Plant Physiol. (1973) *51*, 651–659

Chloroplast Maintenance and Partial Differentiation *in Vitro*[1]

Received for publication June 19, 1972

CONSTANTIN A. REBEIZ,[2] SUSAN LARSON, T. ELLIOT WEIER, AND PAUL A. CASTELFRANCO
Department of Botany, University of California, Davis, California 95616

ABSTRACT

Tissue homogenates, etioplasts, and developing chloroplasts were prepared from cucumber (*Cumucis sativus* L.) cotyledons in tris-sucrose. They were incubated aerobically in the dark or in the light at pH 7.7 in the presence or absence of a cofactor mixture containing coenzyme A, glutathione, potassium phosphate, methyl alcohol, magnesium, nicotinamide adenine dinucleotide, and adenosine triphosphate. These cofactors were previously shown to be essential for protochlorophyll and chlorophyll biosynthesis. Ultrastructural changes were monitored by electron microscopy. The following observations were made. (*a*) Crude homogenates contained agents which degraded etioplasts and developing chloroplasts. (*b*) Added cofactors were essential for the maintenance of the membrane structure; they were also implicated in the transformation of the prolamellar body in the absence and presence of light. (*c*) Light pretreatment of the cotyledons improved the maintenance of the developing chloroplast membranes during subsequent *in vitro* incubation. (*d*) In the presence of the cofactors, grana formation appeared to take place in the absence of nuclear-cytoplasmic control.

In recent years considerable efforts have been devoted to evaluating the nutritional, reproductive, and developmental autonomy of chloroplasts (1, 6, 8, 9). These organelles appear to possess certain amounts of RNA and DNA as well as the machinery for the biosynthesis of lipid, protein, and nucleic acids (9). They seem capable of dividing *in vitro* (17) and of forming their own protochlorophyll and chlorophyll from simple substrates (14, 15, 22). The differentiation of etioplasts into chloroplasts during greening is accompanied by substantial synthesis and accumulation of chlorophyll (11), carotenoids (12, 13), insoluble proteins, and colorless lipids (10). Likewise, dividing chloroplasts must grow before reaching full size (17). An assessment of cellular control during chloroplast differentiation, growth, maintenance, and division is therefore indispensable in evaluating the degree of autonomy of this organelle.

The study of the foregoing developmental phenomena in cell-free preparations is likely to be fruitful. Indeed, an isolated etioplast preparation capable of complete differentiation *in vitro* should constitute an ideal system for these studies (14, 15). Although such a system is presently unavailable, we have found that plastid preparations capable of partial differentiation *in vitro* can yield useful information. As reported earlier, the structure of some plastid preparations capable of chlorophyll biosynthesis *in vitro* was remarkably well preserved after prolonged incubations with cofactors (14, 15). Further examination of this cell-free system revealed that in the absence of organized cellular control various ultrastructural changes took place. Under our best incubation conditions grana formation appeared to take place *in vitro*.

In this communication we describe the ultrastructural changes that take place during incubation of etioplasts and developing chloroplasts *in vitro*.

[1] This work was supported in part by Research Grants GB-12906 and GB-31261 from the National Science Foundation and by funds from the Illinois Agricultural Experiment Station.

[2] Communications concerning this paper should be addressed to: Constantin A. Rebeiz, Department of Horticulture, University of Illinois at Urbana-Champaign, Urbana, Ill. 61801.

MATERIALS AND METHODS

Growing, Harvesting, and Irradiating Cucumber Cotyledons. Cucumber seeds (*Cucumis sativus* L. cv. Alpha Green) were germinated in vermiculite (Terra Lite) at 24 C in complete darkness for 4.5 days (14, 15). Cotyledons to be preirradiated were harvested with full hypocotyl hook as previously described (7). They were placed in beakers with enough distilled H_2O to keep them moist and were illuminated with 240 ft-c of white fluorescent light at 28 C for 2.5 or 4.5 hr (15).

Chemicals. The commercial sources of chemicals were reported elsewhere (14, 15).

Preparation of Unfortified and Fortified Crude Homogenates. "Unfortified" crude homogenates were prepared from 4.5-day-old cotyledons as follows. Four grams of etiolated or greening cotyledons were gently ground with mortar and pestle without sand in 6.0 ml of 0.5 M sucrose and 0.2 M tris-HCl, pH 8.0, at 0 to 5 C. The brei was filtered through four layers of cheesecloth. About 5 ml of unfortified crude homogenates were obtained. "Fortified" crude homogenates were prepared by grinding 4 g of etiolated or greening cotyledons in 6.0 ml of fortified tris-HCl and 0.5 M sucrose, pH 8.0, containing 5 μmoles of GSH, 0.3 μmole of CoA, 0.5 μmole of $MgCl_2$, 50 μmoles of potassium phosphate, 24 μmoles of methanol, 0.4 μmole of ATP, and 0.075 μmole of NAD per ml of fortified buffer. The brei was filtered as described above. About 5 ml of fortified crude homogenates were obtained.

Preparation of Etioplasts and Developing Chloroplasts. "Fortified" and "unfortified" etioplasts or developing chloroplasts refer to isolated organelle preparations obtained from the respective fortified or unfortified crude homogenates. Five milliliters of the fortified or unfortified crude homogenates were centrifuged at 200*g* for 3 min. The pellet was discarded, and the supernatant was centrifuged at 1500*g* for 7 min. The pellet obtained from the unfortified crude homogenate was resuspended in 4 ml of 0.5 M sucrose and 0.2 M tris-HCl, pH 7.7. Such suspensions are referred to as unfortified etioplasts or unfortified developing chloroplasts, as the case may be. The pellet obtained from a fortified crude homogenate was resuspended in 4 ml of fortified 0.2 M tris-HCl and 0.5 M sucrose,

pH 7.7, containing 5 μmoles of GSH, 0.3 μmole of CoA, 0.5 μmole of $MgCl_2$, 50 μmoles of potassium phosphate, 24 μmoles of methanol, 0.4 μmole of ATP, and 0.075 μmole of NAD per ml of fortified buffer. Such plastid suspensions are referred to as fortified etioplasts or fortified developing chloroplasts. All manipulations involving etiolated preparations were performed in the dark under a weak green safelight. Preparations derived from greening cotyledons were handled under subdued laboratory light of about 10 ft-c at bench level.

Incubation. Four milliliters of unfortified and fortified crude homogenates, unfortified and fortified etioplasts, and unfortified and fortified developing chloroplasts were incubated in cylindrical flat bottom glass tubes (2 × 10 cm) on a metabolic shaker operated at about 50 shakes/min. The incubations were performed at 28 C, either in complete darkness or under 10 ft-c of white fluorescent light for 16 hr. Reaction mixtures that were incubated in the absence of light were wrapped in black cloth and covered with aluminum foil, and the incubation was carried out in the darkroom. The unfortified reaction mixture (5.0 ml) at pH 7.7 contained 4.0 ml of unfortified crude homogenates or unfortified plastid preparations, 800 μmoles of tris-HCl, 2 mmoles of sucrose, and 120 nmoles of ALA.[3] Five milliliters of fortified reaction mixture at pH 7.7 contained 4.0 ml of fortified crude homogenates or fortified plastid preparations, 800 μmoles of tris-HCl, 2 mmoles of sucrose, 120 nmoles of ALA, 200 μmoles of potassium phosphate, 2 μmoles of $MgCl_2$, 20 μmoles of GSH, 1.2 μmoles of CoA, 2.4 mmoles of methyl alcohol, 1.6 μmoles of ATP, and 0.3 μmole of NAD. Preirradiated cotyledons excised with full hypocotyl hook were simultaneously incubated in a minimal volume of distilled H_2O under the same light and temperature conditions.

Recovery of the Incubated Plastids from Unfortified and Fortified Reaction Mixtures Containing Crude Homogenates. At the beginning or end of incubation, unfortified or fortified reaction mixtures containing crude homogenates were centrifuged at 200*g* for 3 min to sediment debris. The pellet was discarded. The resulting supernatant was centrifuged at 1500*g* for 7 min to sediment the plastids.

Recovery of the Incubated Plastids from Unfortified and Fortified Reaction Mixtures Containing Isolated Plastic Preparations. At the beginning or end of incubation the unfortified or fortified reaction mixtures containing etioplasts or developing chloroplasts were centrifuged at 1500*g* for 7 min to sediment the plastids.

Electron Microscopy. The plastid pellets were suspended in 1 ml of their respective incubation medium, and equal volumes of 4% glutaraldehyde were added. The suspensions were kept in the dark at room temperature for 45 min, then centrifuged at 1500*g* for 10 min.

Pieces of cotyledons were fixed with 5% glutaraldehyde in 0.15 M phosphate buffer at pH 7.2 for 1 hr and were washed thoroughly in buffer. Both cotyledons and plastid pellets were postfixed in 1% OsO_4 in 0.15 M phosphate buffer, pH 7.2. Dehydration was by an ethanol-propylene oxide series followed by embedding in a low viscosity epoxy resin according to Spurr (18). Sections were cut with a diamond knife in a Porter-Blum MT1 microtrome and stained with a 2% aqueous uranyl acetate solution followed by lead citrate (16). Sections were examined in a Hitachi HU-11 electron microscope. Three grids were prepared from each pellet. Examination on the microscope preceded any evaluation of the final prints which were prepared by a technician without knowledge of the profile types desired. The only selection consisted of photographing etioplasts from each grid that showed some structure. Two hundred etioplasts from each grid were reviewed.

RESULTS

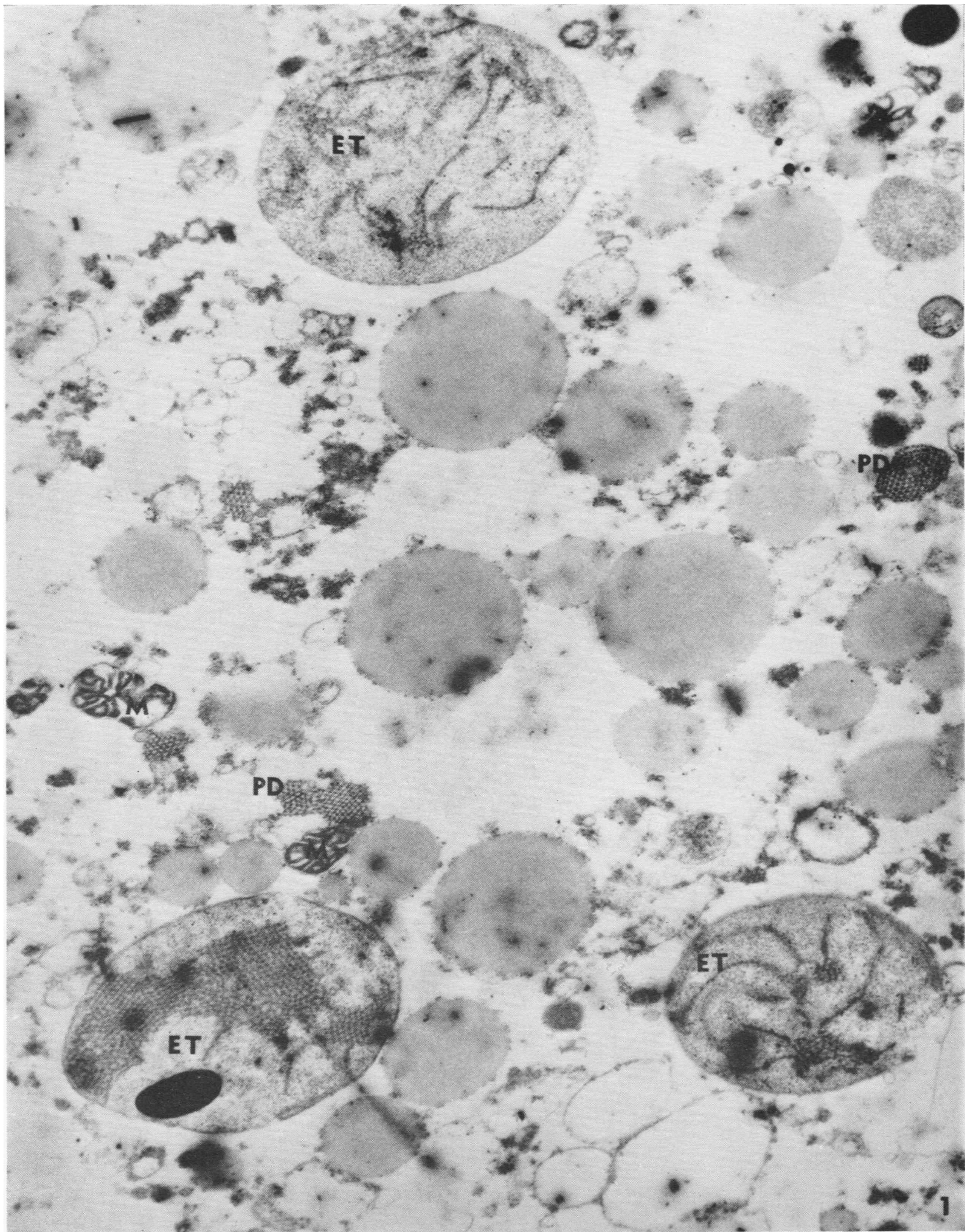
Ultrastructural Changes of Etioplasts after Incubation of Unfortified and Fortified Crude Homogenates in the Dark. Figure 1 depicts the components of an unfortified crude homogenate that sediment with the etioplasts at zero time. Note the presence of intact and damaged etioplasts, prolamellar body debris, and mitochondria as well as other particulate components. Figure 2A depicts a typical etioplast as found in the unfortified crude homogenate at the beginning of incubation. The fixation image of the stroma is uniformly finely gray-granular with some black granules and irregular areas free from electron-dense material. The prolamellar body is in the crystalline phase and has several peripheral lamellae radiating from it. There are occasional membrane overlaps in the peripheral lamellae thought to indicate the initial stacking step in the formation of grana (20). Similar typical etioplasts are also present at zero time in the fortified crude homogenate (Fig. 2C).

After 16 hr of dark incubation in the absence of cofactors the unfortified crude homogenate appeared severely bleached. While etioplasts could not be positively identified, it is thought that the electron-dense homogeneous profiles with heavy osmiophilic centers represent etioplasts with a strongly degraded prolamellar body (Fig. 2B). These profiles indicate a complete loss of the structured molecular organization of the etioplasts. Typical etioplasts were not observed after 16 hr of incubation in the dark in the fortified crude homogenate containing the cofactors required for chlorophyll synthesis (Fig. 2D). However, some degraded etioplasts were tentatively identified as such (Fig. 2D). Many reticular profiles were present, some sectors of which were associated with a double membrane that could be interpreted as a plastid envelope (Fig. 2D). Thus, although the cofactors do not fully protect the etioplasts in the crude homogenate during the 16-hr dark incubation period, they seem to retard the rate of breakdown.

Ultrastructural Changes of Isolated Etioplasts Incubated in the Dark in the Absence and Presence of Cofactors. Unfortified etioplasts incubated in the dark for 16 hr in the absence of cofactors lost much of their structural organization (Fig. 3A). For example, all the plastids in a representative sample of 15 profiles were highly degraded. In most cases there remained only a vesicular stroma (Fig. 3A, right) which generally lacked an outer envelope. There was present in some etioplasts a limiting outer boundary that could be interpreted as a partially degraded outer envelope (Fig. 3A, left). No lamellar structures were observed.

Fortified etioplasts incubated in the dark for 16 hr in the presence of cofactors retained much of their structure. In a representative sample of 15 profiles, 5 etioplasts exhibited a distinct prolamellar body, including peripheral lamellae surrounded by a granular, moderately reticulate stroma (Fig. 3B). The prolamellar body was not crystalline as might be expected since it had received no irradiation, but rather resembled a reacted prolamellar body, that is, one that has lost its paracrystalline structure (Fig. 4A). Reacted prolamellar bodies are normally observed in etioplasts from seedlings that have received a short exposure to light (21). The envelope, while present, only infrequently showed its double nature. The remaining 10 profiles lacking prolamellar bodies were membrane-bound organelles. They are interpreted as sections of regions to one side of the prolamellar body. No irregular reticulate profiles

[3] Abbreviation: ALA: δ-aminolevulinic acid.



FIGS. 1 to 5. E: Envelope; G: granum; I: invagination and/or interaction of inner component of envelope with peripheral lamellae; Ol: overlapping of membranes in peripheral lamellae, possibly incipient grana; Pb: prolamellar body, crystalline or reacted state; PD: prolamellar body debris; Pl: peripheral lamellae; S: stroma; St: starch; ET: etioplast; M: mitochondria.

FIG. 1. Etioplast pellet sedimented from an unfortified crude homogenate at zero time. × 9,000.

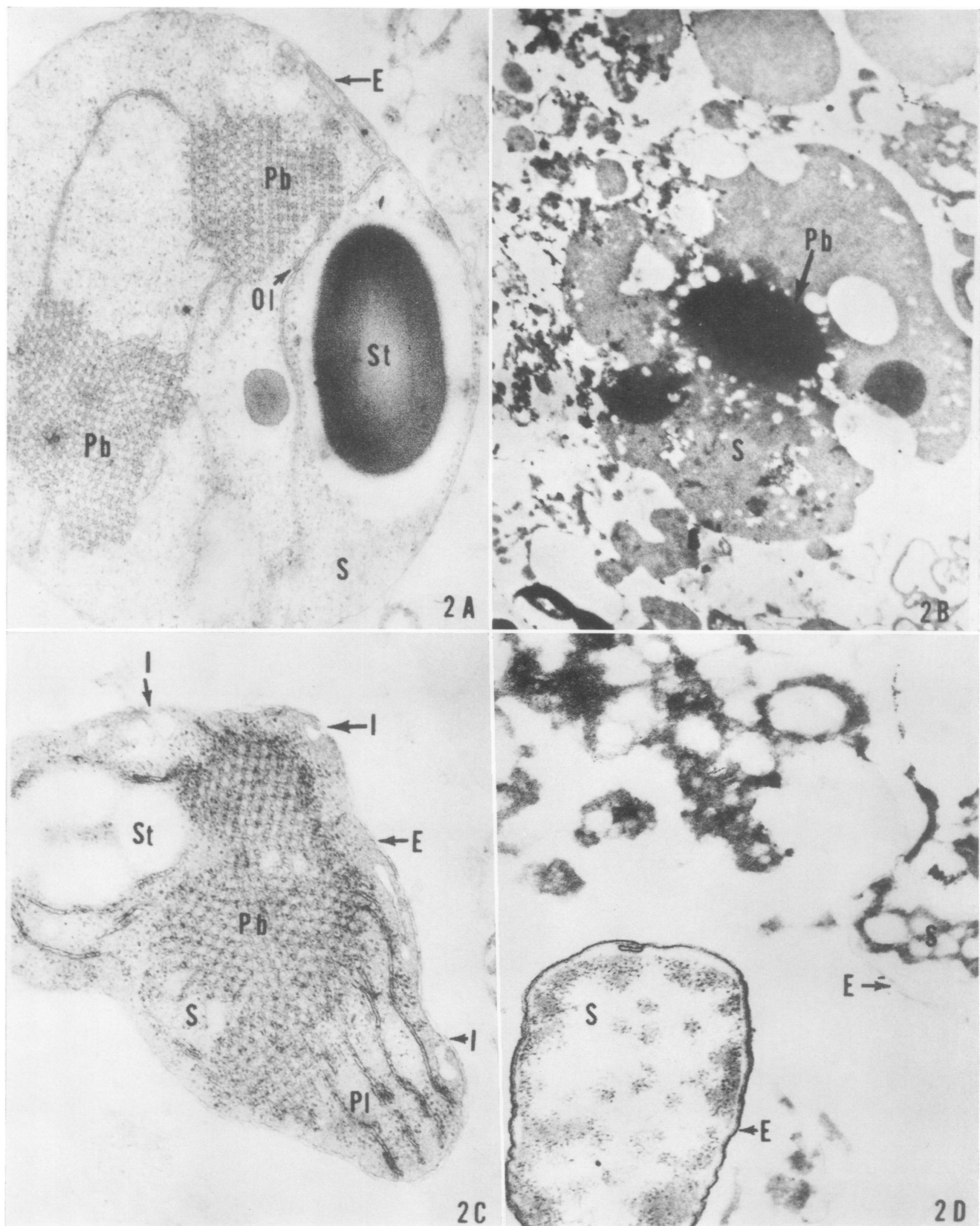


FIG. 2. Etioplasts in the crude homogenate: plastids sedimented from unfortified crude homogenates, A: before incubation; B: after 16-hr dark incubation without cofactors: plastids sedimented from fortified crude homogenates; C: before incubation; D: after 16-hr dark incubation with cofactors. × 18,000.

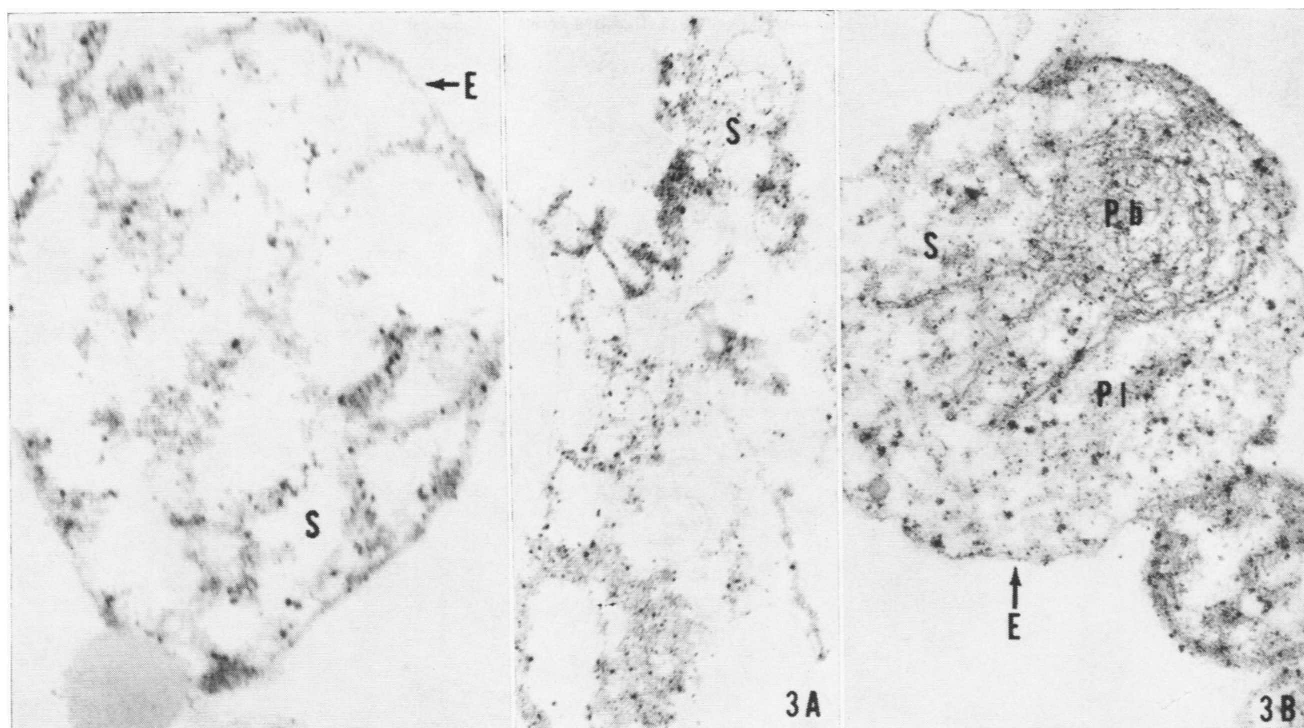


FIG. 3. Isolated etioplasts after 16 hr of dark incubation in the absence and presence of cofactors: A: two profiles of unfortified etioplasts after 16 hr of dark incubation in the absence of cofactors; B: fortified etioplasts after 16 hr of dark incubation in the presence of cofactors. × 18,000.

were observed such as in unfortified etioplasts incubated without cofactors (Fig. 3A, left). The cofactors clearly protect the etioplasts in free suspension from degradation in the dark.

Ultrastructural Changes of Developing Chloroplasts Prepared from 2.5-hr-preilluminated Cotyledons and Incubated in the Light in the Absence and Presence of Cofactors. Greening cucumber cotyledons irradiated for 2.5 hr contained etioplasts with a reacted prolamellar body and incipient two-compartment grana (Fig. 4A) associated with the peripheral lamellae. The plastids in a representative sample of 18 profiles were very similar to the one shown in Figure 4A. The usual picture suggesting membrane proliferation from the inner component of the plastid envelope is also well illustrated. These cotyledons had just emerged from the lag phase and had started forming chlorophyll *b* (11). Fortified homogenates prepared from these cotyledons were able to synthesize chlorophyll *a* but not chlorophyll *b* (15).

Unfortified and fortified plastids isolated from irradiated cotyledons are shown just before incubation (Fig. 4 B, D). They are similar in structure to etioplasts observed in cotyledonary tissue, even to the presence of two-compartmented grana in the peripheral lamellae. No change is induced during isolation whether or not the cofactors are present. However, invaginations from the inner component of the plastid envelope were not distinct in the isolated etioplasts.

Unfortified plastids incubated in the light for 16 hr in the absence of cofactors underwent considerable disorganization (Fig. 4C). In a representative sample of 25 profiles all the plastids were highly degraded. Some degraded etioplasts still possessed an outer envelope or part of one. A much contracted, highly electron-dense prolamellar body is present, embedded in vesicular stroma. Peripheral lamellae are entirely lacking. The vesicular nature of the stroma is difficult to interpret. The individual vesicles appear to be membrane-bound, and they lack association with the prolamellar body.

Greening fortified plastids incubated for 16 hr in the light with the cofactors required for chlorophyll synthesis retained much of their normal structure (Fig. 4E). In a representative sample of 28 profiles, 13 exhibited well preserved structures. The intact outer envelope, reacted prolamellar body with peripheral lamellae, and incipient grana are easily identified. One of the peripheral lamellae is associated with the envelope (Fig. 4E). Invagination of the inner component of the envelope was not observed.

Simple overlaps in the membranes of the peripheral lamellae usually occur at this stage of development, and they do appear in the peripheral lamellae of the etioplasts *in situ* and of the etioplasts isolated both with and without cofactors. Grana formation is usually confined to short (0.5–1 μ) lengths of the peripheral lamellae (Fig. 4, A, B, D). However, during incubation in the presence of the cofactors, grana appear to form over much of the length of the peripheral lamellae in most of the preserved plastids (Fig. 4E).

Ultrastructural Changes of Developing Chloroplasts Prepared from 4.5-hr-irradiated Cotyledons, Incubated in the Light in the Absence and Presence of Cofactors. Greening cucumber cotyledons irradiated for 4.5 hr with 240 ft-c of white fluorescent light contained plastids with reacted prolamellar bodies and two- to four-compartment grana arranged along the numerous radiated peripheral lamellae (Fig. 5A). In a representative sample of 23 profiles the plastids were very similar to the one shown in Figure 5A. The outer ends of several of the peripheral lamellae are closely associated with the outer envelope, giving the usual impression of reaction between envelope and peripheral lamella (Fig. 5A) (20). Such cotyledons had emerged from the lag phase and accumulated chlorophyll *a* and *b* vigorously (11). Homogenates prepared from these cotyledons were also capable of chlorophyll *a* and *b* biosynthesis (15).

When the irradiated cotyledons were moistened with distilled

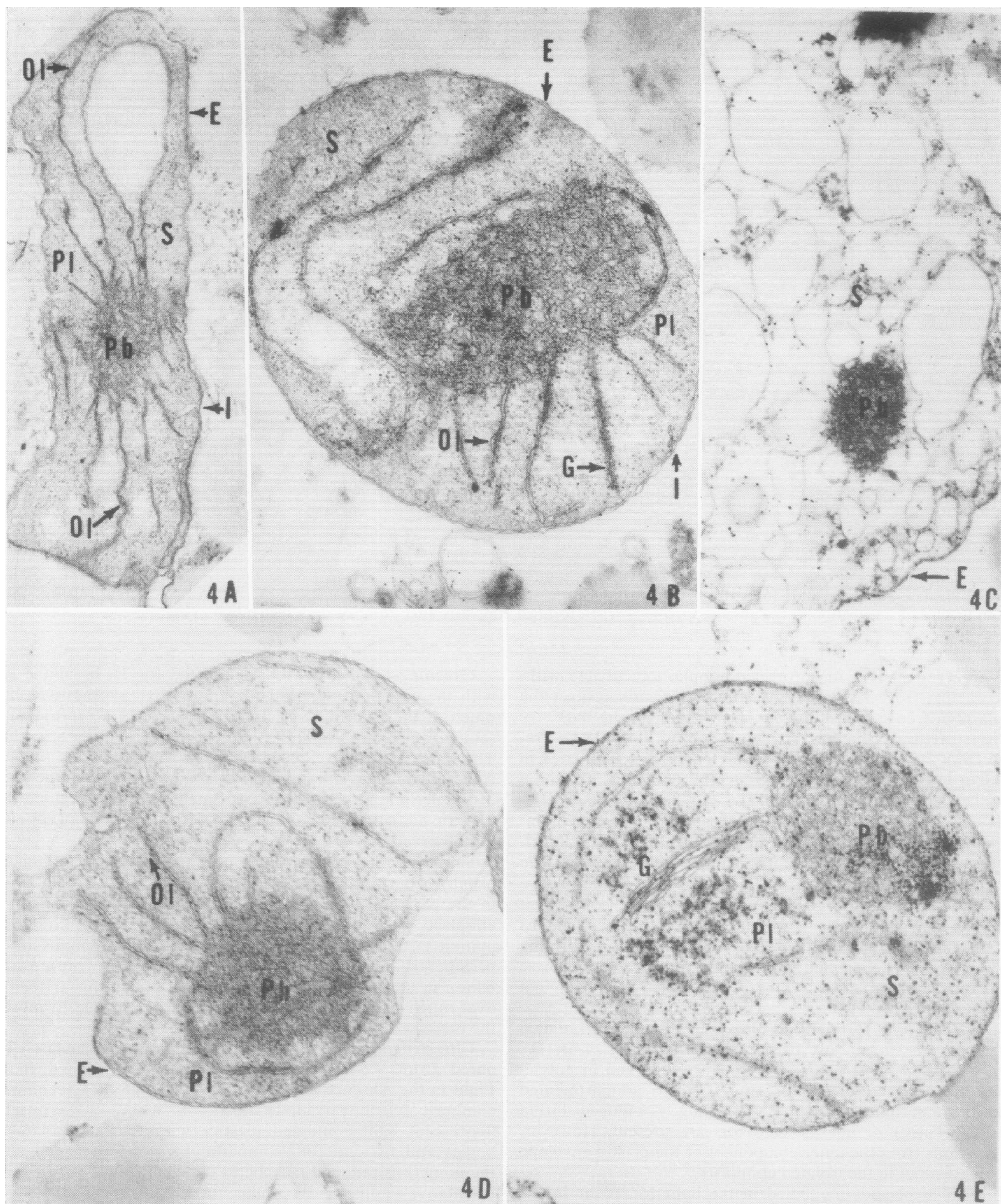


FIG. 4. Developing chloroplasts, isolated from cotyledons after irradiation for 2.5 hr and incubated in low light intensity for 16 hr. A: *In situ* plastid from irradiated cotyledon; B: unfortified plastid at zero time; C: unfortified plastid after 16-hr incubation without cofactors; D: fortified plastid at zero time; E: fortified plastid after 16-hr incubation with cofactors. × 18,000.

H_2O and exposed to 10 ft-c of white fluorescent light for 16 hr, the prolamellar bodies reverted to the crystalline condition and some two- and four-compartment grana grew into six-compartment grana (Fig. 5B).

When unfortified developing chloroplasts were isolated from 4.5-hr-irradiated cotyledons and incubated for 16 hr in subdued light in the absence of the cofactors, extensive degradation of the chloroplast structure occurred (Fig. 5C). In a repre-

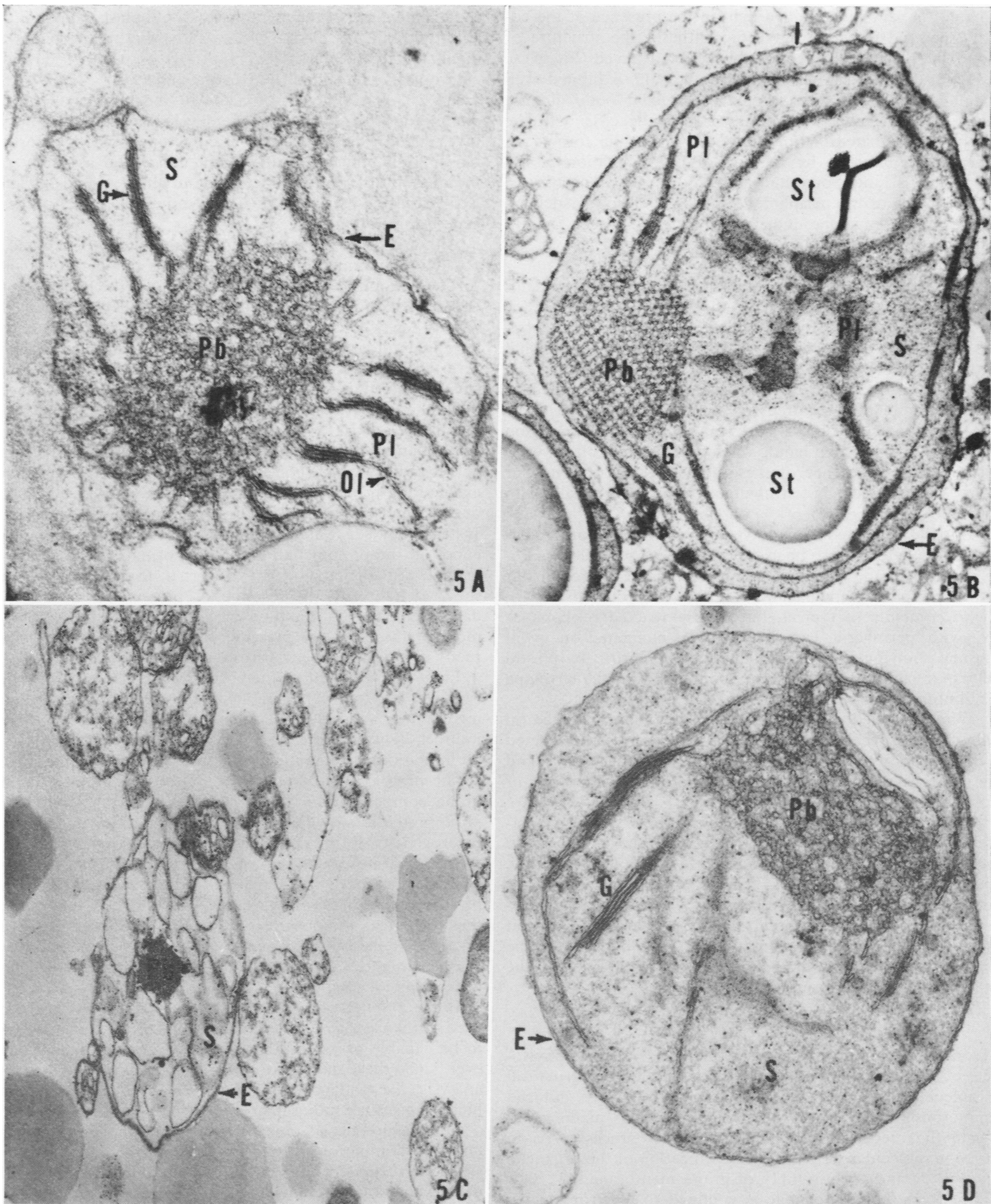


FIG. 5. Developing chloroplasts, isolated from cotyledons irradiated for 4.5 hr and incubated in low light for 16 hr. A: *In situ* plastid from irradiated cotyledon; B: *in situ* plastid from irradiated cotyledons incubated in low light for 16 hr; C: unfortified plastids after 16-hr incubation without cofactors; D: fortified plastid after 16-hr incubation with cofactors. C: × 9,000; all others: × 18,000.

sentative sample of 60 profiles all the plastids were highly degraded. The outer envelope was broken. The tubular structure in the prolamellar body was largely obliterated, and it remained as a very electron-dense body, indicating disintegration. No peripheral lamellae were present. The stroma is vesicular, and although the vesicles appear to be membrane-bound no evidence of their origin is apparent.

When fortified developing chloroplasts were isolated and

incubated for 16 hr in subdued light in the presence of the cofactors required for chlorophyll biosynthesis, the structure of some of the plastids was remarkably well preserved (Fig. 5D). In a representative sample of 29 profiles, 12 exhibited well maintained structures. The reacted prolamellar body did not revert to the crystalline condition, as was the case with the chloroplasts incubated *in situ* (Fig. 5B). The grana formed on the peripheral lamellae by the end of the incubation period were unusually long. Some had as many as 10 compartments. The end of one of the peripheral lamellae is closely associated with the plastid envelope, indicating reaction between the envelope and the peripheral lamellae.

Summary of Electron Micrographs.

1. Incubation of fortified and unfortified crude homogenates with and without the addition of the cofactors required for chlorophyll biosynthesis resulted in the breakdown of the etioplasts (Fig. 2B). The process of degradation was somewhat retarded in the presence of the cofactor mixture (Fig. 2D).

2. When unfortified etioplasts were incubated in the dark without the cofactors required for chlorophyll biosynthesis *in vitro,* the prolamellar body underwent complete disintegration (Fig. 3A). On the other hand, when fortified etioplasts were incubated in the presence of these cofactors, the prolamellar body was maintained, but it changed from the crystalline to the reacted state (Fig. 3B).

3. Etioplasts isolated from cotyledons after 2.5 hr of irradiation and incubated for 16 hr in subdued light showed: (*a*) *without cofactors:* an electron-dense disorganized prolamellar body, no peripheral lamellae, and a vesicular stroma (Fig. 4C); (*b*) *with cofactors:* a typical, but diffuse reacted prolamellar body, with peripheral lamellae bearing elongated incipient grana and showing some terminal association with the plastid envelope; invagination of the inner component of the envelope was not noted (Fig. 4E).

4. Whole cotyledons irradiated 4.5 hr and incubated in distilled H_2O in weak light showed a reversion of the reacted prolamellar body to the crystalline state, a typical stroma, and some evidence of the invagination of the inner component of the plastid envelope (Fig. 5B).

5. Etioplasts isolated from cotyledons after 4.5 hr of irradiation and incubated for 16 hr in the light showed: (*a*) *without cofactors:* an electron-dense disorganized prolamellar body, no peripheral lamellae, a vesicular stroma, and a broken outer envelope (Fig. 5C); (*b*) *with cofactors:* a typical reacted prolamellar body, with peripheral lamellae bearing somewhat elongated grana. Some had as many as 10 compartments. The ends of some peripheral lamellae were associated with the envelope. The stroma was normal (Fig. 5D).

DISCUSSION

Chloroplast breakdown under physiological and nonphysiological conditions is a complex process. The loss of structural and molecular organization of the organelle may be induced by numerous factors. In this study no effort was made to investigate the mechanism of the breakdown processes or to describe in molecular terms the disorganization of the organelle structures (2) and the assembly of grana membranes (3). Our evidence for structural change is confined to electron microscopic observations.

Crude homogenates, containing all cell constituents, had a profound degradative effect on plastids (Fig. 2B). In the presence of the cofactors, this effect was slightly tempered (Fig. 2D). The plastid autodestructive activity was considerably less than that of the rest of the protoplasm.

The cofactors essential for protochlorophyll and chlorophyll synthesis *in vitro* (14, 15) were shown to be essential also for the maintenance of the membranes of etioplasts and developing chloroplasts (Figs. 3–5). This was so whether etioplasts were incubated in the dark (Fig. 3, A, B) or whether developing chloroplasts were incubated in the light (Fig. 4, D, E). The individual role of each cofactor in the maintenance of the membrane structures and in the development of grana was not investigated. The need of these cofactors for both chlorophyll biosynthesis and membrane maintenance and assembly raises the question of whether the action of the required cofactor(s) is a direct one on chlorophyll biosynthesis, on membrane biogenesis, or on both (14).

Although special precautions were taken to exclude any possible contamination by light during the dark incubation, fortified etioplasts incubated in the dark in the presence of the cofactors exhibited a reacted (21, 23) prolamellar body (Fig. 3B). When whole preirradiated cotyledons were incubated at low light intensity for 16 hr, the reacted prolamellar bodies reverted to the crystalline condition (Fig. 5B). Similar reversions were reported by Weier *et al.* (21). However, in developing chloroplasts isolated from preirradiated cotyledons and incubated for 16 hr in the presence of cofactors under the same illumination, the reacted prolamellar bodies failed to revert to the crystalline condition (Fig. 5D). These results tend to exclude the probability of contamination by light as the cause of prolamellar body transformation in incubated etioplasts. Instead they suggest that, *in situ,* light may exert an indirect effect on the prolamellar body transformation. The failure of reacted prolamellar bodies to revert to the crystalline state in subdued light and the reaction of prolamellar bodies in the dark might have a common origin. It is possible that one or more of the cofactors present in the reaction mixture are directly involved in tube transformation.

Light pretreatment of the cotyledonary tissue before plastid isolation improves the maintenance of the outer and inner membranes, as isolated plastids are incubated for 16 hr. Such indirect light effects may be mediated in several ways, *e.g.,* by inhibition of catabolic enzymatic reactions, by induction of repair mechanisms, or by light-induced differences in membrane composition. Evidence derived from the induction of photosynthetic activities (19) and the build-up of chlorophyll heterogeneity during greening (4, 5) suggests that the photosynthetic apparatus is assembled in steps. Therefore, it appears possible that these differences in the preservation of the inner plastid membranes might reflect light-induced differences in their chemical composition. Likewise, differences in the preservation of the etioplast and developing chloroplast outer envelopes might indicate that these two membranes are not necessarily identical.

In the absence of a complete cellular entity, but in the presence of exogenous cofactors, developing chloroplasts appear to be capable of assembling elongated grana (Fig. 4E). However, since these data were not subjected to detailed statistical analysis, the evidence for grana formation *in vitro* might be highly suggestive rather than conclusive.

Finally, no special precautions were taken to exclude bacterial contamination. Bacteria other than the anaerobic photosynthetic forms do not synthesize chlorophyll, and even these do not elaborate prolamellar bodies. The contribution of bacterial contamination to the maintenance of the membranes in the fortified incubations is unlikely since the contamination is probably identical in the fortified and unfortified plastid preparations.

LITERATURE CITED

1. Bogorad, L. 1968. *In:* Control Mechanisms in Developmental Processes, Suppl. 1. Academic Press, New York. pp. 1-31.

2. Chapman, D. and D. F. H. Wallach. 1968. *In:* D. Chapman, ed., Biological Membranes. Academic Press, New York. pp. 125–202.
3. Dawson, R. M. C. 1968. *In:* D. Chapman, ed., Biological Membranes. Academic Press, New York. pp. 203–232.
4. DeGreef, J., W. L. Butler, and T. F. Roth. 1971. Greening of etiolated bean leaves in far red light. Plant Physiol. 47: 457–464.
5. French, C. S., J. S. Brown, and M. C. Lawrence. 1972. Four universal forms of chlorophyll *a*. Plant Physiol. 48: 421–429.
6. Givan, C. V. and R. M. Leech. 1971. Biochemical autonomy of higher plant chloroplasts and their synthesis of small molecules. Biol. Rev. 46: 409–428.
7. Hardy, S. I., P. A. Castelfranco, and C. A. Rebeiz. 1970. Effect of the hypocotyl hook on greening in etiolated cucumber cotyledons. Plant Physiol. 46: 705–707.
8. Kirk, J. T. O. 1970. Biochemical aspects of chloroplast development. Annu. Rev. Plant Physiol. 21: 11–42.
9. Kirk, J. T. O. and R. A. E. Tilney-Basset. 1968. *In:* The Plastids. Freeman, London and San Francisco. pp. 302–398.
10. Luersen, K. 1970. Volumen, Trockengewicht und Zusammensetzung von Etioplasten und ihren Ergrünungsstadien. Z. Naturforsch. B. 25: 1113–1119.
11. Rebeiz, C. A. 1967. Studies on chlorophyll biosynthesis in etiolated excised cotyledons of germinating cucumber at different stages of seedling development. Magon, Sér. Sci. 13: 1–21.
12. Rebeiz, C. A. 1968. The chloroplast pigments of etiolated and greening cucumber cotyledons. Magon, Sér. Sci. 21: 1–25.
13. Rebeiz, C. A. 1968. Dark and light carotenoids accumulation in etiolated and greening cucumber cotyledons. Magon, Sér. Sci. 23: 1–10.
14. Rebeiz, C. A. and P. A. Castelfranco. 1971. Protochlorophyll biosynthesis in a cell free system from higher plants. Plant Physiol. 47: 24–32.
15. Rebeiz, C. A. and P. A. Castelfranco. 1971. Chlorophyll biosynthesis in a cell free system from higher plants. Plant Physiol. 47: 33–37.
16. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 18: 208–212.
17. Ridley, S. M. and R. M. Leech. 1970. Division of chloroplasts in an artificial environment. Nature 227: 463–465.
18. Spurr, A. Q. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31–43.
19. Thorne, S. W. and N. K. Boardman. 1971. Formation of chlorophyll *b*, and the fluorescence properties and photochemical activities of isolated plastids from greening pea seedlings. Plant Physiol. 47: 252–261.
20. Weier, T. E. and D. L. Brown. 1970. Formation of the prolamellar body in 8-day dark-grown seedlings. Amer. J. Bot. 57: 267–275.
21. Weier, T. E., R. D. Sjoland, and D. L. Brown. 1970. Changes induced by low light intensities on the prolamellar body of 8-day, dark-grown seedlings. Amer. J. Bot. 57: 276–284.
22. Wellburn, F. A. M. and A. R. Wellburn. 1971. Chlorophyll synthesis by isolated intact etioplasts. Biochem. Biophys. Res. Commun. 45: 747–750.
23. Wettstein, D. Von. 1967. Chloroplast structure and genetics. *In:* A. San Pietro, F. A. Greer, and T. D. Army, eds., Harvesting The Sun. Academic Press, New York. pp. 153–190.