cause nonenzymatic hydrolysis of ATP (105), suggested that the lead method might not be suitable for localization of small amounts of ATP-ase encountered in the EDTA extracts of etioplasts.

A simple staining procedure which utilizes the formation of calcium phosphate precipitate at the site of ATP-ase activity was, therefore, devised. The precipitate formation is brought about by placing the gels after electrophoresis into a solution containing 100 mM TES-NaOH, 5 mM ATP and 50 mM $CaCl_2$ at pH 8. At this high $CaCl_2$ concentration, the phosphate liberated from ATP by the action of the ATP-ase precipitates as a white band of calcium phosphate on the gel. The band appears within 15-60 minutes at room temperature depending on the amount of ATP-ase present. Its density increases with time. Although some fogging of the gels occurs within a few days, the bands are still clearly visible after several months. No precipitate formation takes place if the gels are boiled after electrophoresis, indicating that the staining is the result of enzymatic action.

This method of ATP-ase localization has the advantage that it does not inactivate the enzyme and that it can also be used as a rapid and

simple qualitative test for ATP-ase activity in a test tube. In this case a solution containing the ATP-ase activity is used instead of the gel. Concentration of the reagents is the same as for staining of the gels. An amount of enzyme which liberated 1.1 μmole Pi per hr at 37° caused formation of a visible precipitate after 3 minutes of incubation at room temperature. The amount of precipitate increased with time while no precipitate formed in a control containing boiled enzyme.

2. Similarities between the bean etioplast and chloroplast ATP-ase

Coupling factor 1 of spinach chloroplasts was shown to be required for restoration of photophosphorylation and for light and DTT activated, Mg^{2+}-dependent ATP-ase in partially deficient chloroplast preparations (37, 38). The isolated coupling factor could be converted to a Ca^{2+}-dependent ATP-ase. Bean chloroplasts were found to contain a coupling factor with similar properties (53).

It was of considerable interest to establish whether such a coupling factor was also present in bean etioplasts isolated from dark grown plants. The inducible ATP-ase activity of the coupling factor is simpler to assay than the restoration of photophosphorylation. Moreover the ATP-ase activity in plastid extracts represents enzymic activity of a soluble protein whereas both photophosphorylation and light-induced ATP-ase are activities which probably involve a number of components of the chloroplast membrane including the coupling factor. It follows that the latter activities, being more complex, were less suitable for an initial search for the coupling factor in etioplasts.

Ca^{2+}-dependent ATP-ase in etioplast and chloroplast extracts

Existence of a latent Ca^{2+}-dependent ATP-ase in EDTA extracts of both chloroplasts and etioplasts is demonstrated in Table 9. The properties of the chloroplast and etioplast enzyme are very similar. Both require dithiothreitol for activation and have a higher activity in the presence of Ca^{2+} ions than Mg^{2+} ions. These properties as well as the inactivation of the enzyme by cold treatment are similar to those of the spinach chloroplast ATP-ase (35, 38). The specific activity of the etioplast enzyme based on the amount of soluble protein in the EDTA extracts is about seven times lower than the chloroplast enzyme indicating that the ATP-ase known to be present in chloroplasts is also present in etioplasts, although in a relatively smaller amount.

Because of the differences in the inner structure and overall composition of etioplasts as compared to chloroplasts, it was expected that the optimal EDTA concentration for extracting the Ca^{2+}-dependent etioplast ATP-ase might differ from that of chloroplasts. Figure 4 demonstrates, however, that the optimal EDTA concentration for extracting both the bean chloroplast and etioplast enzymes was 1 mM. This suggests that the mode of binding of the factor by the membrane may be similar in both types of plastids. The optimal EDTA concentration found in these experiments is close to the 0.5-1 mM EDTA required for extraction of the spinach coupling factor (35) while the optimal EDTA concentration required for the enzyme extraction from maize etioplasts and chloroplasts was recently reported to be 0.15 mM (54).

Disc gel electrophoresis of plastid extracts

Further evidence of the similarity of the etioplast enzyme with the

Table 9. Properties of a solubilized chloroplast and etioplast ATP-ase from bean plastids.

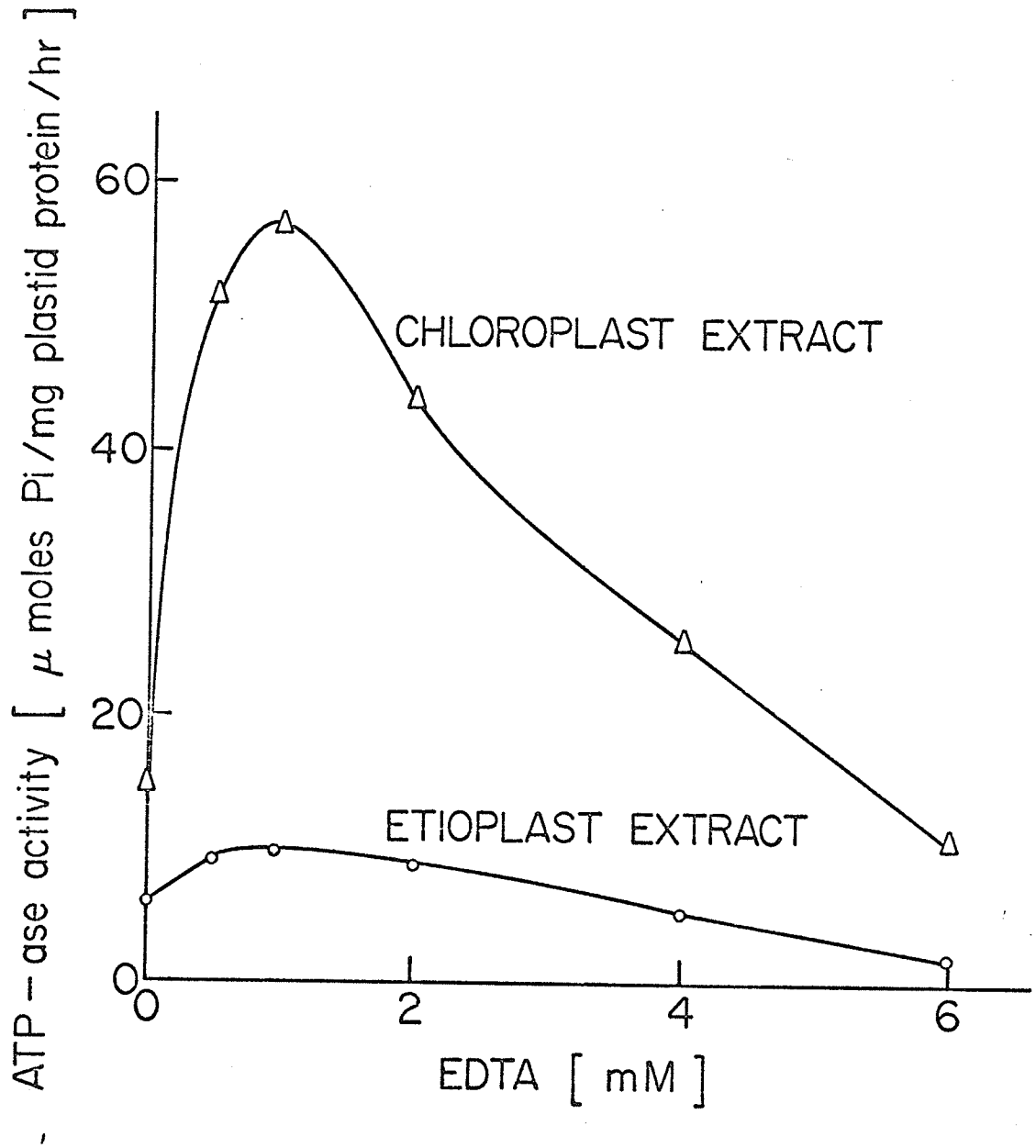
EDTA extracts of plastids were prepared, activated and incubated as described in Methods. Before the activation step with DTT, one part of the plastid extracts was stored at room temperature for 19 hours while another portion was kept for the same time at 2° to measure the cold inactivation of the ATP-ase.

Treatment	Activating ions	ATP-ase activity (μmoles Pi released/mg protein/hr)	
		Chloroplasts	Etioplasts
Room temperature, No DTT	Ca^{2+}	4.4	0.61
Room temperature, DTT	-	2.5	0.34
Room temperature, DTT	Mg^{2+}	19.4	2.4
Room temperature, DTT	Ca^{2+}	39.2	5.8
2°, DTT	Ca^{2+}	7.6	1.1

Figure 4

Effect of EDTA concentration upon the extraction of ATP-ase from bean plastids.

Etioplasts containing 472 µg protein were extracted with 2 ml of an EDTA solution (pH 8) of varying concentration. The ATP-ase was measured in the extracts after DTT activation as described in Methods. Chloroplasts containing 310 µg protein were used for extraction with 4 ml of EDTA solution.


ATP-ase activity [μ moles Pi/mg plastid protein/hr]
60
40
20
0
0
2
4
6
CHLOROPLAST EXTRACT
ETIOPLAST EXTRACT
EDTA [mM]

chloroplast enzyme is given by the results of disc gel electrophoresis shown in Fig. 5. The etioplast enzyme has an identical R_f to the chloroplast enzyme as can be seen from the gels stained for ATP-ase activity. The amount of ATP-ase in etioplast extracts, however, is several times lower than that in chloroplast extracts as can be seen from both the staining patterns for ATP-ase and protein as well as from the specific activity of the extracts.

The ATP-ase band on the gels was identified by the specific staining for this activity. In addition to the ATP-ase and BSA which is the fast moving band visible close to the front, a third strong protein band that migrated more slowly than the ATP-ase was found in the gels after electrophoresis of EDTA extracts of plastids (Fig. 5). A similar extraction pattern was observed by Howell and Moudrianakis (45) with EDTA extracts of spinach chloroplasts. They identified the slowly moving band as belonging to ribulose diphosphate carboxylase and observed that this enzyme was released upon hypotonic rupture of chloroplasts with H_2O while the ATP-ase was released by the subsequent EDTA wash.

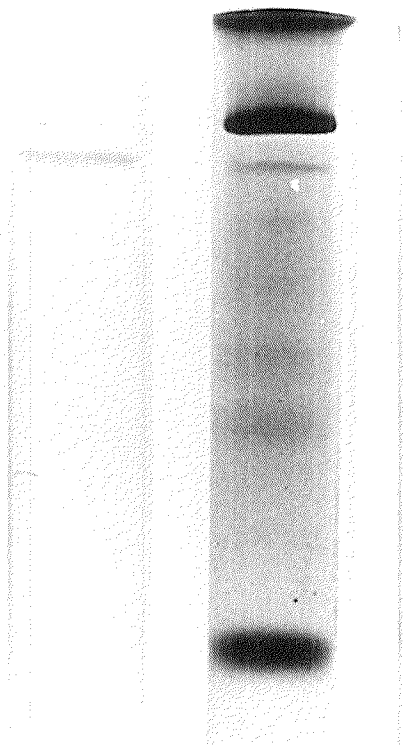
Results of similar experiments carried out with bean chloroplasts and etioplasts are reported in Fig. 6 which shows extraction patterns obtained when plastids isolated in a hypertonic isolation medium are exposed to H_2O or 1 mM EDTA pH 8. As with the spinach chloroplasts (45), hypotonic rupture of bean chloroplasts by H_2O removes a protein which migrates slowly upon disc gel electrophoresis. Direct extraction of the chloroplasts with 1 mM EDTA causes release of an additional protein species which migrates somewhat faster upon disc gel electro-

Figure 5

ATP-ase and protein patterns obtained upon disc gel electrophoresis of EDTA extracts of bean plastids.

Concentrated EDTA extract of etioplasts (117 µg protein/0.1 ml) and a concentrated EDTA extract of chloroplasts (150 µg protein/0.1 ml) were applied on each gel and after electrophoresis stained for ATP-ase and protein as described in Methods. The fast moving protein band is BSA from the homogenization medium. The figures indicate specific activity of the ATP-ase in EDTA extracts in µmoles Pi released/mg soluble protein/hr. The origin (cathode) is at the top of the gels.

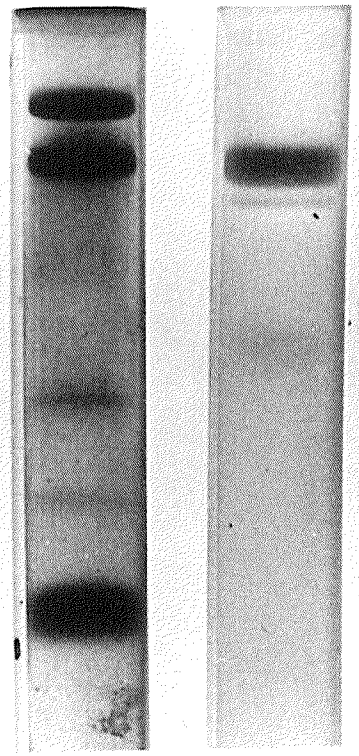
ETIOPLAST



ATPase Protein

11.5

CHLOROPLAST



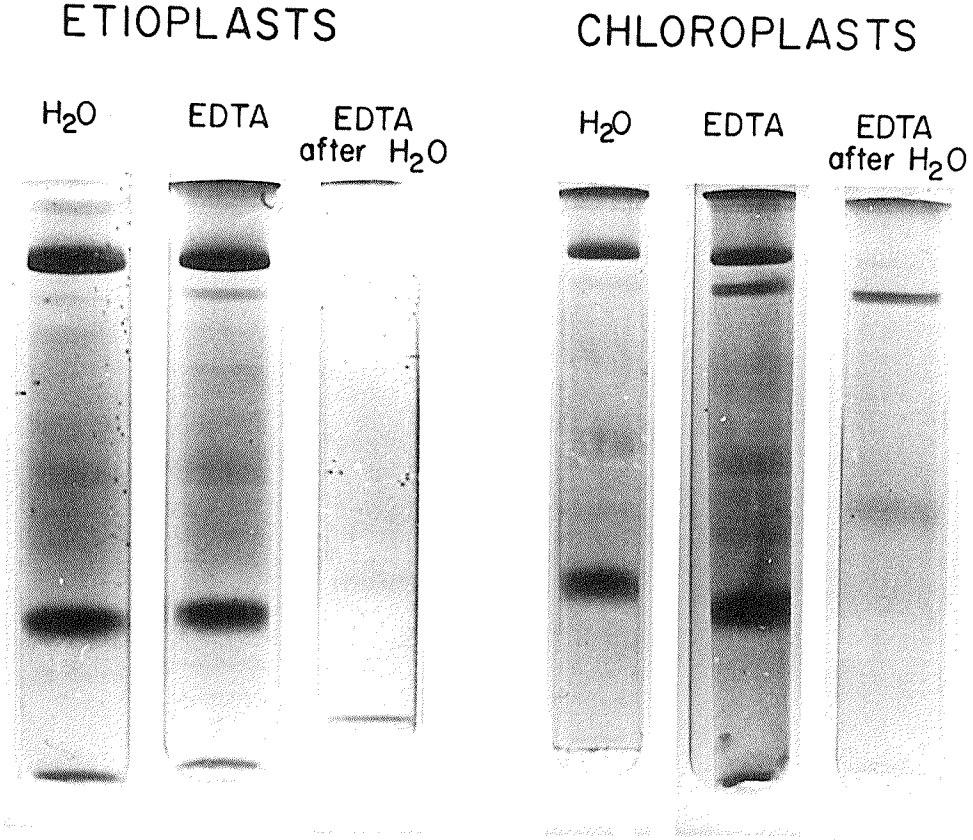
Protein ATPase

49

Figure 6

Disc gel electrophoresis patterns, RuDP carboxylase and ATP-ase activities of bean plastid extracts.

Plastids prepared from 6 g of etiolated and 2 g of green leaves were extracted with 30 ml H_2O or EDTA and the extracts, after removal of membranous material by centrifugation, were concentrated 10-fold as described in Methods. The concentrated extracts (0.1 ml) were applied to the gels and after electrophoresis the gels were stained for protein. The ATP-ase was determined using concentrated extracts while the RuDP carboxylase was measured in a separate experiment using freshly prepared plastid extracts. ATP-ase activity is expressed in μmoles Pi released/mg protein/hr and RuDP carboxylase activity in μmoles CO_2 fixed/mg protein/hr.



RuDP carboxylase :	1.8	–	0.2	2.0	–	0.1
ATP-ase :	2	12	22	7	49	68

phoresis in a position identical with the ATP-ase band (see also Fig. 5). When plastid membranes subjected to H_2O extraction are subsequently extracted with EDTA, only the latter protein band is released. A similar extraction pattern was also found in the case of etioplasts except that the ATP-ase band was weaker than in chloroplast extracts.

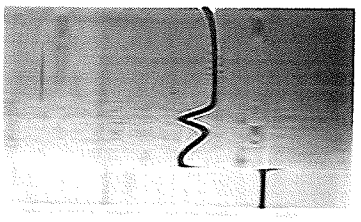
The specific activities of the ATP-ase and ribulose diphosphate carboxylase were measured in all these extracts. The results are indicated in Fig. 6 together with the corresponding disc gel electrophoresis patterns. The appearance of the slow moving electrophoretic band is associated with the appearance of increased ribulose diphosphate carboxylase activity while the faster moving band is associated with increased ATP-ase activity.

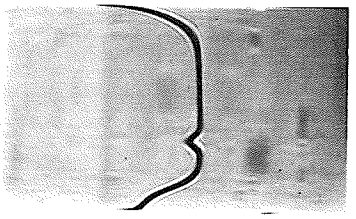
Analytical ultracentrifugation

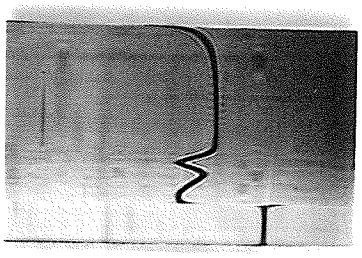
The conclusion that ribulose diphosphate carboxylase activity is associated with the slow moving electrophoretic band while the faster one possesses ATP-ase activity is further supported by the observation that the H_2O extract of bean chloroplasts contained a protein species which sedimented with an S value of 16.8 upon analytical ultracentrifugation while the EDTA extract of H_2O washed chloroplast membranes contained a protein with an S value of 12.1 (Fig. 7). The former value is close to the S values of 16.2 to 19.5 reported for ribulose diphosphate carboxylase from various sources (106) while the latter approaches the S values of 12.7 (45) and 13.8 (50) reported for the spinach chloroplast ATP-ase.

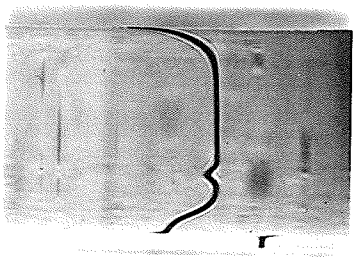
Figure 7

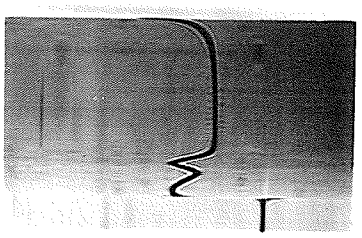
Analytical ultracentrifuge patterns of bean plastid extracts. The upper photograph shows the sedimentation pattern of a concentrated (3.3 mg protein/ml) EDTA extract of H_2O washed chloroplasts. The lower photograph shows the sedimentation pattern of a concentrated (2.6 mg protein/ml) H_2O extract of chloroplasts. The pictures were taken at 4 minute intervals at 48,000 r.p.m. using a Beckman Model E analytical ultracentrifuge. To obtain a sufficiently high protein concentration, the plastid extracts were concentrated about 50-fold by blowing air over dialysis tubing before the ultracentrifuge runs.

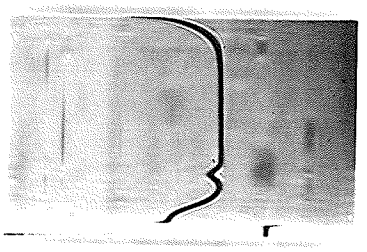


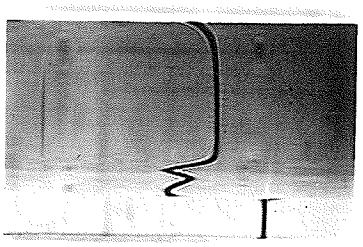


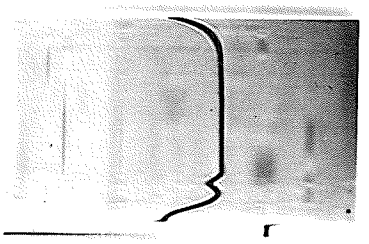


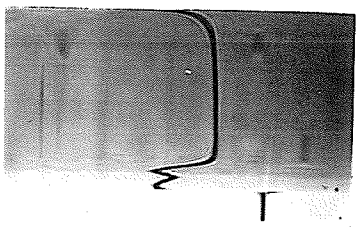


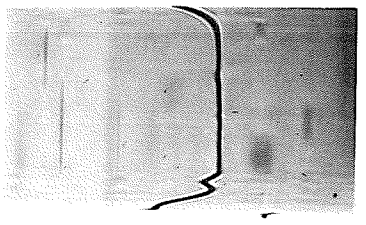












3. Identity of the ATP-ase with the modified coupling factor

In the previous section, it was demonstrated that EDTA extracts of bean etioplasts and chloroplasts contained an ATP-ase with properties similar to the ATP-ase of the spinach chloroplast coupling factor. Attempts were made to demonstrate that the bean plastid ATP-ase represented a modified coupling factor for photophosphorylation. The results of these experiments are reported in this section.

Light and DTT activated, Mg^{2+}-dependent ATP-ase in plastids

The results of Table 10 demonstrate that bean chloroplasts contain a light and DTT activated, Mg^{2+}-dependent ATP-ase of the type described in spinach chloroplasts (38) indicating that they probably contain the coupling factor which was shown to be required for this reaction by McCarty and Racker (38). No comparable ATP-ase could be demonstrated in bean etioplasts incubated under the same conditions. This result does not mean, however, that the coupling factor is not present in bean etioplasts. The membrane to which the enzyme is bound in etioplasts is probably different from that of chloroplasts and does not respond to illumination in the same way since it lacks chlorophyll and possibly other components as well. This might prevent light activation of the ATP-ase. The fact that the ATP-ase activated upon 2 hours of incubation with 50 mM DTT was found in EDTA extracts of etioplasts (Table 9) together with the finding that incubation of etioplasts directly with 50 mM DTT for 2 hours in the dark (without EDTA extraction) caused activation of an ATP-ase (Table 11) indicated that the coupling factor probably was present in etioplasts although in a smaller amount than in chloroplasts. Chloroplasts catalyzed hydrolysis of ATP at a rate

Table 10. Light and DTT activation of the Mg^{2+}-dependent ATP-ase of bean plastids.

Assay conditions are described in Methods.

Conditions of activation	Dark		Light	
	-DTT	+DTT	-DTT	+DTT
ATP-ase activity in chloroplasts (μmoles Pi released/mg chlorophyll/hr)	52	63	66	162
ATP-ase activity in etioplasts (μmoles Pi released/mg protein/hr)	0.6	0.6	0.8	0.5

Table 11. DTT activation of an ATP-ase in bean etioplasts. Etioplasts containing 200 µg of protein were incubated in the dark for 2 hours with or without 50 µmoles DTT in a volume of 0.75 ml at pH 8 and room temperature. This activation was followed by addition of 50 µmoles TES-NaOH, 5 µmoles ATP and 5 µmoles $CaCl_2$ or $MgCl_2$ as indicated in a volume of 0.25 ml at pH 8. The test tubes were incubated for 30 minutes at 37° in the dark. Deproteination and Pi estimation were performed as described in Methods for the light and DTT activated, Mg^{2+}-dependent ATP-ase.

Conditions for activation	Activating ion	ATP-ase activity (µmoles Pi released/mg protein/hr)
No DTT	Ca^{2+}	1.4
No DTT	Mg^{2+}	0.8
DTT	Ca^{2+}	9.2
DTT	Mg^{2+}	9.9

of 244 μmoles Pi released/mg chlorophyll/hr when activated with DTT and assayed in the presence of Ca^{2+} ions as described in Table 11.

Dio-9 inhibition

Dio-9 is known to be both an inhibitor of photophosphorylation (107) and an inhibitor of the solubilized spinach chloroplast ATP-ase (38, 107). Dio-9 caused strong inhibition of photophosphorylation of bean chloroplasts and also inhibited the ATP-ase solubilized from them by EDTA (Table 12). The ATP-ase of etioplast extracts was also inhibited by Dio-9 and its inhibition increased with increasing Dio-9 concentration. These data provide additional evidence that the ATP-ases derived from the two types of plastids are similar. In addition, the data also suggest that the ATP-ase is a modified coupling factor for photophosphorylation.

Restoration of photophosphorylation by plastid extracts

Evidence for the presence of coupling factor activity in extracts of both types of plastids, chloroplasts and etioplasts, is presented in Table 13. Extraction of chloroplasts with a solution containing 10 mM NaCl, 0.2% BSA and 1 mM EDTA at pH 8 (NBE solution) yielded chloroplast residues with low rates of photophosphorylation. Photophosphorylation was stimulated over three fold upon addition of an NBE extract of etioplasts and over six fold upon readdition of an NBE extract of chloroplasts (Experiment 1) indicating that both extracts contained coupling factor activity. It is evident from Experiment 2 in Table 13 that the chloroplast coupling factor is heat labile and therefore probably proteinaceous.

Although the use of the NBE solution for the extraction of the coupling factor was necessary to prevent irreversible loss of photophosphorylation activity in the chloroplast residue (as discussed in

Table 12. Inhibition of photophosphorylation and ATP-ase activity by Dio-9.

Photophosphorylation was measured at 20° using bean chloroplasts containing 34 µg chlorophyll. ATP-ase activity was measured in EDTA extracts of chloroplasts after DTT activation using 20 µg protein per test tube. To measure the inhibition of the ATP-ase of the etioplast extract, 26 µg protein were used.

Dio-9 (µg/ml)	ATP formation (µmoles/mg chlorophyll/hr)	ATP-ase activity (µmoles Pi released/mg protein/hr)	
		Chloroplasts	Etioplasts
0	270	92	12.6
4.6	-	-	11.6 (8)
7.0	30 (89)[1]	53 (42)	-
9.2	-	-	7.9 (37)
14.0	-	-	4.8 (62)

[1] Figures in brackets give % inhibition.

Table 13. Restoration of photophosphorylation in bean chloroplast residues by NBE extracts of plastids.

Experimental conditions are described in Methods. Incubation was performed at 20°. In Experiment 1 the NBE extract of chloroplasts was prepared by extracting plastids containing 230 µg of chlorophyll with 14 ml of NBE. Etioplasts containing 3.25 mg of protein were extracted with 4 ml of NBE solution to obtain the etioplast extract. A chloroplast residue containing 23 µg of chlorophyll per test tube was used for the restoration experiment. In Experiment 2, chloroplasts containing 360 µg of chlorophyll were extracted with 12 ml of NBE solution and part of the extract was heated for 10 minutes on a boiling water bath. Chloroplast residue used in this experiment contained 36 µg of chlorophyll per test tube.

Component	ATP formation (µmoles /mg chlorophyll/hr)
Experiment 1	
Chloroplast residue	7
Chloroplast residue + etioplast extract	25
Chloroplast residue + chloroplast extract	45
Experiment 2	
Chloroplast residue	14
Chloroplast residue + boiled chloroplast extract	17
Chloroplast residue + chloroplast extract	48

section II, 1, Restoration of photophosphorylation), it was found in the course of the experiments that a simple EDTA extract (1 mM EDTA pH 8) could be used for the restoration of photophosphorylation in a chloroplast residue prepared by NBE extraction. This finding enabled the measurement of coupling factor activity in H_2O and EDTA extracts of plastids. This activity could then be correlated with ATP-ase activity as well as with disc gel electrophoresis patterns of these extracts. The results shown in Table 14 demonstrate the restoration of photophosphorylation in chloroplast residues after NBE extraction by chloroplast and etioplast extracts. Addition of a H_2O extract of chloroplasts stimulated photophosphorylation of the chloroplast residue to some extent. However, EDTA extracts of water-washed chloroplasts gave a far greater stimulation indicating that most of the coupling factor activity is released from the plastid membranes by the EDTA wash. A similar although not as marked pattern was found with the etioplast extracts indicating that at least part of the coupling factor is also membrane-bound in etioplasts. A boiled EDTA extract of etioplasts did not stimulate photophosphorylation in chloroplast residues, suggesting the protein nature of the coupling factor activity.

Comparing the results in Table 14 with those given in Fig. 6, it is apparent that the EDTA extracts of both chloroplasts and etioplasts have a higher coupling factor activity as well as a higher latent ATP-ase activity than the H_2O extracts. This observation together with the inhibition of both types of activities by Dio-9 suggest that the ATP-ase may be identical to a modified coupling factor for photo-phosphorylation.

Further evidence that bean etioplasts and chloroplasts contain a

Table 14. Restoration of photophosphorylation in bean chloroplast residues by H_2O and EDTA extracts of plastids. Chloroplasts isolated from 1.5 g of green leaves were extracted with 20 ml H_2O and subsequently with 20 ml 1 mM EDTA pH 8. Etioplasts isolated from 6 g of etiolated leaves were extracted with 8 ml of each solution. After addition of 0.1 ml of a solution containing 120 mM NaCl and 2.4% BSA to 1.1 ml of the extract, the extracts were combined with a chloroplast residue obtained by NBE extraction as described in Methods. The chloroplast residue contained 37 µg chlorophyll per tube of incubation in Experiment 1 and 34 µg chlorophyll in Experiment 2. Photophosphorylation was measured at 5°. At this temperature the chloroplasts used in Experiment 1 formed 79.2 µmoles ATP/mg chlorophyll/hr before NBE extraction.

Additions to chloroplast residue	ATP formation (µmoles/mg chlorophyll/hr)
Experiment 1	
None	9.4
H_2O extract of chloroplasts	14.6
EDTA extract of H_2O washed chloroplasts	36.0
Experiment 2	
None	5.3
H_2O extract of etioplasts	9.2
EDTA extract of H_2O washed etioplasts	13.4
Boiled EDTA extract of etioplasts	5.6

coupling factor similar to the spinach chloroplast coupling factor 1 is provided by the work of P. Silvanovich in this laboratory who has observed that the antibody against spinach chloroplast coupling factor 1 caused formation of lines of identity with EDTA extracts of bean etioplasts and chloroplasts upon immunodiffusion (personal communication).

Restoration of photophosphorylation by extracts of disc gel electrophoresis zones containing ATP-ase activity

The preceding results strongly suggest the identity of the plastid ATP-ases with a coupling factor for photophosphorylation. However, final proof of this identity can be given only by demonstrating that a single protein species can catalyze both ATP-ase and coupling factor activities.

Since disc gel electrophoresis of plastid extracts resulted in separation of the ATP-ase band from other proteins, it was assumed that the portion of the gel containing ATP-ase activity would represent a partially purified enzyme preparation. Extraction of the gel zones containing ATP-ase activity could provide extracts which should be active in restoration of photophosphorylation in partially deficient chloroplast residues. It can be seen from Table 15, however, that rather little restoration of photophosphorylation was obtained when the chloroplast residues after NBE extraction were combined with various amounts of the extract of disc gel electrophoresis zones containing ATP-ase activity. This result was not caused by lack of extraction of the enzyme from the gels, since the gel extract contained 410 µg protein per ml and considerable amounts of ATP-ase activity as judged by the qualitative test described earlier. The lack of coupling

Table 15. Restoration of photophosphorylation in chloroplast residues by extracts of disc gel electrophoresis zones containing ATP-ase activity.

Conditions of the experiment are described in Methods. Incubation was performed at 5°.

Addition to chloroplast residue	ATP formation (μmoles/mg chlorophyll/hr)
None	2.5
5 μl of gel extract	2.9
10 μl of gel extract	2.9
25 μl of gel extract	3.7
50 μl of gel extract	4.6
100 μl of gel extract	3.7
200 μl of gel extract	3.7
1.2 ml of an NBE extract of chloroplasts	11.2

factor activity in the gel extract containing ATP-ase activity could be due to heat development in the gels during electrophoresis since Farron and Racker (51) have found the spinach chloroplast coupling factor to be very sensitive to heat treatment. Although it is not very probable in view of the other results, there is a possibility that the coupling factor and the ATP-ase are not identical. The final proof of the identity of the two proteins will require purification of the ATP-ase by other methods followed by a demonstration that the purified enzyme possesses coupling factor activity.

4. Biosynthesis of the latent plastid ATP-ase in beans

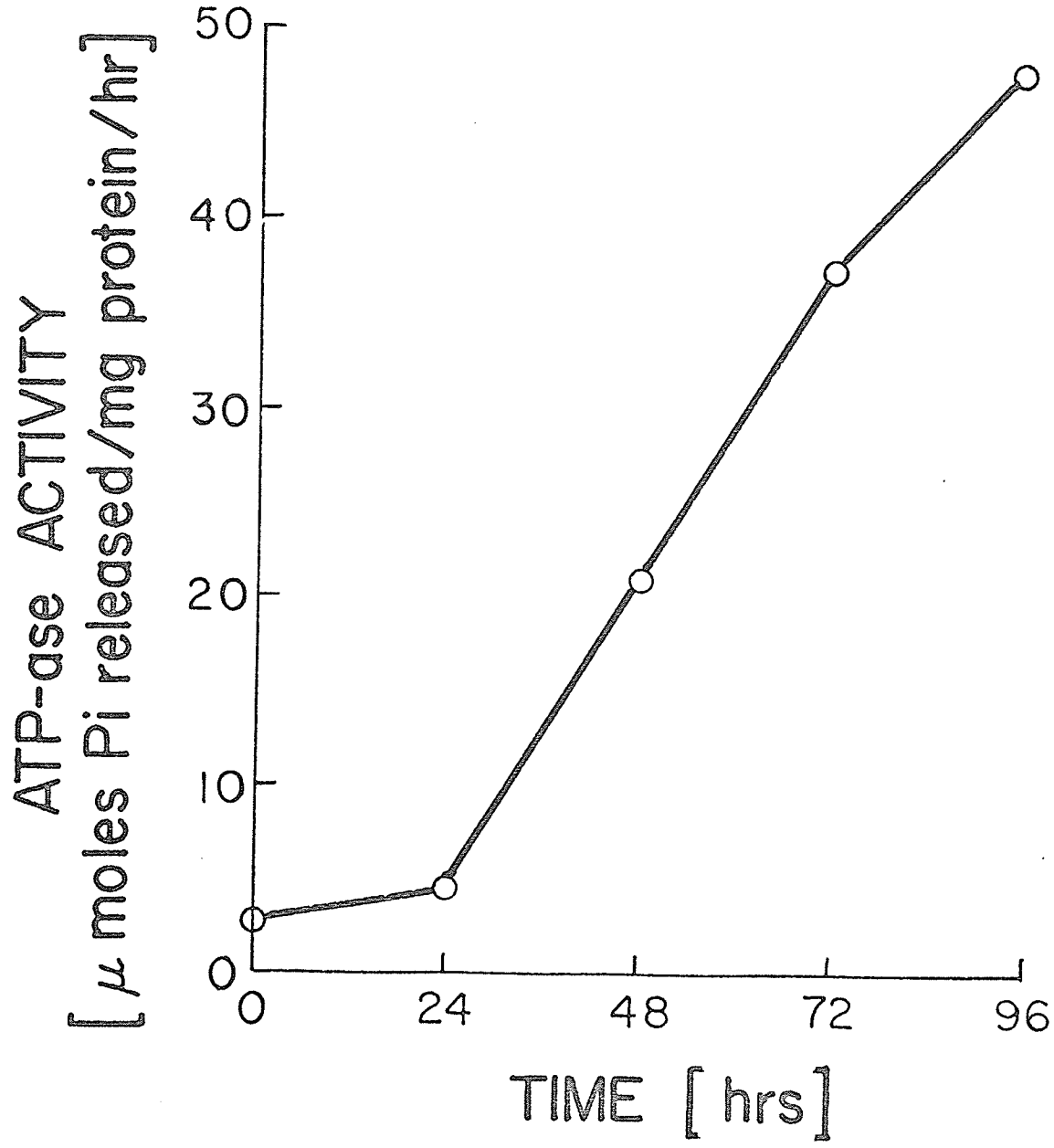
Increase in latent ATP-ase activity upon greening

The preceding data indicated that although the ATP-ase was present in etioplasts, the level of activity was considerably lower than that present in chloroplasts. As is evident from Fig. 8 the specific activity of the ATP-ase in plastids increases considerably during greening. There is an approximately 10-fold increase in specific activity of the ATP-ase during the 96 hours of illumination of dark grown plants. In contrast, Lockshin *et al.* (54) have suggested that most if not all of the ATP-ase/coupling factor activity of maize chloroplasts may be present in etioplasts. This is based mainly on the lack of change of ATP-ase activity with respect to membrane protein during greening.

In the present work the specific activity of the ATP-ase measured in EDTA extracts of plastids is expressed on a per mg of soluble protein basis. As the amount of protein increases upon greening of etiolated

Figure 8

Time course of the increase in specific activity of ATP-ase upon greening of bean plants. Etiolated plants were greened, plastids isolated and ATP-ase activity in the EDTA extracts of plastids measured as described in Methods.


ATP-ase ACTIVITY
[μ moles Pi released/mg protein/hr]
50
40
30
20
10
0
0
24
48
72
96
TIME [hrs]

plants it was suggested that a better quantitative expression of the increase of plastid enzymes upon greening is obtained by expressing the activity on a per leaf basis (91). However, the yield of chloroplasts varied from one homogenization to the other and therefore the expression of ATP-ase activity on a per leaf basis would not have been accurate in this work. The expression of the activity on a per plastid basis would be ideal but the counting of plastids in a hemocytometer was not accurate. It was therefore concluded that the changes in ATP-ase activity during greening are best expressed on a per mg of soluble protein basis although the actual increase in the amount of ATP-ase activity during greening is probably larger due to the increase in the amount of total soluble plastid protein.

Effect of chloramphenicol and cycloheximide on ATP-ase activity in plastid extracts

To determine the cellular site of the ATP-ase synthesis during greening D-threo chloramphenicol, a specific inhibitor of protein synthesis on the 70S ribosomes of chloroplasts (80), and cycloheximide, a specific inhibitor of protein synthesis on the 80S ribosomes of the cytoplasm (84), were used. These antibiotics sometimes affect not only protein synthesis at the ribosomal level but also the energy metabolism of the cells, causing secondary inhibition of protein synthesis by limiting the energy supply (80). To be sure that the effect of the inhibitors was at the ribosomal level and not on cyclic photophosphorylation -- which has been shown to be the energy source for chloroplast protein synthesis (108) -- or on the ATP-ase assay itself, the effect of chloramphenicol and cycloheximide on these two activities was measured.

It can be seen from Table 16 that neither of these activities was significantly inhibited at 10 µg of chloramphenicol or cycloheximide per ml. Slight inhibition of photophosphorylation was observed at 100 µg of chloramphenicol per ml.

Inhibition of the light induced increase in the specific activity of the ATP-ase of greening plastids as a function of chloramphenicol (CAP) and cycloheximide (CHI) concentration is shown in Fig. 9. About 50% inhibition was reached at 2 µg CAP/ml and 1 µg CHI/ml. The strong inhibition of the light induced increase in the specific activity of the ATP-ase by both inhibitors indicated that both the chloroplastic and the cytoplasmic ribosomal systems were required for the formation of the plastid bound ATP-ase.

Further proof that chloramphenicol inhibited ATP-ase synthesis by inhibiting protein synthesis at the ribosomal level and not by inhibiting the energy supply for the synthesis comes from experiments with D- and L-threo chloramphenicol. Like the D-threo isomer, L-threo CAP is known to inhibit some energy dependent processes of higher plants such as ion uptake and oxidative phosphorylation but contrary to D-threo CAP, the L-threo isomer does not inhibit protein synthesis at the ribosomal level (80).

Table 17 shows that while D-threo CAP completely abolished the light induced increase in specific activity of the ATP-ase upon greening at 10 µg/ml, L-threo CAP had little effect on the increase even at 100 µg/ml. From these results it was concluded that D-threo CAP inhibited ATP-ase synthesis at the ribosomal level.

Disc gel electrophoresis of postribosomal supernatants

Postribosomal superantants of both dark and light grown plants

Table 16. Effect of D-threo chloramphenicol and cycloheximide on photophosphorylation and ATP-ase activity *in vitro*. Antibiotics were added to the incubation mixtures in amounts specified in the table. Photophosphorylation was measured at 20° using bean chloroplasts containing 17 μg chlorophyll per test tube. The DTT activated EDTA extract of chloroplasts used for the ATP-ase assay contained 67 μg soluble protein.

Antibiotic (μg/ml)	ATP formation (μmoles/mg chlorophyll/hr)	ATP-ase (μmoles Pi released /mg protein/hr)
D-threo CAP		
0	498	39.5
10	475	40
100	418	40
CHI		
0	498	37.1
1	519	37.5
10	492	37.1

Figure 9

Inhibition of ATP-ase formation during greening of bean plants as a function of CAP and CHI concentration.

Detached etiolated bean shoots were floated and illuminated on the inhibitor solutions as described in Methods. The graphs indicate percent inhibition of the light induced increase in specific activity of the ATP-ase in EDTA extracts of plastids.

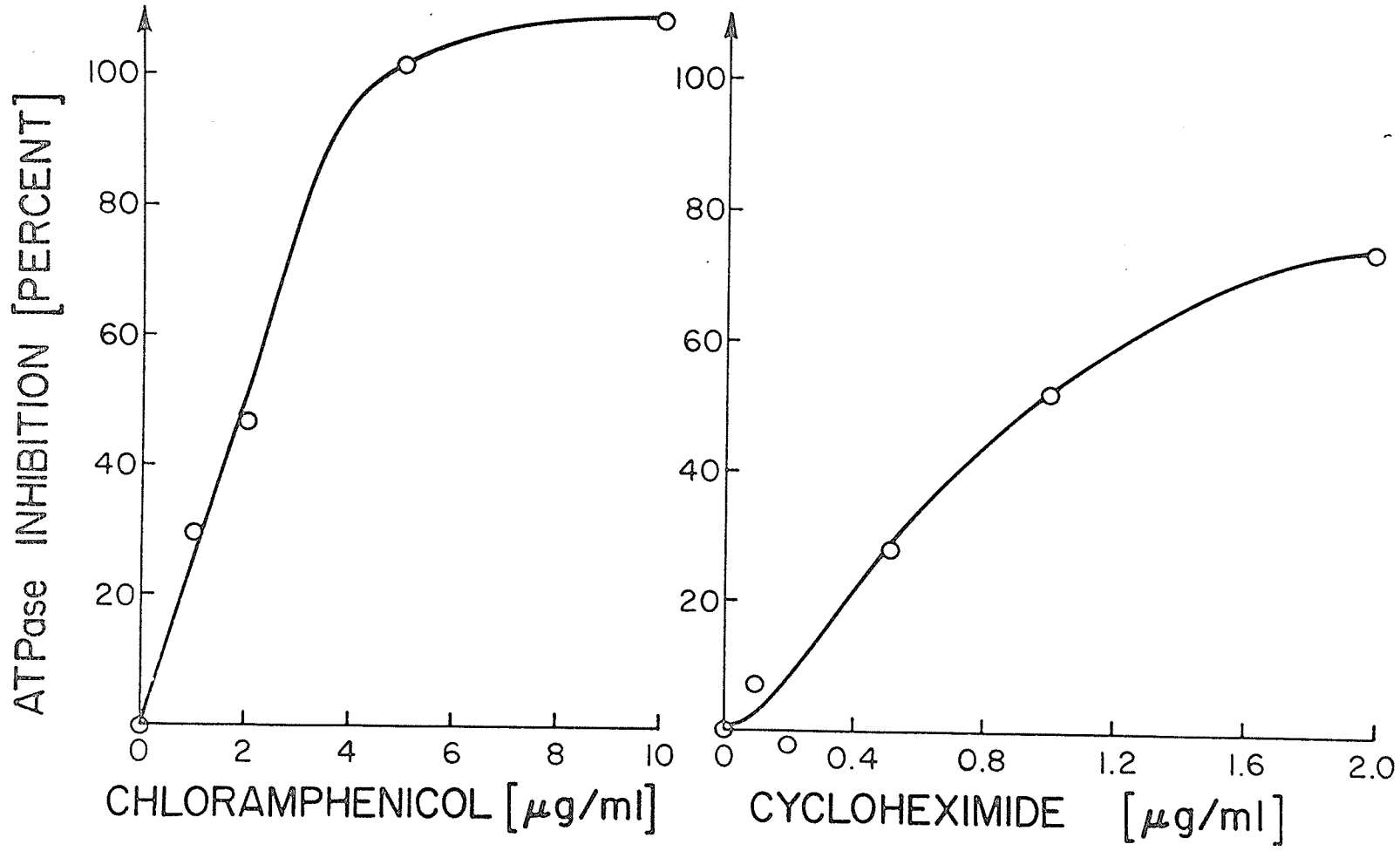

ATPase INHIBITION [PERCENT]
100
80
60
40
20
0
2
4
6
8
10
CHLORAMPHENICOL [μg/ml]
100
80
60
40
20
0
0.4
0.8
1.2
1.6
2.0
CYCLOHEXIMIDE [μg/ml]

Table 17. Effect of D- and L-threo CAP on the light induced increase in the specific activity of the ATP-ase of greening bean plastids.

Excised etiolated plants were greened for 72 hours on 20 ml of inhibitor solution as described in Methods and the ATP-ase activity was measured in EDTA extracts of plastids isolated from the treated leaves.

Treatment	ATP-ase activity (μmoles Pi released /mg protein/hr)
Dark grown	6.1
72 hrs light; H_2O	31.2
" " 10 μg D-CAP/ml	4.3
" " 100 μg D-CAP/ml	2.8
" " 10 μg L-CAP/ml	29.5
" " 100 μg L-CAP/ml	27.0

were found to contain small amounts of an ATP-ase which migrated on disc gel electrophoresis with an identical R_f value to the plastid ATP-ase. This ATP-ase could have been released from the plastids during homogenization of the leaves or it could have been a product of the cytoplasmic ribosomal system. If the latter were true, one should find accumulation of the ATP-ase in the postribosomal supernatant upon chloramphenicol treatment of the plants as is the case in the mitochondrial ATP-ase of yeast (109). Fig. 10 shows, however, that no substantial accumulation of the enzyme in the postribosomal supernatant occurred either in the presence of chloramphenicol or cycloheximide during the greening of etiolated bean plants.

Since the ATP-ase level in plastids and in the postribosomal supernatant does not increase upon greening in the presence of either of the two inhibitors of protein synthesis, the function of both ribosomal systems must be required for the ATP-ase formation.

Sequential exposure of greening plants to chloramphenicol and cycloheximide

To determine whether one ribosomal system forms a component which would enable formation of the ATP-ase by the other ribosomal system, plants were greened for 24 hours on H_2O or CAP, then transferred to a solution containing CHI and further illuminated. It can be seen from Fig. 11 that a transfer of the plants from H_2O to CHI results in severe inhibition of the ATP-ase formation as compared to the control plants left on H_2O. However, transfer of the plants that were first greened on CAP to a solution containing CHI results in no CHI inhibition initially when compared to control plants transferred from CAP to H_2O. This reversal of the CHI inhibition by CAP pretreatment might mean

Figure 10

Disc gel electrophoresis of postribosomal supernatants isolated from bean plants treated with CAP or CHI. Postribosomal supernatants were prepared from dark grown plants (D), plants greened for 96 hours on H_2O (L), 10 μg CAP/ml (CAP) or 10 μg CHI/ml (CHI) by homogenizing 50 leaves after each treatment in 10 ml of homogenizing medium and centrifuging for 1 hour at 150,000 g at 20° to remove particulate material. Disc gel electrophoresis was performed on 50 μl of the supernatant. The ATP-ase band is indicated by the arrow.

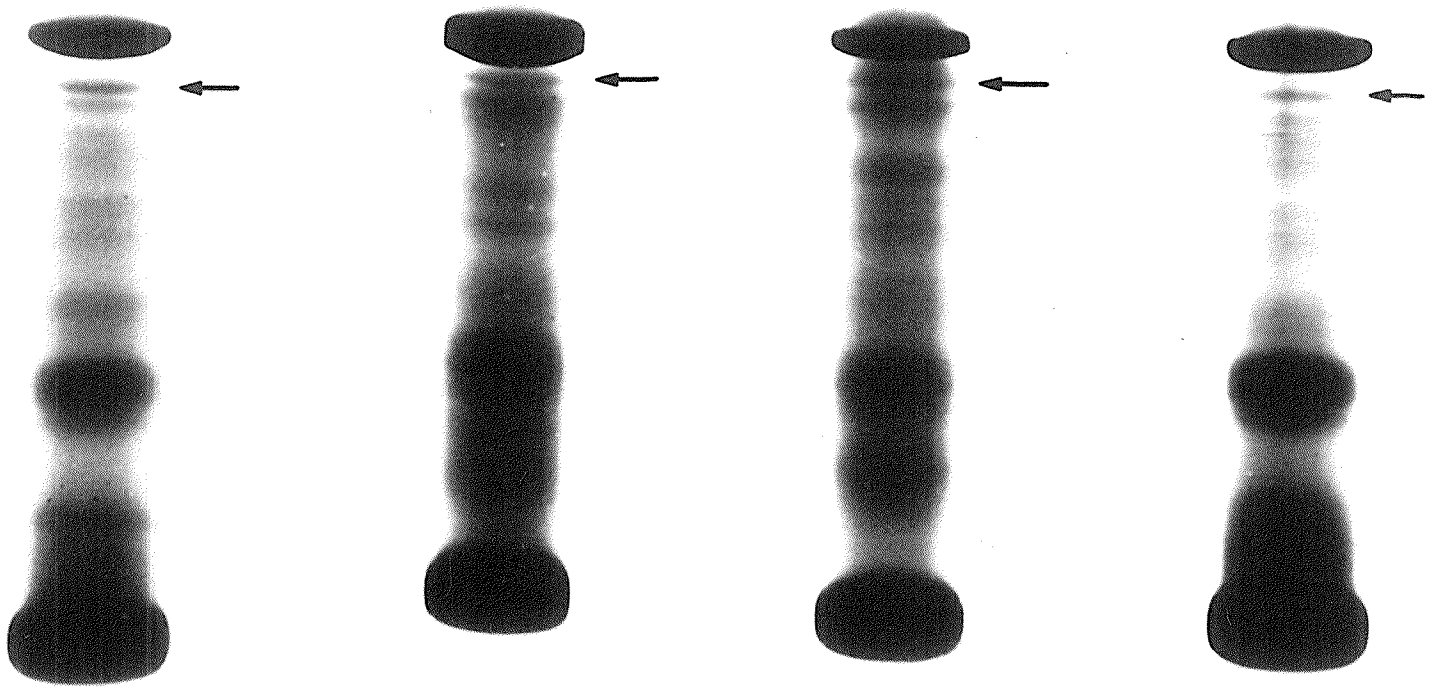
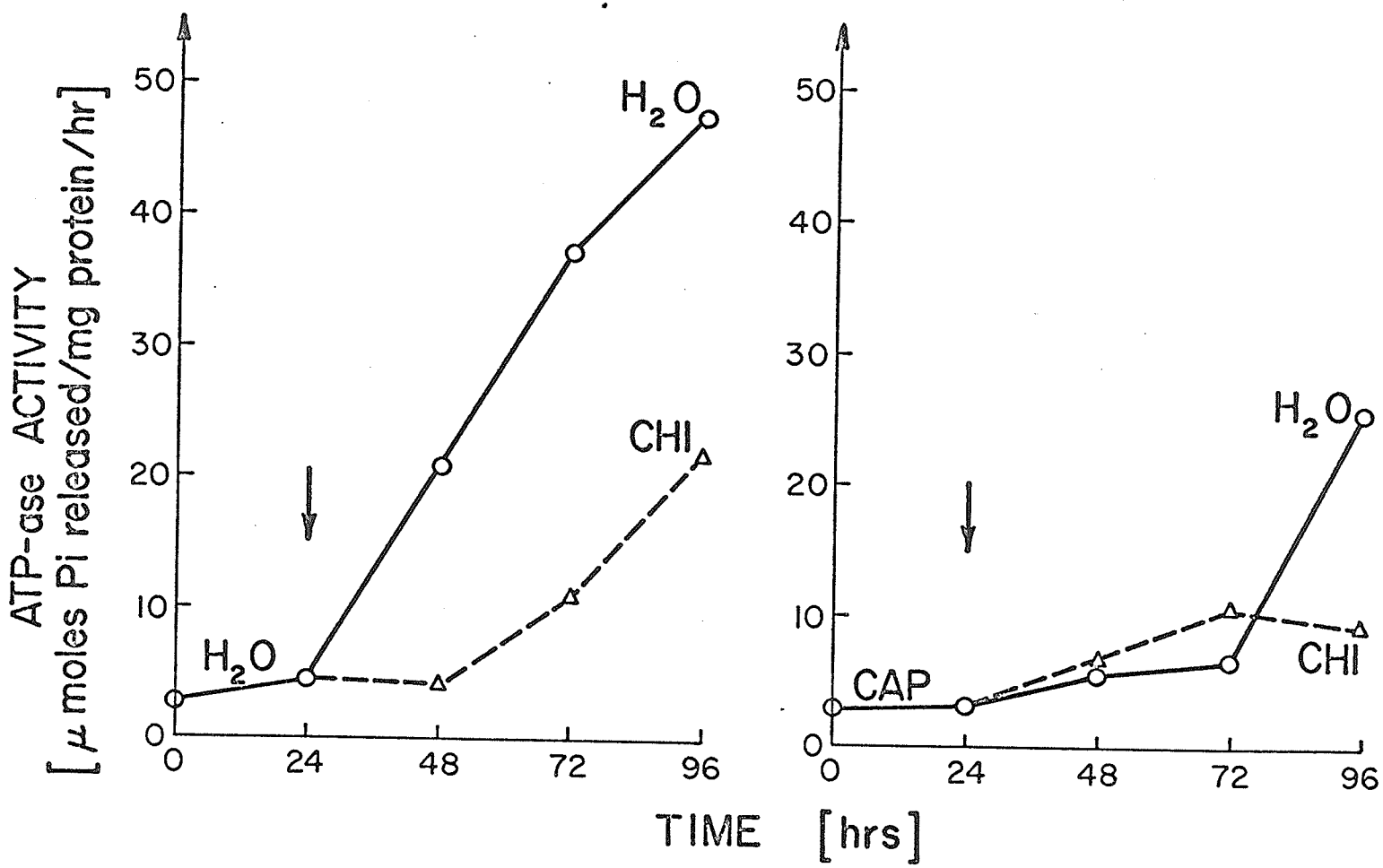

D
L
CAP
CHI

Figure 11

The effect on the ATP-ase activity in plastids of sequential exposure of greening plants to CAP and CHI.

Etiolated bean plants were greened for 24 hours on H_2O or 5 µg CAP/ml as described in Methods. After 24 hours of illumination the plants of both treatments were transferred to H_2O or 5 µg CHI/ml and illuminated further. ATP-ase activity was measured in EDTA extracts of plastids isolated at 24 hour intervals.


ATP-ase ACTIVITY
[μ moles Pi released/mg protein/hr]
50
40
30
20
10
0
0
24
48
72
96
H2O
CHI
H2O
CAP
CHI
TIME [hrs]

that a product of the cytoplasmic system accumulates in the presence of CAP. After transfer to CHI and partial removal of CAP this product could stimulate the formation of the ATP-ase by the chloroplast system.

Unfortunately the removal of CAP upon transfer of the plants to a CAP-free medium is slow in bean plants as is evident from the CAP to H_2O transfer in Fig. 11. Consequently the increase in ATP-ase activity after the transfer to the CAP-free medium is small.

GENERAL DISCUSSION

Spinach chloroplasts are known to contain a coupling factor for photophosphorylation (36, 37). This protein is required for coupling of ATP formation to light-driven electron flow. The coupling factor does not seem to be peculiar to spinach chloroplasts. It is also found in other higher plant chloroplasts such as bean (53, 110, 111); maize (54), and pea[1]; in the algae chloroplasts of Euglena gracilis (55) and Anabaena variabilis (56), as well as in photosynthetic bacteria (57, 58). The presence of a coupling factor with an identical function and some similar properties in such a variety of photosynthetic organisms suggests that at least some of the steps in the photosynthetic conversion of solar energy to chemical energy are probably similar in all these organisms. The possible universal presence of the coupling factor in photosynthetic organisms points out the importance of this protein.

The finding of the coupling factor in plastids of dark grown plants (54, 110, 111) is a rather interesting observation. In etioplasts the enzyme cannot carry out the function for which it seems to be designed. It cannot couple ATP formation to light-driven electron transport, as the latter does not take place in etioplasts due to the lack of chlorophyll. It is possible that the enzyme has in addition to its coupling factor activity other yet unrecognized physiological roles for which it may be required in the etiolated plant. Alternatively, the etiolated plant may not be able to eliminate the synthesis of proteins that are essential under normal growth conditions (i.e. in green plants), as other photosynthetic enzymes (e.g. enzymes of

[1]This thesis

CO_2 fixation cycle) are also found in etioplasts (76).

The finding of the coupling factor in etioplasts eliminates the possibility that it would be the factor limiting photophosphorylation in etiolated plants. Comparing the increase in the ATP-ase/coupling factor observed during the greening of bean plants with the evolution of photophosphorylation ability of plastids from greening bean leaves (17), it cannot be excluded that the coupling factor might become limiting for photophosphorylation at some stages of the greening. In chloroplasts isolated from greening bean plants, cyclic photophosphorylation could be detected after 10 hours of illumination whereas noncyclic photophosphorylation with ferricyanide was not found until 15 hours of illumination (17). These two reactions are dependent on different segments of the electron transport chain but are presumed to require the same coupling factor since an antibody against CF_1 inhibited both cyclic and noncyclic photophosphorylation (35). It would seem therefore that the enzymes of the electron transport path are more probably the limiting factors for light-dependent ATP formation.

The considerable light-induced increase in ATP-ase activity of bean plastids upon greening enabled the investigation of the cellular site of ATP-ase formation using chloramphenicol -- a specific inhibitor of protein synthesis on the 70S ribosomes of chloroplasts, and cycloheximide -- a specific inhibitor of protein synthesis on the 80S ribosomes of the cytoplasm. It was found that the antibiotics inhibited the light induced increase of ATP-ase activity extractable from the plastids of greening plants. In addition, disc gel electro-

phoresis of postribosomal supernatants prepared from leaves of plants grown on either chloramphenicol or cycloheximide showed no substantial accumulation of the ATP-ase. This should exclude the possibility that either: (1) the ATP-ase is formed by the plastid ribosomes in the presence of cycloheximide and is released from the plastids upon homogenization; or (2) that the ATP-ase is formed by the cytoplasmic ribosomes in the presence of chloramphenicol and accumulates in the cytoplasm as is the case with yeast mitochondrial ATP-ase (109).

It would appear that both ribosomal systems are required for synthesis of the ATP-ase/coupling factor in bean plants. Similar results (i.e. inhibition of the light-induced enzyme increase by both antibiotics) were observed with other membrane bound photosynthetic enzymes by Smillie et al. (73) in greening Euglena cells. They have observed inhibition of three enzymes of the electron transport system by both antibiotics and concluded that the enzymes were made by the chloroplast ribosomes. They suggested that the inhibition by cycloheximide might have been indirect by blocking synthesis of structural elements essential for formation of the electron transport enzymes and for their incorporation into the plastid membranes. This might be also true for the bean plastid ATP-ase/coupling factor as sequential exposure of greening plants to chloramphenicol and cycloheximide has indicated formation of a cytoplasmic product in the presence of chloramphenicol. This product might have caused the observed stimulation of the ATP-ase synthesis on the chloroplast ribosomal system upon transfer to cycloheximide and partial removal of chloramphenicol. The product of the cytoplasmic ribosomal system could be a membrane

component required for the formation and binding of the ATP-ase to the plastid membrane; it could be a factor influencing the functioning of the chloroplastic ribosomal system; or, it could be a subunit of the ATP-ase itself. Unfortunately the removal of chloramphenicol upon transfer of the plants to a chloramphenicol-free medium is only partial and slow. More conclusive results could probably be obtained by greening the plants in the presence of chloramphenicol or cycloheximide, isolating the plastids from these plants and measuring the capacity of the plastid membranes to bind radioactively labeled ATP-ase/coupling factor. Attempts should also be made to identify the component(s) accumulating in the presence of chloramphenicol.

The possibility of partial formation of the ATP-ase/coupling factor by each ribosomal system could be investigated by immunological methods as well as by disc gel electrophoresis. Such a partial formation of an enzyme by each ribosomal system was shown by Criddle et al. (92), who have observed differential synthesis of the two types of subunits of ribulose diphosphate carboxylase by the chloroplastic and cytoplasmic ribosomal systems.

Hoober et al. (93) have observed that chloramphenicol inhibited photoreductive activities in the greening y-1 mutant of Chlamydomonas reinhardi while plastid membranes formed at normal rates. However, the membranes formed in the presence of chloramphenicol rarely fused into grana. A similar observation concerning the grana formation in the presence of chloramphenicol was made in pea chloroplasts by Srivastava et al. (90). As the present study has shown that chloramphenicol strongly inhibits coupling factor formation and since the

coupling factor is known to be attached to the outer surface of the thylakoid membrane (47, 48, 49), it is possible that it plays a role in the interaction of the thylakoids to form grana. The lack of grana formation in the presence of chloramphenicol could be due to the lack of coupling factor molecules on the thylakoid surface. It would be, therefore, interesting to investigate whether there is a correlation between the presence of the coupling factor on the thylakoid surface and aggregation of the thylakoids into grana.

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