FINE STRUCTURE AND PIGMENT CONVERSION IN ISOLATED ETIOLATED PROPLASTIDS

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ABSTRACT

Proplastids containing a prolamellar body were isolated from leaves of etiolated bean plants. The isolation methods do not necessarily lead to destruction of their submicroscopic structure and most of the isolated proplastids show well preserved outer membranes, lamellar strands, and the prolamellar body. Morphological intactness of the proplastids varies; certain leaf fractions contain single prolamellar bodies as well as proplastids. Since pellets after centrifugation between 350 *g* and 1000 to 3000 *g* contain intact proplastids and, as was shown by quantitative experiments, the same fractions show photoconversion of protochlorophyll to chlorophyll, it is supposed that the isolated particles probably retain many of the properties which are characteristic of them *in situ*. Isolated proplastids may thus be a valuable tool in investigations on the development of the photosynthetic apparatus.

In a preliminary note (5), we reported the isolation of proplastids from etiolated bean leaves and showed that contrary to the suggestion made by others (13) isolation does not necessarily cause destruction of the submicroscopic structure of these particles. The availability of proplastids isolated at various stages of development provides new possibilities for the study of their chemical composition, enzymatic activities, and the physiological factors affecting their development.

In previous studies with intact leaves, effects of light (1, 2, 10, 14) and temperature (4) on the development of plastid structure have been described. Such *in situ* studies, valuable as they are, provide no clear cut evidence of the direct effect of these factors on the plastids, since almost nothing is known about the interaction between these particles and the surrounding cytoplasm. Work with isolated plastids should overcome this difficulty.

In this paper we will report additional details on the isolation of the proplastids, some further observations on their internal structure, and some qualitative experiments on the photoconversion of protochlorophyll to chlorophyll in the proplastid-containing fractions.

MATERIAL AND METHODS

Etiolated bean seedlings of the local variety "Bulgarian" were grown in the dark and proplastids isolated from their first leaves as previously described (5). The isolation method consists mainly of homogenizing the leaf tissue in a phosphate buffer containing 0.7 M sucrose, squeezing the homogenate through cheesecloth, and isolating the various fractions by differential centrifugation. The phosphate buffer was adjusted to various pH values.

For pigment determinations the pellet containing the proplastids was extracted with 80 per cent acetone and the absorption spectrum of the extract was determined in the range of 600 to 700 mμ in a Beckman DU spectrophotometer. In a few cases, the pigments were transferred from acetone to ether. All these procedures were carried out in a cold room at 2 to 4°C, under weak green light. (Incandescent light filtered through a Chance filter O Gr 1.)

For photoconversion of the pigments the whole homogenate cleared of heavy debris was centrifuged at 350 *g*, and the entire supernatant fraction poured

into Petri dishes, brought up to 10°C, and illuminated for various periods of time. The particles were then sedimented by centrifugation either at 1500 *g* or at 3000 *g*, and the pellet was used for pigment extraction or fixation for electron microscopy as described previously (5). Where further washing was required the particles were sedimented, resuspended in new buffer, illuminated and resedimented again for pigment extraction and/or fixation.

RESULTS

The methods used in these investigations were adapted to yield sufficient material for electron microscopic investigations and qualitative pigment determination. No attempt was made to achieve quantitative isolation of the proplastids from the leaves. After the material retained in the cheesecloth and the sediment remaining after centrifugation at 350 *g* were discarded, the suspension contained 15 to 25 mg protein per 1 gm initial fresh weight of tissue. After further fractionation, the protein content of this suspension was distributed as follows:

Sediment after further centrifugation at 1000 *g*, 3 to 5 per cent

Sediment after further centrifugation at 3000 *g*, 3 to 5 per cent

Final supernatant fraction 90 per cent

The discarded sediment after centrifugation at

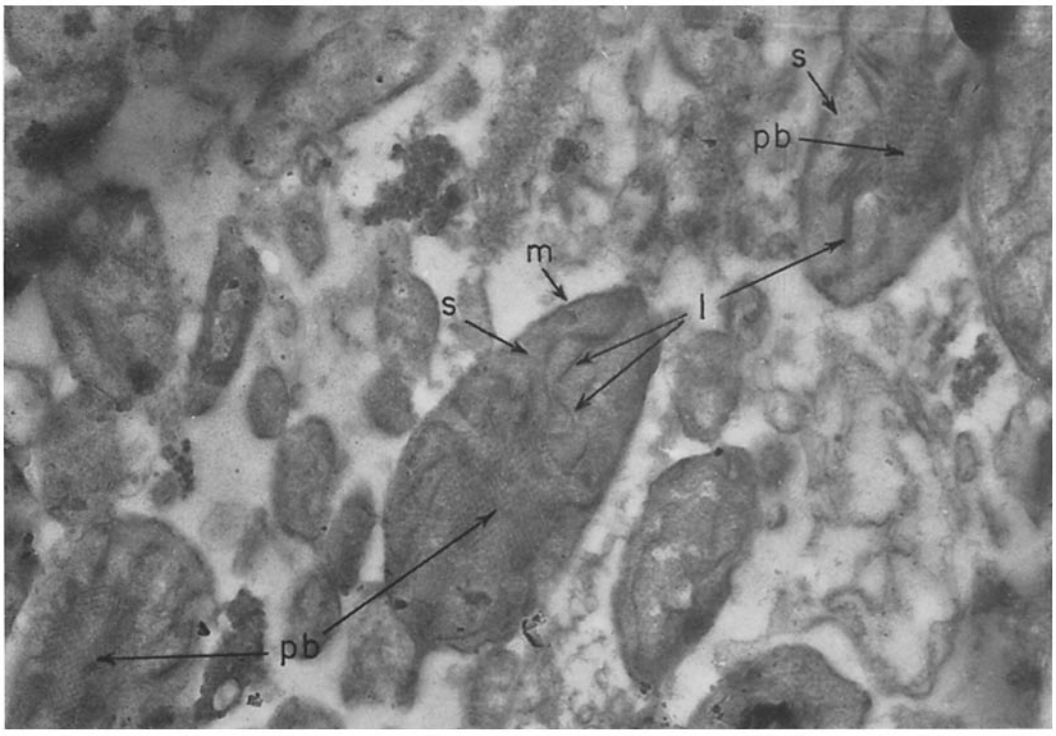

FIGURE 1

Section through pellet (3000 *g*) containing proplastids isolated from etiolated bean leaves. Plastid membrane *m;* prolamellar body *pb;* lamellar strand *l;* stroma *s.* × 19,000.

The light source was a white fluorescent tube giving 150 ft-c at the site of the suspensions. Before illumination the temperature of the suspension was brought up to 10°C.

Protein determinations were made according to Lowry *et al.*, using a Folin-Ciocalteu reagent (9).

The material was fixed in a 2 per cent Veronal-buffered OsO_4 solution at pH 7.4, carried through graded alcohols, and embedded in a 4:1 butyl-methyl methacrylate mixture. A Porter-Blum ultramicrotome equipped with a diamond knife was used for sectioning and the sections were investigated under an RCA EMU 3 electron microscope.

350 *g* contained unbroken cells, cell debris, hairs, and isolated particles. The fraction sedimented at 1000 *g* and 3000 *g* contained a large amount of intact and broken proplastids together with particulate contamination which could not be identified with certainty (Fig. 1). No hairs, unbroken cells, or identifiable cell walls occurred in them. Although no count was made of intact particles in the 1000 *g* and 3000 *g* pellets, more of the intact proplastids appeared to be in the 1000 *g* pellet. When the supernatant fraction after 3000 *g* was again centrifuged at 20,000 *g*, the pellet contained a large amount of mitochondria, but no recognizable proplastids. This electron microscopic finding was confirmed by determining α-ketoglutaric acid oxidation in the various fractions, using the Warburg method. Whereas almost no α-ketoglutaric acid oxidation occurred in the 1000 *g* and 3000 *g* fractions, such oxidation was very strong in the 20,000 *g* pellet. It may thus be assumed that only very low mitochondrial contamination occurred in the lower fractions.

The appearance of recognizable proplastids was dependent on the pH of the isolating medium. Thus no such particles could be seen after isolation at pH 5.5 and pH 6, but they occurred abundantly at pH 7 and 8. Therefore the 1000 *g* fraction of the leaf tissue homogenized at pH 8 was chosen for investigations into the structure of isolated proplastids. The higher pH was adapted taking into consideration that experiments on photoconversion of protochlorophyll usually are carried out at higher pH values (6, 11, 12).

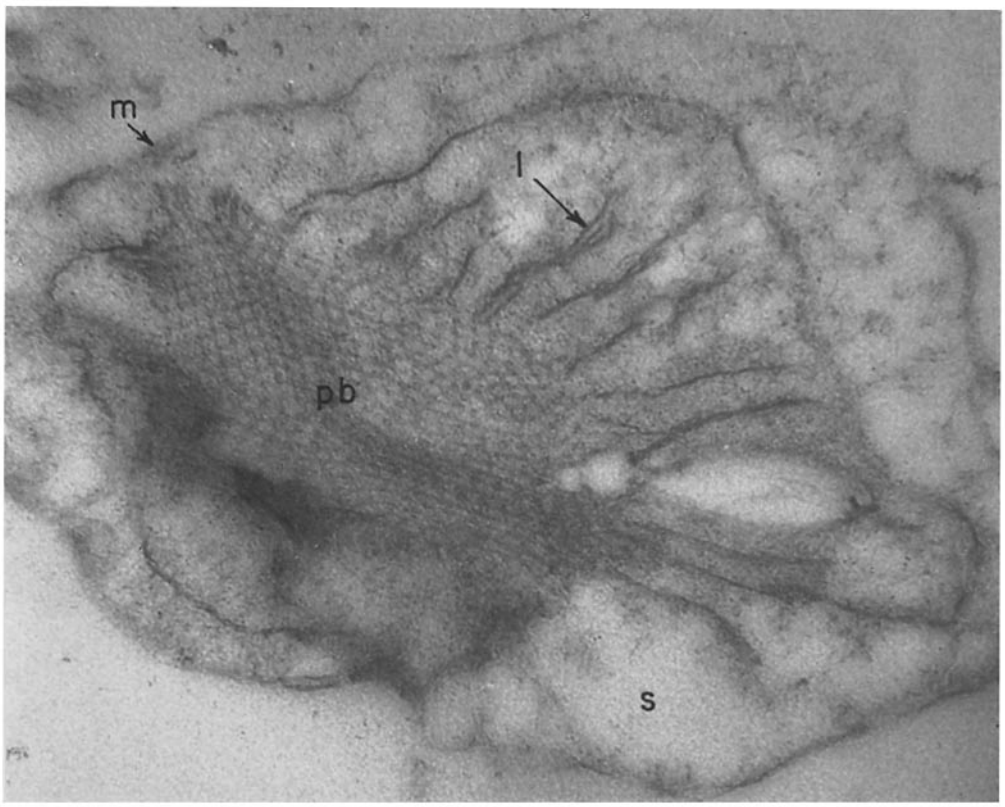

FIGURE 2

Isolated etiolated proplastid (1000 *g*) at higher magnification. Symbols as in Fig. 1. × 53,000.

As was stated in our preliminary note (5), no significant changes in the internal structure can be found between the isolated proplastids and proplastids *in situ*. The external membrane, the prolamellar body, and single strands are well preserved (Figs. 1 and 2). The size of the proplastids is less constant than in leaf tissue, indicating a

varying degree of swelling. Differences among the various proplastids could also be found in the density of the stroma, *i.e.*, that part of the plastid which is enclosed by the plastid membrane and which surrounds the prolamellar body and the strands. In Fig. 3 the stroma of the plastids marked *a* is significantly darker than the background outside the plastid, whereas in the plastids designated *b* this is not so. This indicates that in the various plastids the density of the stroma has changed

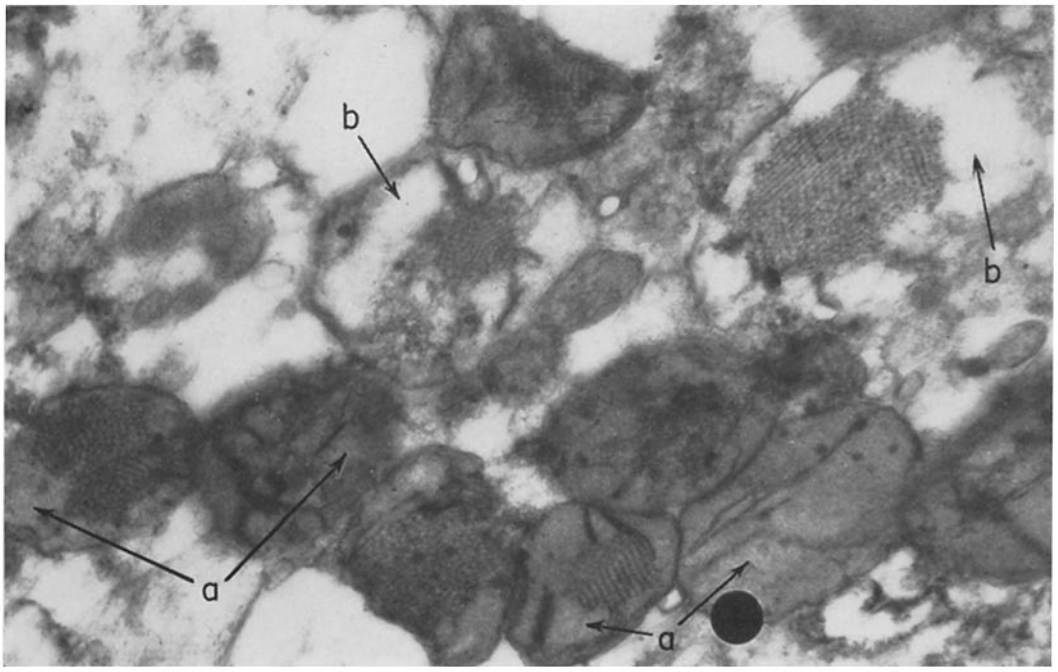

FIGURE 3

Isolated proplastids (3000 *g*) showing differences in stroma densities. Stroma in proplastids *a* is much darker than in proplastids designated *b*. Size of latex particle 0.34 μ. × 21,000.

either by swelling or contraction or by leakage of material from the plastid.

Fig. 4 gives the detail of the prolamellar body as it appears in at least part of the isolated proplastids. It may be interpreted as a lattice of fused vesicles, forming a tridimensional system of tubes, the interior of which is more dense than the intervesicular area. Although this structure appears quite frequently, it cannot be said that this is the only form of the prolamellar body in the isolated proplastids, since not all our preparations allowed this resolution.

The degree of intactness of the isolated proplastids varied. It is of special interest that "free" prolamellar bodies are frequently preserved without any surrounding membranes (Fig. 5). The "free" prolamellar bodies were found mostly in the precipitate after centrifugation at 1000 *g*, but occurred also in the precipitate after 3000 *g*. These structures have approximately the same dimensions as the prolamellar bodies inside the proplastids. The prolamellar body seems to be quite sturdy and less prone to destruction than the proplastid as a whole. In unpublished experiments on swelling of isolated proplastids in hypotonic media we have also found that whenever "vacuoles" were formed in the proplastids they usually occurred outside the prolamellar body.

Structures which may be interpreted as ghosts of proplastids can be seen too, although, since no serial sections were made, they may represent proplastids which were cut without intersecting the prolamellar body.

Protochlorophyll was found in all the fractions after centrifugation at 1000 *g* and 3000 *g* as well as in the remaining supernatant and, when exposed

to light, all fractions showed photoconversion to chlorophyll. In the proplastid fractions photoconversion occurred whether the particles were suspended in the primary extraction medium or in pure medium. For example, material spun down at 3000 *g* (discarding the sediment after centrifugation at 350 *g*) and resuspended in pure buffer prior to illumination showed, after exposure to light, a protochlorophyll-chlorophyll conversion. Though it cannot be excluded that contaminating particles in the proplastid fractions contributed to the conversion, these results nevertheless suggest that the conversion occurred in the proplastids themselves.

Varying the time of light exposure from ½ minute to 10 minutes did not affect the amount of chlorophyll formed. Although the ratio of initial protochlorophyll to chlorophyll formed in the light varied in different experiments, various light exposures carried out in the same experiment gave the same amount of chlorophyll (Fig. 6).

Investigations into the internal structure of the isolated proplastids illuminated for ½ to 10 minutes gave varying results. Frequently the plastids did not change; sometimes they seemed less well preserved than in the dark, and in some experiments the structure of the prolamellar body changed as shown in Fig. 7. The network which in the "dark" proplastids is seen to be more or less symmetrical in three dimensions seems to be less so after illumination, and already at low magnifications it can be seen that the prolamellar body is made up of elongated vesicles.

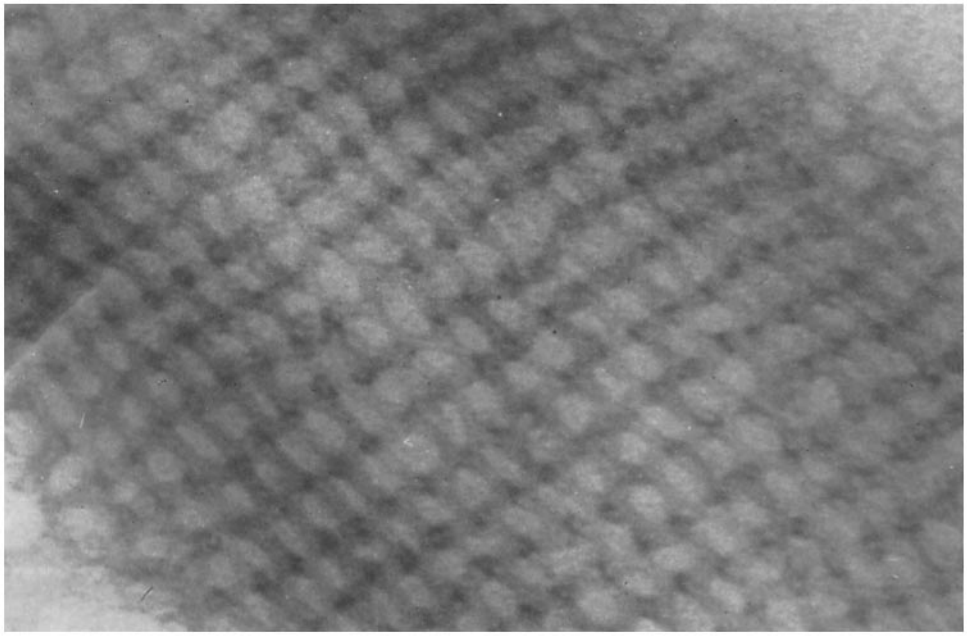

FIGURE 4

Detail from the prolamellar body of an isolated proplastid (1000 *g*). The structure is interpreted as part of a tridimensional network composed of fused tube-like vesicles. × 151,000.

DISCUSSION

The development of the submicroscopic structure of proplastids in etiolated plants has been described previously for a number of plants. In the bean plants, the material used here, development is similar to that described by Gerola *et al.* (2) for etiolated peas. Prior to the development of the prolamellar body, strands of vesicles are formed in the proplastids, which when viewed in the electron microscope at low magnifications may give the impression of lamellae. These strands are congruent, and at a certain stage a crystalline center is formed which grows to be the prolamellar body. In contrast with the pea proplastid, in which

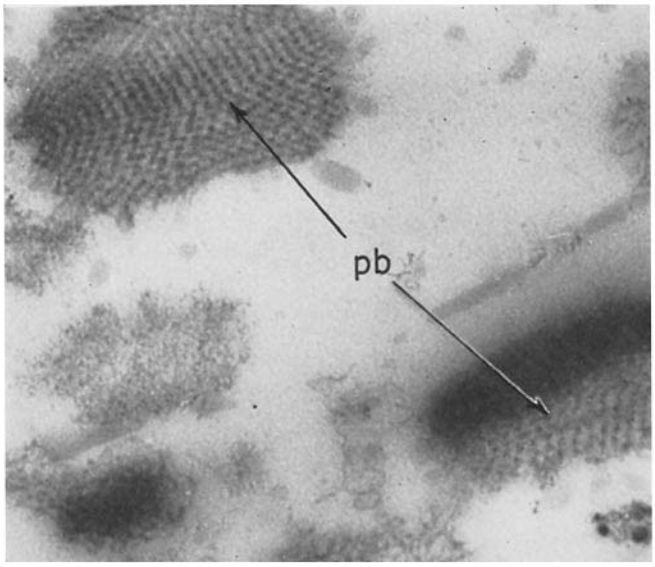

FIGURE 5

Prolamellar bodies (*pb*) as found in the proplastid-containing fractions (1000 *g*). These structures may remain as entities even after disruption of the outer membranes of the proplastid. × 27,000.

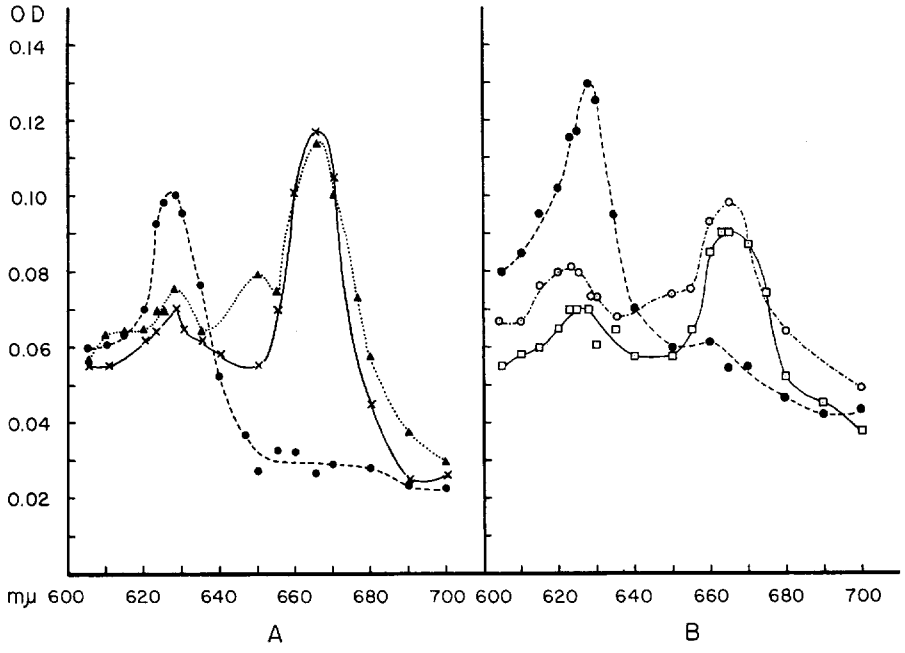

FIGURE 6

Absorption spectra of pigments from isolated material before and after illumination. Pellets after centrifugation at 3000 *g* of etiolated bean leaf extracts were resuspended, illuminated for various periods, and extracted with acetone. *A:* an experiment where extracts were illuminated for ½ min. and 5 min. *B:* illumination for 1 and 10 min. ●-----● before illumination; ×——× after illumination for ½ min.; ▲·····▲ after illumination for 5 min.; □——□ after illumination for 1 min.; ○·–·–·○ after illumination for 10 min.

the prolamellar body remains of a limited size, in the bean the prolamellar body may ultimately fill the whole proplastid (Shimoni and Klein, unpublished).

The proplastids in the material used here were therefore in a stage prior to the ultimate enlargement of the prolamellar body. The vesicular strands converging into the prolamellar body must be considered not as outgrowths of these not of single units but by interconnected tube-like vesicles. This network is probably a result of fusion of vesicles. A similar fusion of vesicles has been found by Von Wettstein in the prolamellar body of etiolated barley plants (14). It may be plausible to assume that at this stage the structure, similar to other membrane structures in the cell, is made up of lipoprotein compounds, although it probably also contains protochlorophyll.

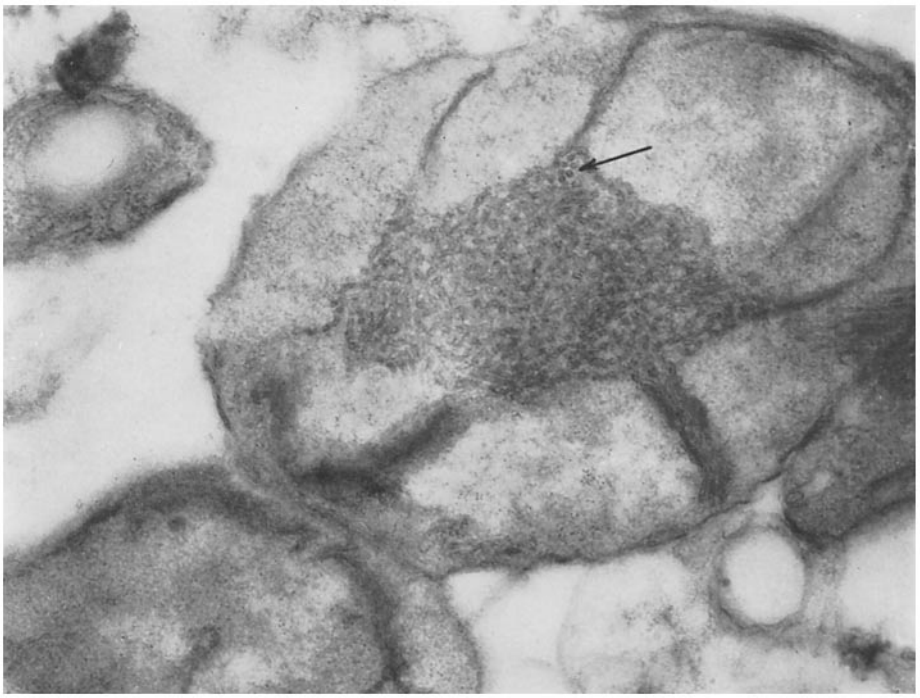

FIGURE 7

Isolated proplastids (3000 *g*) from etiolated plants after exposure to white light for ½ minute. Note the changes in the prolamellar body. The vesicles have elongated and their arrangement is less regular than before illumination. Most of the vesicles have been cut in a longitudinal direction. The arrow points towards a group which has been cut crosswise. × 49,000.

structures but as remnants of preformed strands. In a few cases, however, instead of these strands there occurred lamellae with double membrane structure. It cannot be ascertained whether this occurs usually in the dark or whether this is due to the influence of the weak green light used in the preparative procedure.

Smith has mentioned the possibility that the single units in the prolamellar body of *Eranthemum Leuconorum* (3) may actually be single protein-chlorophyll complexes (12). According to Fig. 4, however, the prolamellar body seems to be formed

The fact that the prolamellar body may remain as an entity even after disruption of the outer membranes of the proplastid is further evidence that its units are firmly connected. In some experiments, as shown in Fig. 7, a short light exposure seemed to cause a reorganization of the prolamellar body, resulting in an elongation of the vesicles and a change in their orientation. That the units still remain as tubes can be seen in the same figure in which most of the vesicles are cut longitudinally but at the arrow are cut crosswise. The fact that both the protochlorophyll-chlorophyll conversion

and the change in structure occur in resuspended pellets suggests that these changes are not dependent on extraplastid material. To what degree the pigment changes and structural differences after the light treatment are interlinked remains an open question.

The fact that the photoconversion of protochlorophyll was demonstrated also in fractions containing the intact isolated particles (sedimented at 1000 *g*) suggests that the holochrome-complex which is supposed to be the smallest entity capable of such photoconversion is actually a part of the organized structure of the proplastid and therefore probably located in the prolamellar body and/or in the strands connected with it.

Photochemical conversion of protochlorophyll to chlorophyll has already been observed in cell free material. Krasnovsky and Kossobutskaya (6, 7) and Smith (11, 12) have studied this conversion in supernatant fractions after high speed centrifugation. Krasnovsky *et al.* studied it also in whole leaf homogenates and in supernatant fractions after centrifugation at 1000 *g* and 3000 *g* (8). Except for the experiments with whole leaf homogenates, the conversion has therefore been studied in plastid free fractions, which contained chlorophyll-protein complexes or these complexes together with plastid fragments. In this work the emphasis has been on studying fractions containing intact particles, and therefore pellets formed after centrifugations only up to 3000 *g* were used. As not much definite information is available on the properties of etiolated proplastids, the only obvious criteria for their intactness up to now are the preservation of the structure and the possibility of photoconversion of protochlorophyll to chlorophyll. According to these criteria it appears that the isolated particles described here have retained their properties.

The further study of the chemical composition or physiological activity of isolated proplastids requires further refinements in isolation methods, since both the yield and the purity of the fractions are still low. The isolation methods employed here have made it possible only to carry out qualitative work with isolated proplastids. Experiments are in progress to achieve better information on the physiology of isolated proplastids.

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BIBLIOGRAPHY

1. GEROLA, F. M., CHRISTOFORI, F., and DASSIN, G., *Caryologia*, 1960, **13**, 164.
2. GEROLA, F. M., CHRISTOFORI, F., and DASSIN, G., *Caryologia*, 1960, **13**, 179.
3. HEITZ, E., *Experientia*, 1956, **12**, 476.
4. KLEIN, S., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 529.
5. KLEIN, S., and POLJAKOFF-MAYBER, A., *Exp. Cell Research*, 1961, **24**, 143.
6. KRASNOVSKY, A. A., and KOSSOBUTSKAYA, L. M., *Doklady Akad. Nauk. SSSR.*, 1952, **87**, 177.
7. KRASNOVSKY, A. A., and KOSSOBUTSKAYA, L. M., *Doklady Akad. Nauk. SSSR.*, 1953, **91**, 343.
8. KRASNOVSKY, A. A., and BYSTROVA, M. I., *Biokhymia*, 1960, **25**, 168.
9. LOWRY, O. H., ROSEBROUGH, N. R., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.
10. MÜHLETHALER, K., and FREY-WYSSLING, A., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 507.
11. SMITH, J. H. C., *Proc. 2nd. Intern. Congr. Photobiology*, Turin, 1957, Minerva Medica, Turin, 333.
12. SMITH, J. H. C., *Brookhaven Symposia in Biology*, 1958, **11**, 296.
13. STRUGGER, F., and KRIGER, L., *Protoplasma*, 1960, **52**, 230.
14. VON WETTSTEIN, D., *Brookhaven Symposia in Biology*, 1958, **11**, 138.