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# A COUPLING FACTOR FOR PHOTOSYNTHETIC PHOSPHORYLATION FROM PLASTIDS OF LIGHT- AND DARK-GROWN MAIZE

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SUMMARY

1. Maize chloroplasts contain a trypsin-, dithiothreitol-, and $Ca^{2+}$-activated ATPase. This enzyme, which can serve as a coupling factor for photosynthetic phosphorylation, differs slightly in a few properties but in general resembles a similar one in spinach plastids which was described earlier by others.

2. Maize etioplasts (immature plastids in dark-grown plants) also contain this ATPase, and it is shown that NaCl–EDTA extracts of etioplasts can restore photosynthetic phosphorylation activity to depleted green membranes of chloroplasts.

3. Electron microscopy of maize etioplast and chloroplast membranes demonstrates the presence of protruding knobs, approx. 90 Å in diameter. Removal and reassociation of knobs with membranes can be correlated with the ability to carry on photosynthetic phosphorylation.

4. Most or possibly all of the coupling factor (measured as ATPase) activity of a chloroplast may be present in the etioplast from which it develops. The photosynthetic membrane of the chloroplast can be formed in stages.

5. The significance of these observations is discussed with regard to membrane formation in general and plastid membrane development in particular.

## INTRODUCTION

Seedlings of angiosperms germinated and grown in darkness are yellow. Chlorophyll is lacking and the plastids (etioplasts) are developed to a characteristic stage of immaturity. The most conspicuous structural feature of an etioplast is the presence of one or two prolamellar bodies, each of which has a regular, angular, paracrystalline appearance when viewed in cross-section with the electron microscope. Structural rearrangement of the prolamellar body; production of proteins, RNA and large amounts of chlorophyll; and the acquisition of photosynthetic competence all occur within the first few hours of illumination (*e.g.* ref. 1).

Chloroplast lamellae contain chlorophyll and are capable of carrying on photo-

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; PMS, phenazine methosulfate.

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synthetic phosphorylation *in vitro*, as well as *in vivo*. The detailed molecular architecture of these membranes is not known but it has been shown principally by RACKER and his associates[2–6] (a) that $Ca^{2+}$-activated ATPase activity is associated with spinach chloroplast membranes, (b) that this activity can be washed from the membranes by dilute EDTA solution, (c) that EDTA-extracted membranes are incapable of carrying on photosynthetic phosphorylation, and (d) that photophosphorylating capacity can be restored by adding the EDTA extract to preparations of EDTA-washed membranes. A factor, which can exhibit $Ca^{2+}$-activated ATPase activity but which normally probably serves to couple photosynthetic electron transport to phosphorylation, has been shown to occur as particles about 90 Å in diameter which are attached to the surface of spinach photosynthetic lamellae and can be removed by EDTA solution[7–9].

Relatively little is known about the composition of the prolamellar body of the etioplast or about the changes required for its membranes to become capable of photosynthesis. The present investigation was undertaken to establish some properties of prolamellar body membranes, particularly in the interest of studying membrane development. Knob-like structures approx. 90 Å in diameter attached to membranes were observed during an electron microscopic examination of negatively stained maize etioplast membranes. These and similar structures observed on maize chloroplast lamellae resembled coupling factor particles previously described in spinach[7–9].

It has been found that maize chloroplasts contain a coupling factor for photosynthetic phosphorylation which is similar in many properties to that in spinach plastids. Furthermore, it is shown that maize etioplasts also contain material which can substitute for coupling factor of green membranes.

## MATERIALS

ADP and ATP were obtained from Calbiochem, Los Angeles. Trypsin (bovine pancreas) and soybean trypsin inhibitor were obtained from Worthington Biochemical Corporation, Freehold, N.J. Maize (single cross WF9Tms × B37) was obtained from Illinois Foundation Seeds, Inc., Champaign, Ill.

## METHODS

### *Preparation of plastids*

Maize (*Zea mays*, L.) was grown in light or in total darkness at 28°. Leaves of the dark-grown plants were harvested usually 7–9 days after planting.

Plastids and plastid fragments for most ATPase and photophosphorylation experiments were obtained from light-grown or etiolated maize by chilling and then homogenizing leaves in a Waring Blendor with 2 ml of a solution of 0.4 M sucrose, 0.01 M NaCl and 0.05 M Tris–HCl or *N*-tris(hydroxymethyl)methylglycine (Tricine)–NaOH buffer (pH 8.0) per g of tissue. Tricine buffer was used exclusively in photophosphorylation experiments. Tris buffer was usually employed for preparation of plastids for ATPase experiments.

The homogenate was squeezed through muslin to remove cell debris and then centrifuged at 3000 × *g* for 6 min. The supernatant was discarded and the precipitate was resuspended in about 1/4 the volume of the homogenizing solution used and

centrifuged at 12000 × *g* for 10 min. The pellet was resuspended in a small volume of homogenizing solution and used for experimentation.

Purified etioplasts were used in a few experiments. These were prepared by homogenizing etiolated leaves in a Waring Blendor with 1.5 ml of a medium containing 0.5 M sucrose, 0.5 M Tris–HCl (pH 8.0) and 0.001 M $MgCl_2$ per g of tissue. The homogenate was squeezed through muslin and then centrifuged at 200 × *g* for 3 min. The pellet was discarded and the supernatant fluid recentrifuged at 1000 × *g* for 10 min. The pellet so obtained was resuspended in homogenizing medium and centrifuged at 1000 × *g* for 15 min. The supernatant fluid was discarded and the pellet was resuspended in 6 ml of 0.05 M Tris–HCl (pH 8.0). A discontinuous sucrose gradient was made in the following manner: 10 ml of 1.37 M sucrose was layered onto 10 ml of 2 M sucrose. The plastid suspension was layered on top of the 1.37 M sucrose to fill the 50 ml centrifuge tube. The gradients were then centrifuged at 25000 × *g* in a Sorvall HB-4 rotor for 20 min. Etioplasts collected at the top of the 2 M sucrose layer and were removed with a Pasteur pipette. The etioplasts were then resuspended in the solution appropriate for the particular experiment in which they were to be used.

*Assay for ATPase activity*

An aliquot of plastid suspension or extract was incubated at 37°, and a solution containing 50 μmoles Tris–HCl (pH 8.0), 5 μmoles ATP (pH 8.0) and 5 μmoles $CaCl_2$ was added to start the ATPase reaction. The final volume was 1.0 ml. The reaction was stopped after 20 min by adding 0.5 ml of 16% (w/v) trichloroacetic acid. For "0" time controls, the trichloroacetic acid was added before incubation.

After centrifugation at 12000 × *g* for 10 min, the supernatant fluid was assayed for inorganic phosphate by the colorimetric method of AMES[10] modified by incubating for 5 min at 45°.

*Assay of coupling factor activity for photophosphorylation*

The assay for coupling factor activity followed the general approach employed by MCCARTY AND RACKER[3]. Maize chloroplasts were mixed with a NaCl–EDTA solution (pH 8.0) to give a final concentration of 0.01 M NaCl and 0.15 mM EDTA. The suspension was allowed to stand for 20 min in the cold (4°) and was then centrifuged for 20 min at 35000 × *g*. NaCl–EDTA washed chloroplast fragments containing 100–200 μg chlorophyll were incubated in an ice bath with the following reagents added in the order given: an aliquot of the NaCl–EDTA extract (coupling factor) of either chloroplast or etioplast membranes, 1.0 μmole EDTA (pH 8.0) and 10 μmoles $MgCl_2$. After incubation for 15 min in the ice bath, 1.4 ml of a reaction mixture containing 50 μmoles *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.0) and approx. 2 μC of [$^{32}$P]phosphate were added. Finally, 0.1 ml of $2 \cdot 10^{-4}$ M phenazine methosulfate (PMS) was added to the mixture. The final volume of the reaction mixture was 3.0 ml. The tubes were illuminated for 15 min at 15–17° with about 2500 ft-candles of white light. After illumination, 0.5 ml of 16% trichloroacetic acid was added to each tube and the suspension was centrifuged for 10 min at 12000 × *g* to remove insoluble plastid material. An aliquot of the supernatant fluid was taken for analysis of radioactive phosphate ester.

Inorganic phosphate was separated from organic phosphate by the method of AVRON[11] and the $^{32}$P in the organic fraction was measured with a Model 3375 Packard

Liquid Scintillation counter by measuring[12] the Cereknov radiation produced in water by the $^{32}P$.

Protein concentrations were determined according to the method of LOWRY *et al.*[13]. When necessary, proteins were first solubilized by heating in KOH. The chlorophyll content of plastid suspensions was estimated colorimetrically in 80 % acetone extracts[14].

The chlorophyll content of whole leaves was determined by extracting in 24 vol. of absolute ethanol, measuring the optical densities of the extracted chlorophyll at 649 nm and 655 nm and calculating the chlorophyll concentration[15].

*Electron microscopy*

Some suspensions of membrane fractions (Figs. 3 and 4) were mixed with an equal volume of either 2 % potassium phosphotungstate or 4 % ammonium molybdate at about pH 7 and the mixture was immediately sprayed on to grids coated with carbon on formvar. After drying the grids were examined with a Hatachi IIc electron microscope at an accelerating potential of 50 kV.

In other cases (Figs. 5–7), samples were applied to grids coated with carbon floated off of mica which had been cleaved and then coated by evaporation. Negative staining was with 2 % potassium phosphotungstate at pH 7. These specimens were examined with a Phillips 300 electron microscope at an accelerating voltage of 60 kV.

## RESULTS

*Maize chloroplast ATPase*

Mature maize chloroplasts have readily demonstrable ATPase activity. Rates as high as 3 $\mu$moles ATP hydrolyzed per mg chlorophyll per h can be obtained by incubating chloroplasts at 37° with ATP, $Ca^{2+}$ and pH 8 buffer. Inorganic phosphate is liberated at a constant rate for up to at least 20 min. No enhancement of ATPase activity comparable to that reported with spinach plastids[16] is found by transferring maize chloroplasts from an "acid" (pH 4.0) bath to a "base" (final pH 8.4) or *vice versa* ("base to acid").

*Activation by dithiothreitol and trypsin.* Maize chloroplast ATPase can be activated by dithiothreitol and by trypsin much in the same way as has been shown for spinach chloroplast ATPase[5]. This activation is accomplished by incubating the chloroplasts with 0.05 M dithiothreitol, followed by solubilizing ATPase from the chloroplast fragments with dilute EDTA. The solubilized ATPase is activated further by incubation with trypsin. The combined treatments result in a more than 70-fold increase in activity per mg protein (Table I).

*The effect of divalent cations on activity.* ATPase activity in crude maize chloroplast preparations can be observed without the addition of any divalent cation, although $Mg^{2+}$ and especially $Ca^{2+}$ can be shown to increase the activity. However, trypsin-treated preparations are almost completely inactive without added divalent cation; $Ca^{2+}$ is many times more effective than $Mg^{2+}$ in activating the ATPase (Table I).

Much of the ATPase activity can be removed from chloroplast fragments by water or EDTA solutions, but activity equivalent to 1/3–1/2 of the original remains in the plastid residues after 4 consecutive washes (Table I).

TABLE I

EXTRACTION AND ACTIVATION OF ATPase FROM MAIZE CHLOROPLASTS

Chloroplast membranes obtained from 48 g of green corn leaves were suspended in 6.0 ml of 0.05 M Tris–HCl buffer (pH 8.0). 3.1 ml of the suspension was extracted and centrifuged 4 times consecutively: twice with water (yielding supernatants of 8.75 and 8.3 ml, respectively) and twice with 0.75 mM EDTA, pH 7.0 (yielding supernatants of 10.5 and 12.0 ml, respectively). After each extraction with water or EDTA, the material was centrifuged for 20 min at 35000 × *g*. The 4-times washed chloroplast membranes were resuspended in 4.0 ml of 0.05 M Tris–HCl. To obtain the trypsin-activated extract, 1.2 ml of a solution containing 12 μmoles ATP, 100 μmoles Tris–HCl (pH 8.0) and 600 μg trypsin was added to 6.0 ml of the first EDTA extract. The mixture was incubated at room temperature for 6 min, and then 1.2 ml of a 2 mg/ml solution of soybean trypsin inhibitor was added to a final volume of 8.4 ml to stop the trypsin reaction. To prepare dithiothreitol-treated chloroplasts 0.5 ml of 0.25 M dithiothreitol was added to 2.0 ml of the original chloroplast suspension and the mixture was incubated at room temperature for 65 min before assay and further treatment. 1.6 ml of the dithiothreitol-treated chloroplast suspension was extracted once with 0.75 mM EDTA and centrifuged for 20 min at 35000 × *g* to yield a supernatant of 13.3 ml. The trypsin-activated dithiothreitol extract was prepared in the same manner as the non-dithiothreitol extract. ATPase was assayed as described in METHODS. "Total activity" is calculated on the basis of the total activity of each fraction obtained from 3.1 ml of the original chloroplast suspension. For the dithiothreitol fractions, the corresponding calculation is on the basis of 3.83 ml of dithiothreitol-treated chloroplast suspension. The specific activities related to chlorophyll concentration are based on the chlorophyll concentration of the extracts before centrifugation to remove the washed chloroplast residues.

| *Fraction* | *Total activity (μmoles ATP hydrolyzed/ total fraction per h)* | | *Specific activity* | |
|---|---|---|---|---|
| | | | *(μmoles ATP hydrolyzed/ mg protein per h)* | *(μmoles ATP hydrolyzed/ mg chlorophyll per h)* |
| *Minus dithiothreitol* | | | | |
| Chloroplasts | 17.5 | | 1.09 | 2.65 |
| 1st water extract | 15.0 | | 11.0 | 2.27* |
| 2nd water extract | 15.2 | | 4.38 | 2.30* |
| 1st EDTA extract | 4.95 | 36.3 | 3.26 | 0.75* |
| 2nd EDTA extract | 1.14 | | | 0.17* |
| Washed chloroplast residue | 6.24 | | 0.51 | 0.94 |
| 1st EDTA extract and trypsin | | | | |
| + 0.5 M $CaCl_2$ | | | 9.49 | 16.2* |
| + 0.5 M $MgCl_2$ | | | 0.39 | 0.59* |
| No divalent cation | | | 0.04 | 0.07* |
| *Plus dithiothreitol* | | | | |
| Chloroplasts | 21.3 | | 1.33 | 3.22 |
| EDTA extract | 31.2 | | 3.80 | 4.72* |
| EDTA extract and trypsin | | | | |
| + 0.5 M $CaCl_2$ | | | 73.8 | 91.9* |
| + 0.5 M $MgCl_2$ | | | 15.7 | 19.5* |
| No divalent cation | | | 1.48 | 1.85* |

* Determined on basis of amount of chlorophyll in plastid before extraction with water or EDTA solution.

Washing the chloroplast fragments also results in an increase in the total ATPase activity. The total activity measured in the extracts *plus* activity remaining with the plastid residue is about 2.5 times greater than the ATPase activity originally detected in the unwashed plastid fragments.

*The effect of storage temperature and dithiothreitol on ATPase activity.* The ATPase activity of trypsin-treated EDTA extracts of maize chloroplast fragments declined slightly less at room temperature than at 4° and dithiothreitol had a protective effect

at either temperature. About 23 % of the activity remained after 24 h when the extract was kept at room temperature with dithiothreitol.

*Matze etioplast ATPase*

The ATPase of maize etioplasts is similar in many respects to that found in maize chloroplasts. The activity does not depend on an "acid–base" or "base–acid" transition.

TABLE II

EXTRACTION AND ACTIVATION OF ATPase FROM MAIZE ETIOPLASTS

Etioplast membranes obtained from 108 g of dark-grown corn were suspended in 5.5 ml of 0.05 M Tris–HCl buffer (pH 8.0). 2.6 ml of the suspension was extracted and centrifuged 4 times consecutively: twice with water (yielding supernatants of 8.75 ml and 8.3 ml, respectively) and twice with 0.75 mM EDTA, pH 7.0 (yielding supernatants of 9.4 and 10.2 ml, respectively). After each extraction with water or EDTA, the material was centrifuged for 20 min at 35000 × *g*. The 4-times washed chloroplasts were resuspended in 4.0 ml of 0.05 M Tris–HCl. To obtain the trypsin-activated extract, 12 μmoles ATP, 100 μmoles Tris–HCl buffer (pH 8.0) and 600 μg trypsin were added to 6.0 ml of the first EDTA extract. The mixture was incubated for 6 min at 37°, and then 1.2 ml of a 2 mg/ml solution of soybean trypsin inhibitor was added to a final volume of 8.6 ml to stop the trypsin reaction. To prepare dithiothreitol-treated etioplast membranes, 0.5 ml of 0.25 M dithiothreitol was added to 2.0 ml of the plastid suspension, and the mixture was incubated at room temperature for 160 min before assay. ATPase was assayed as described in METHODS. From the time of harvesting the leaves to assaying all fractions, experimentation was carried out under green light. White light was excluded. "Total activity" is calculated on the basis of the total activity of each fraction obtained from 2.6 ml of the etioplast suspension.

| *Fraction* | *Total activity (μmoles ATP hydrolyzed/ total fraction per h)* | | *Specific activity (μmoles ATP hydrolyzed/ mg protein per h)* |
|---|---|---|---|
| *Minus dithiothreitol* | | | |
| Etioplasts | 12.18 | | 0.42 |
| 1st water extract | 7.14 | | 3.00 |
| 2nd water extract | 0.33 | | 0.22 |
| 1st EDTA extract | 1.41 | 11.19 | 1.20 |
| 2nd EDTA extract | 2.31 | | 3.78 |
| Washed etioplast residue | 5.85 | | 0.49 |
| 1st EDTA extract and trypsin | | | |
| + 0.5 M $CaCl_2$ | | | 8.34 |
| + 0.5 M $MgCl_2$ | | | 2.86 |
| *Plus dithiothreitol* | | | |
| Etioplasts | 16.9 | | 0.54 |

Etioplasts purified by sucrose density gradient centrifugation (see METHODS) hydrolyzed 8.86 μmoles ATP per mg protein per h compared to rates of 4.40 for crude etioplasts and 0.10 for the sucrose density gradient supernatant.

*Activation by dithiothreitol and trypsin.* Etioplast ATPase, like that of chloroplasts, can be activated with dithiothreitol and with trypsin, as shown in Table II. Dithiothreitol increases the activity of the unwashed etioplast fragments about 30 %. In a separate experiment, dithiothreitol enhanced the activity of trypsin-treated EDTA extracts by almost 100 %. The activity of the first EDTA extract was increased almost 3-fold by treatment with trypsin.

The optimal EDTA concentration for extracting ATPase from washed etioplasts

is about 0.15 mM (Fig. 1). The variation in amount of ATPase extracted with EDTA concentration is not striking, however, in comparison with the results reported for spinach[3]. McCARTY AND RACKER[5] also found that when an EDTA extract of plastid fragments was incubated with EDTA-washed chloroplast membranes and $Ca^{2+}$, the ATPase activity of the extract was decreased by about 70 %. With maize etioplasts, however, the ATPase activity of a mixture of an EDTA-washed plastid suspension and EDTA extract incubated with $Ca^{2+}$ was only 10–16 % lower than the sum of the activities of the 2 fractions incubated separately.

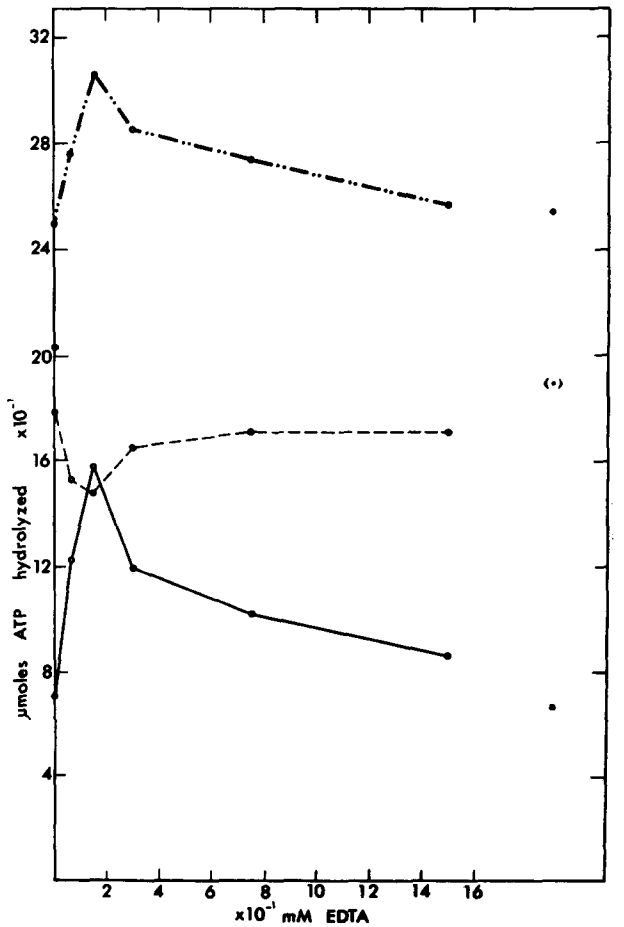



Fig. 1. Effect of EDTA concentration on corn etioplast ATPase. Etioplast membranes from 294 g of dark-grown corn were washed twice with 10 mM NaCl. The plastids were resuspended in 10 ml distilled water. 1.0-ml aliquots of the plastid suspension were mixed with appropriate aliquots of 15 mM EDTA, pH 7.0 (or 10 mM NaCl) and water to give a final volume of 5.0 ml. The mixtures were shaken gently at 37° in a water bath and were then centrifuged for 20 min at 35000 × *g*. The supernatant was decanted, and the plastid precipitate was resuspended in a small volume of distilled water. The supernatants and resuspended precipitates were assayed for ATPase as described in METHODS. O–·–O, total activity (reaction rate in 10 mM NaCl: ◆); O---O, plastid pellet (reaction rate in 10 mM NaCl: (·)); O—O, supernatant (reaction rate in 10 mM NaCl: ■).

The time course of trypsin activation is shown in Fig. 2. In an experiment testing the effect of dithiothreitol on the trypsin-activated extract, an increase of 86 % in ATPase activity resulted from preincubating the extract for 30 min at room temperature with 0.05 M dithiothreitol.

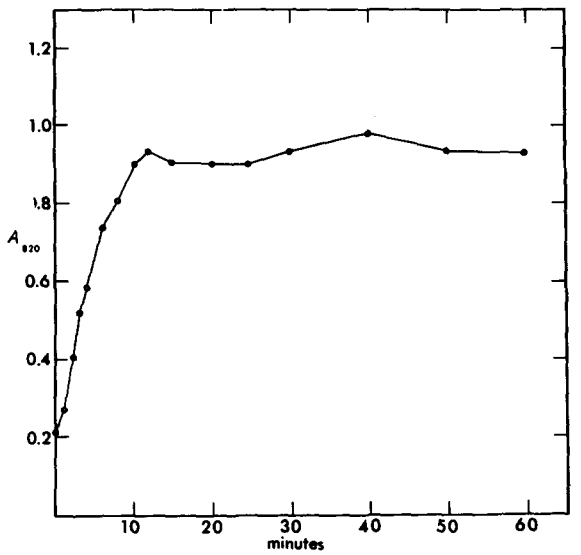



Fig. 2. Time course of activation of etioplast ATPase by trypsin. Etioplasts were prepared from 140 g of dark-grown corn and plastid fragments were suspended in 0.15 mM EDTA (pH 8.0) to give 24.0 ml of extract after centrifugation at 35000 × *g* for 20 min. 100 μmoles Tris–HCl buffer (pH 8.0), 7.5 μmoles EDTA and 5 μmoles ATP were added to 6.0 ml of etioplast EDTA-extract, and then 800 μg trypsin was added to start the activation (final volume 8.0 ml). 0.3-ml aliquots were removed from the reaction vessel at intervals and added to the tubes each containing 0.2 ml soybean trypsin inhibitor (2 mg/ml). ATP, $CaCl_2$ and Tris buffer were added to each tube to assay for ATPase as described in METHODS. Activation was carried on at room temperature.

As observed with maize chloroplast membrane preparations, repeated washing of the etioplast membranes left a substantial amount of ATPase activity on the membranes. Even sonication did not allow subsequent separation of all the ATPase activity from the etioplast residues. Etioplasts were suspended in a solution containing 0.15 mM EDTA (pH 7.0) and 0.05 M dithiothreitol. Aliquots of the suspension were sonicated for 1, 3, and 6 min and then centrifuged for 20 min at 35000 × *g*. Up to 60 % of the original activity remained in the plastid membrane precipitates.

*The effect of divalent cations on activity.* As with maize chloroplasts, ATPase activity of etioplasts is observed without addition of a divalent cation to crude preparations. However, inclusion of 0.05 M $CaCl_2$ in the assay mixture results in almost 2.5 times greater activity than when the divalent cation is omitted and about 60 % higher activity than when 0.05 M $MgCl_2$ is included instead. Similarly, as shown in the data of Table II, the trypsin-treated EDTA extract of etioplast fragments was nearly 3 times more active with 0.05 M $CaCl_2$ than with 0.05 M $MgCl_2$. The results, therefore, indicate that both chloroplasts and etioplasts of maize contain an ATPase which requires $Ca^{2+}$ for optimal activity.

*Effect of pH on ATPase activity.* The pH value for maximum activity of etioplast

ATPase is around 8.0 when Tris–HCl buffer at a concentration of 0.05 M is used. With Tricine buffer at the same concentration, the optimum is shifted to about pH 8.4.

*The effect of storage temperature on activity.* Etioplasts which were stored at room temperature for 24 h, extracted with 0.15 mM EDTA (without dithiothreitol) and treated with trypsin retained 87 % of the ATPase activity of the original trypsin-treated extract.

TABLE III

THE EFFECT OF EDTA EXTRACTS OF CHLOROPLAST OR ETIOPLAST MEMBRANES ON PHOTOPHOSPHORYLATION BY EXTRACTED CHLOROPLAST MEMBRANES

A corn chloroplast suspension containing 1.8 mg chlorophyll per ml was divided into 2 portions. 1.0 ml of the suspension was mixed with 4.0 ml of NaCl solution to give a final concentration of 0.01 M NaCl. 4.0 ml of the suspension was mixed with 16.0 ml of a NaCl–EDTA (pH 8.0) solution to give a final concentration of 0.01 M NaCl and 0.15 mM EDTA. Both mixtures were allowed to stand for 20 min at 4°. The mixtures were centrifuged for 30 min at 20000 × *g*, the supernatants were decanted, and the latter supernatant was saved. 1.5 ml of a maize etioplast suspension was mixed with 6 ml of NaCl–EDTA (pH 8.0) to give a final concentration of 0.01 M NaCl and 0.15 mM EDTA. The supernatant extract was obtained in the same manner as the chloroplast extracts. The chloroplast precipitates obtained after extraction and centrifugation were resuspended in the homogenizing medium, and 0.2 ml of the chloroplast suspensions containing 350 μg chlorophyll were incubated with either 1.1 ml of chloroplast NaCl–EDTA extract, 1.1 ml of etioplast NaCl–EDTA extract, or 1.1 ml water. Duplicates were run for each mixture, and photophosphorylation was assayed as described in METHODS. The chloroplast and etioplast NaCl–EDTA extracts contained 140 and 286 μg protein per 1.1 ml, respectively.

| *Treatment* | *μmoles [$^{32}P$]phosphate ester formed/ mg chlorophyll per h* | *% of activity of unwashed chloroplasts* |
|---|---|---|
| Chloroplasts washed with 0.01 M NaCl | 22.57 | 100 |
| Chloroplasts washed with 0.01 M NaCl + 0.15 mM EDTA | 4.98 | 22.1 |
| EDTA-treated chloroplasts + 1.1 ml chloroplast NaCl–EDTA extract | 14.69 | 65.1 |
| EDTA-treated chloroplasts + 1.1 ml etioplast NaCl–EDTA extract | 6.25 | 27.7 |
| 1.1 ml chloroplast NaCl–EDTA extract | — | 0.2 |
| 1.1 ml etioplast NaCl–EDTA extract | — | 0.1 |

In a separate experiment (using 0.05 M $MgCl_2$ instead of $CaCl_2$), it was found that 82 % of the ATPase activity in crude etioplast preparations was preserved after 24 h in the cold (4°). Thus, both the chloroplast and etioplast ATPases are more readily inactivated at 4° than at room temperature.

*Photophosphorylation coupling factors from maize chloroplasts and etioplasts*

AVRON[17], MCCARTY AND RACKER[3] and HOWELL AND MOUDRIANAKIS[8] have demonstrated that extraction with dilute EDTA solutions markedly inhibits the photophosphorylating capacity of swiss chard or spinach chloroplast preparations. These workers also have shown that some photophosphorylation activity is recovered in mixtures of EDTA extracts of chloroplasts with EDTA-extracted chloroplast membranes.

Maize and spinach plastids do not respond to EDTA solutions in the same way. Photophosphorylation by maize chloroplast preparations is reduced to less than 0.2 %

of the controls after extraction with 0.15 mM EDTA at 4° for 20 min. Activity is not recovered by adding the EDTA extract and $Mg^{2+}$ (see METHODS) to extracted membranes. On the other hand, when chloroplast lamellae were extracted instead with a solution of 0.01 M NaCl in 0.15 mM EDTA the photophosphorylating activity of the lamellae was reduced only to about 20%. However, the most significant feature of the latter procedure was that the NaCl–EDTA extract could restore significant amounts of activity to depleted membranes (Table III). JAGENDORF AND SMITH[18] reported a beneficial effect of cations in a study of uncouplers of photophosphorylation in spinach chloroplasts.

TABLE IV

THE RESTORATION OF THE CAPACITY FOR PHOTOSYNTHETIC PHOSPHORYLATION TO EXTRACTED MAIZE CHLOROPLAST MEMBRANES BY SOLUBLE EXTRACTS OF MAIZE ETIOPLASTS

EDTA–NaCl-extracted maize chloroplasts and etioplast EDTA–NaCl extract were prepared as described in Table III. The extracted chloroplast membranes containing 115 μg chlorophyll, were incubated with aliquots of the etioplast extract or boiled (control) etioplast extract and assayed for photophosphorylation activity. The etioplast extract contained 1.52 mg protein per ml. No incorporation of $^{32}P$ above the amount in the dark control was obtained when the unboiled etioplast extract was assayed without chloroplast membranes.

| *Experiment* | *[$^{32}P$]ATP (counts/min)* | *[$^{32}P$]ATP minus boiled control (counts/min)* |
|---|---|---|
| *Expt. 1a* | | |
| EDTA-extracted chloroplast membrane | | |
| + 0.5 ml etioplast extract | 22 000 | 6 800 |
| + 1.0 ml etioplast extract | 27 700 | 12 500 |
| + 1.5 ml etioplast extract | 34 500 | 19 300 |
| + 1.0 ml boiled etioplast extract | 15 200 | (0) |
| *Expt. 1b* | | |
| EDTA-extracted chloroplast membrane | | |
| + 2.0 ml etioplast extract | 37 300 | 25 400 |
| + 2.0 ml boiled etioplast extract | 11 900 | (0) |

When 1.1 ml of chloroplast extract (containing 140 μg protein) was added to washed chloroplast membranes containing 350 μg chlorophyll, the $^{32}P$ incorporation by the chloroplast preparation increased by about 9.7 μmoles/h. When 1.1 ml of etioplast extract (containing 286 μg protein) was added to the washed chloroplast membrane preparation, $^{32}P$ incorporation increased by about 1.3 μmoles/h. (The rate of esterification is directly proportional to the amount of etioplast extract added as is shown below.) When chloroplast extract was added to washed or unwashed etioplast membranes, no photophosphorylation was observed. Etioplast membranes are incapable of catalyzing photophosphorylation; they lack chlorophyll.

Table IV shows that the NaCl–EDTA extract of etioplast membranes enhances the photophosphorylation activity of washed chloroplast membranes in proportion to the amount of extract added. The etioplast extracts used in these experiments contained 5.3 times more protein than did the preparation used in the experiments described in Table III. Heating the etioplast extract to 100° renders it inactive. Thus, etioplasts contain a coupling factor for photophosphorylation.

*Electron microscopy and centrifugation of maize plastid membranes*

Fig. 3 is an electron micrograph of negatively stained maize etioplast membranes. This material was prepared as described in METHODS. Similar preparations of chloroplast membranes were indistinguishable from etioplast membranes. The protruding knobs are approx. 90 Å in diameter and, as shown, exhibit 5-fold symmetry by optical reinforcement techniques[19]. (see Figs. 4a–4c).

Electron micrographs of NaCl–EDTA washed chloroplast lamellae, of coupling factor particles in an EDTA–NaCl extract of maize etioplasts, and of etioplast coupling factor–chloroplast membrane hybrids are shown in Figs. 6,5 and 7, respectively.

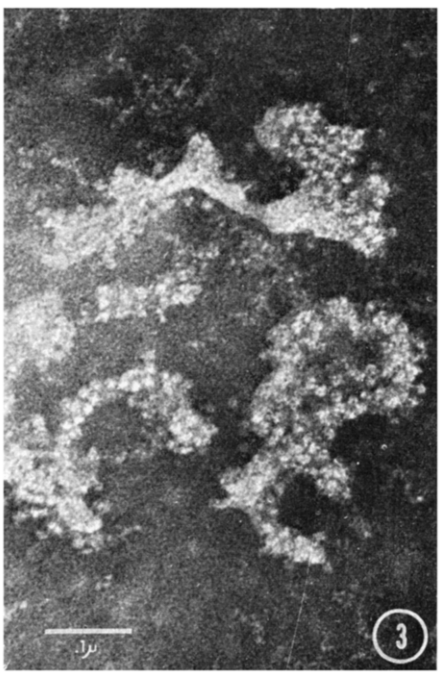
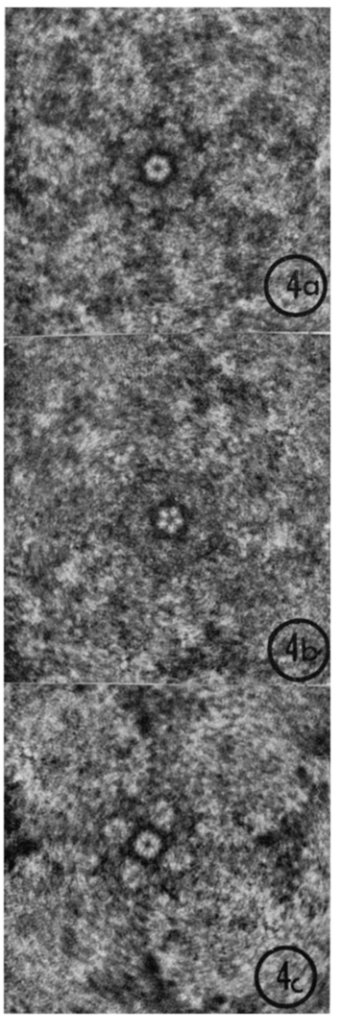



Fig. 3. Electron micrograph of negatively stained maize etioplast membranes showing arrays of protruding knobs. Magnification is about 110000. The knobs are about 90 Å in diameter.

The washed chloroplast membranes shown in Fig. 6 were prepared by mixing chloroplasts obtained as described in METHODS with a NaCl–EDTA solution to a final concentration of 10 mM NaCl and 0.15 mM EDTA. After 1 h at room temperature the suspension was centrifuged at 17000 × *g* for 20 min. The clear supernatant fluid was discarded and the membranes in the loose green layer above the tightly packed pellet was removed and was precipitated by centrifugation at 34000 × *g* for 20 min. These membranes were further purified by sucrose density centrifugation essentially according to a method previously used for spinach membranes[20]. (The loose layer contained large envelopes. The material which was packed tightly by centrifugation at 17000 × *g* consisted mainly of smaller thylakoid fragments.) The washed membrane suspension was made 40 % (w/w) with respect to sucrose and 10 mM with regard to Tris–HCl (pH 8.0). Continuous linear density gradients using 40 % sucrose–Tris–plastid membrane preparation and 50 % (w/w) sucrose–Tris were generated in Spinco

SW 25.2 cellulose nitrate tubes. The gradients were centrifuged at 106000 × *g* for 16 h at 5°. The chloroplast membranes shown in Fig. 6 were obtained from the lower part of the single broad green zone which formed during centrifugation. Membranes were sedimented from the sucrose solution after the suspension was diluted with 1 mM $MgCl_2$; membranes were rewashed twice with 1 mM $MgCl_2$ and then prepared for electron microscopy as described in METHODS.



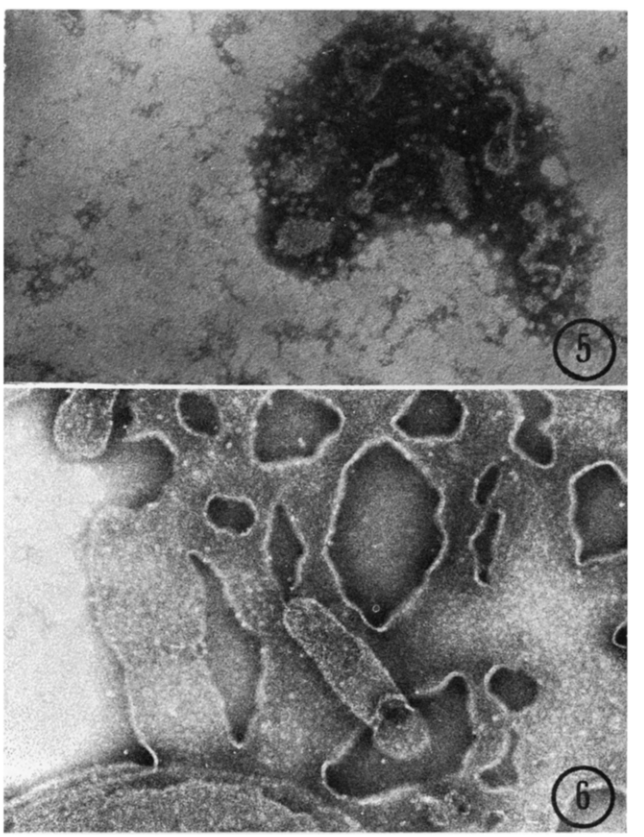




Fig. 4. Image rotation electron micrographs of one of the knobs of the type seen in Fig. 3: (a) 6-fold rotation; (b) 5-fold rotation; (c) 4-fold rotation.

Fig. 5. Electron micrograph of particles in an EDTA–NaCl extract of maize etioplast membranes. Negatively stained. Shown at magnification of about 110000.

Fig. 6. Electron micrograph of negatively stained EDTA–NaCl extracted maize chloroplast membranes obtained from sucrose density gradient after centrifugation as described in the text. Shown at a magnification of about 80000.

The material shown in Fig. 7 was obtained by mixing NaCl–EDTA washed chloroplast membranes (Fig. 6) with NaCl–EDTA extract of etioplasts (Fig. 5) in the following proportions: 0.2 ml washed chloroplast membranes in 1 mM $MgCl_2$; 2 ml etioplast EDTA–NaCl extract; 0.1 ml 10 mM EDTA (pH 8.0); and 1 ml 50 mM $MgCl_2$. After 20 min at room temperature the suspension was centrifuged to separate the membranes from particles which remained free. The membranes were washed two additional times using a solution containing EDTA, NaCl, and $MgCl_2$ in the same proportions as in the incubation mixture. After this the thrice sedimented membranes were prepared for electron microscopy as described in METHODS.

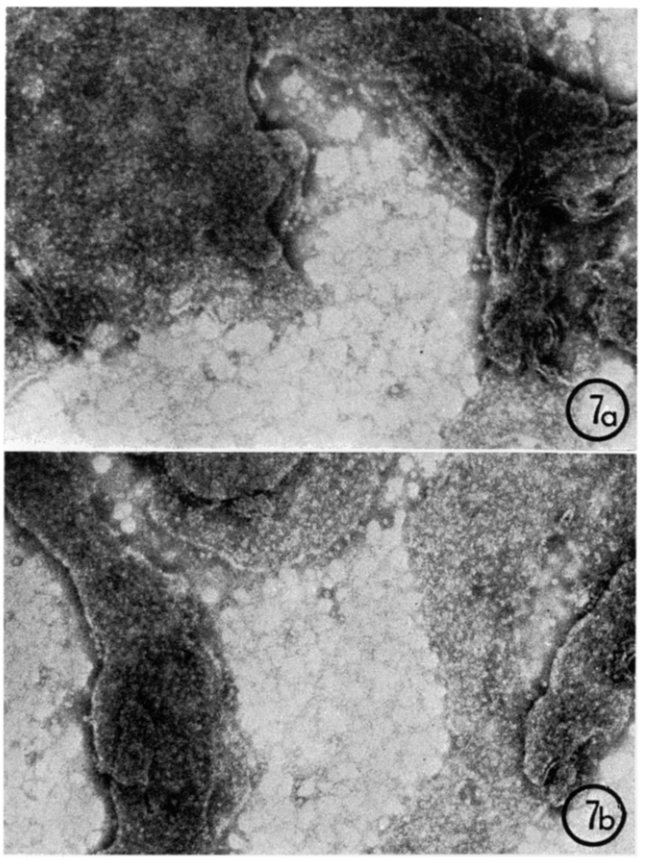



Fig. 7. Electron micrographs of twice washed negatively stained "hybrid" maize plastid membranes. Prepared by mixing aliquots of preparations shown in Figs. 5 and 6 as described in the text. Shown at a magnification of about 80000.

*ATPase activity during greening of etiolated maize and plastid maturation*

Plastids of dark-grown maize can perform detectable PMS-mediated photosynthetic phosphorylation (and probably complete photosynthesis) only after plants have been illuminated for 90 min (J. FORGER AND L. BOGORAD, unpublished results). Judging from patterns of chlorophyll accumulation, maize chloroplasts attain maximum development at 28° after dark-grown plants have been illuminated for about 25 h.

An attempt was made to determine whether plastid ATPase (coupling factor) activity parallels development of etioplasts into photosynthetically active chloroplasts. Results are presented in Table V.

The chlorophyll per g fresh weight of leaf tissue increased about 100-fold during the 25.5 h of illumination. During this same period the ATPase activity per g of leaf tissue dropped by a factor of about 5. The reason for the particularly sharp drop in this ratio between hours 2 and 5 is unknown but it varies relatively little between hours 7 and 12 during which the chlorophyll per g of leaf tissue rises more than 6-fold.

The variations noted cannot be fully understood until several possible compensatory changes during illumination are studied but the data tend to support the impression gained from electron micrographs and some experiments cited here that a good deal (if not all) of the ATPase/coupling factor activity of a chloroplast can be present in its etioplast precursor.

TABLE V

ATPase ACTIVITY DURING GREENING OF ETIOLATED MAIZE LEAVES

7-day-old dark-grown maize seedlings were harvested according to the schedule shown in the left-hand column. Illumination, where indicated, was with cool white fluorescent lamps at 200 ft-candles and at 28°. The chlorophyll concentration in leaf tissues was determined[15]. Crude plastids were prepared and ATPase was assayed as described in METHODS.

| *Time in light (h)* | *μg chlorophyll/ g leaf tissue* | *nmoles ATP hydrolyzed/20 min* | |
|---|---|---|---|
| | | *per mg of leaf tissue* | *per mg membrane protein* |
| 0 | 9.8* | 2.80 | 0.158 |
| 0.5 | 12.9 | 2.51 | 0.217 |
| 1 | 10.2 | 1.30 | 0.158 |
| 2 | 29.9 | 2.85 | 0.149 |
| 5 | 79.4 | 0.90 | 0.156 |
| 7 | 158.8 | 0.58 | 0.141 |
| 12 | 428.9 | 0.78 | 0.150 |
| 25.5 | 1065.0 | 0.55 | 0.073 |
| 12 (*plus* 13.5 h dark) | 511.5 | 0.54 | 0.087 |
| 25.5 (continued darkness) | 8.0* | 2.12 | 0.193 |

* μg protochlorophyllide measured as chlorophyllide.

The ATPase activity per mg of membrane (insoluble) protein is relatively constant through 12 h of illumination. It has not been determined whether this results from (a) synthesis of neither more membrane nor more ATPase, or (b) parallel synthesis of membrane and ATPase, but it is clear that this value drops by a factor of about 2 sometime during the next 13.5 h regardless of whether or not the plants are illuminated. Perhaps much more membrane, but little new coupling factor, is being formed during this period.

DISCUSSION

The data presented here show that a coupling factor for photosynthetic phosphorylation is associated with thylakoids of maize chloroplasts. The maize factor is similar to that previously described in spinach[3,5] in most properties which have been studied, including possession of latent ATPase activity. The most conspicuous differences are: (a) maize coupling factor is partially removed from membranes by washing them with distilled water—the spinach factor, on the other hand, is not easily released under these conditions[8]; (b) after washing maize chloroplast fragments with water, additional ATPase activity can be removed with EDTA but the optimal EDTA concentrations was found to be 0.15 mM while spinach ATPase-coupling factor is reported[3] to be best solubilized by 0.75 mM EDTA; in addition, the spinach factor is removed over a much narrower range of EDTA concentrations[5] than the maize enzyme; (c) although some of the maize ATPase is very readily removed from membranes, about 20–50 % remains bound even after 4 consecutive water and EDTA washings; and (d) the ATPase activity of the maize coupling factor in crude preparations seems less dependent on the addition of $Ca^{2+}$ than does the spinach enzyme. Also, conditions required for demonstrating photosynthetic phosphorylation coupling activity in maize are slightly different from those for spinach. Maize chloroplast membranes extracted with EDTA solution loose their capacity for photophosphorylation but it cannot be restored by adding back the EDTA extract. However, if extraction is with NaCl–EDTA solution, the membranes retain about 1/5 of their original photophosphorylating activity but, more important, the NaCl–EDTA extract can restore activity to the extracted membranes. The function of the NaCl is not known nor is it clear whether the cation "protects" the membrane, the dislodged coupling factor, or both.

It is not possible to ascribe particular significance to any of the differences in properties between maize and spinach coupling factors. Perhaps the meanings of such differences will become clear after photosynthetic coupling factors from a larger number of plants are studied.

Etioplasts lack chlorophyll and are, at least for this reason, incapable of carrying on photosynthesis. The present investigation was partially directed at the question of the mode and pattern of membrane assembly and function. OHAD *et al.*[21] conclude that in the mutant strain Y-1 of *Chlamydomonas reinhardi* all of membrane synthesis is closely integrated and that all functions and components are acquired in parallel. Unless it is assumed, contrary to previous observations (*e.g.* refs. 22, 23) that prolamellar body membranes in higher plant etioplasts are discarded and that functional photosynthetic membranes arise separately, it appears reasonable that partial synthesis of angiosperm plastid membranes can occur. This conclusion is strongly supported and extended by the present work.

The $Ca^{2+}$-activated ATPases from maize etioplast and chloroplast membranes seem the same. It is particularly interesting that material with latent ATPase activity can be removed from etioplast membranes and be used to restore photophosphorylating capacity to EDTA-extracted green chloroplast membranes. Experiments using etioplast ATPase as a coupling factor for photophosphorylation with washed chloroplast membranes show that a stoichiometric relationship exists between the amount of enzyme added to chloroplast membranes and amount of ATP produced

over the range examined (see Table VI). (HOWELL AND MOUDRIANAKIS[8] failed to find such a relationship. In their reconstitution experiments, using spinach chloroplast ATPase and washed chloroplast membranes, large amounts of coupling factor inhibited photophosphorylation. They suggested that this might have resulted from ATPase activity of the coupling factor in the assay system used.)

The present experiments reveal that the membraneous elements of the prolamellar body in maize etioplasts as well as the thylakoids of maize chloroplasts are coated with 90 Å particles. (It seems most likely that this is true for etioplast membranes generally.) The membraneous elements of the prolamellar body are arranged in a striking paracrystalline array but when maize etioplasts are isolated, broken osmotically, and spread on a grid for electron microscopy they appear as vesicles with coupling factor particles attached. Thus, the association of vesicles and the organization of the paracrystalline array may result from weak interactions between coupling factor particles attached to separate membranes or of "outer" faces of particles with adjacent membranes. *In vivo*, the elements of the prolamellar body can dissociate into separate vesicles after a few seconds of illumination with bright light. This change has been correlated spectrally and temporally with the photoreduction of protochlorophyllide to chlorophyllide[22,23] but the significance of the correlation is not clear. It could be coincidental or very indirectly casual. A change in the ionic environment might result in a change in affinity between the "outer" edges of coupling factor particles and adjacent membranes or between coupling factor particles.

Similarly the presence (or absence) of coupling factor particles must be taken into account in considering the mechanism of stacking of thylakoids in chloroplasts—a phenomenon which has many similarities to membrane association in prolamellar bodies.

Large amounts of chlorophyll are deposited in or on membranes during thylakoid maturation. Some pigment molecules must become associated with specific electron carriers, *etc.* at photosynthetic reaction centers; other chlorophyll molecules, which serve primarily as light absorbers, go to different but probably specific sites. The experiments described in this paper show that both etio- and chloroplast membranes are coated with coupling factor particles and suggest that the membranes also have some other surface properties in common—the conditions for removing coupling factor are the same for both types of membranes and coupling factor particles from etioplasts can become associated and function with green chloroplast membranes. Where in the developing membrane does the chlorophyll go? If some chlorophyll molecules are finally located on particles deep in the membrane, how do they get there? Do membranes constantly or repeatedly break and fuse thus providing frequent opportunities to incorporate new parts? Or, do chlorophyll molecules (and others?) migrate through the membrane to functional sites? What is the unit of synthesis and assembly of plastid membranes? None of these questions can be answered at present.

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