STUDIES ON THE GREENING OF DARK-GROWN BEAN PLANTS

I. FORMATION OF CHLOROPLASTS FROM PROPLASTIDS

By N. K. BOARDMAN* and JAN M. ANDERSON*

[*Manuscript received October* 3, 1963]

Summary

Bean plants were grown in the dark for 10–14 days and then transferred to continuous artificial light for varying periods. After illumination, the plastids were isolated and purified by differential and density-gradient centrifugation. The structure of the plastids was studied by phase-contrast and fluorescence microscopy.

Fluorescence microscopy showed that protochlorophyll was localized in discrete regions within the proplastid of the dark-grown plant. These regions appeared dark when viewed under phase-contrast and because of their size they have been named "1 μ centres". On illumination of dark-grown plants, there was a lag period of about 3 hr before chlorophyll was observed in regions of the plastid other than the 1 μ centres. However, the fluorescent 1 μ centres observed within the proplastid persisted for up to 10 hr after the dark-grown plants were illuminated.

Grana-like structures were seen in plastids isolated from plants which had been illuminated for at least 6 hr.

These observations are discussed in relation to published work on the fine structure of developing chloroplasts.

I. INTRODUCTION

The electron microscope has been used by several groups (see review by Granick 1955) to study the structural changes occurring during the development of proplastids into mature chloroplasts. The lamellated structure normally observed in higher plant chloroplasts is absent from the plastids of dark-grown leaves or from the young plastids of meristematic tissue. Instead, the proplastids may contain one or more dense vesicular centres, which have been termed prolamellar bodies (Hodge, McLean, and Mercer 1956).

The sites where chlorophyll is deposited within the plastid cannot be determined by electron microscopy, since chlorophyll is readily extracted during the fixation and embedding procedures. But chlorophyll may be detected in sections of living tissue or in isolated plastids by its characteristic red fluorescence under ultraviolet light.

In the work reported in this paper, the structural changes of developing bean plastids were studied by phase-contrast microscopy, while the sites of chlorophyll deposition within these plastids were determined by fluorescence microscopy. Concurrently, we have been studying both the development of photochemical activity (Anderson and Boardman 1964) and changes in the properties of the chlorophyll–protein complex during the greening of dark-grown plants in an attempt to obtain some correlation between the physical and chemical properties of chlorophyll and its role in photosynthesis.

* Division of Plant Industry, CSIRO, Canberra.

II. Materials and Methods

(a) *Plant Material*

Bean plants (*Phaseolus vulgaris* cv. Brown Beauty) were grown in the dark at 25°C and 80% R.H. in trays of Perlite which had been mixed with Hoagland nutrient solution. After 10–14 days, the trays were transferred to continuous artificial light and held at 25°C for varying periods. White fluorescent tubes provided an average intensity of 400 f.c. at the level of the leaves.

(b) *Isolation of Plastids*

The primary leaves were harvested, divided into 6-g lots, and ground in a sucrose–phosphate buffer in a Servall Omni-Mixer (Boardman and Wildman 1962). The plastids were isolated by sedimentation at 1000 *g* and purified further by centrifugation in a sucrose-gradient column, as described for the purification of proplastids (Boardman and Wildman 1962). The modified method was used in which 2-ml aliquots of plastid suspension were placed on aqueous sucrose columns prepared from 1·0, 1·2, and 1·0 ml, respectively, of solutions containing 240, 500, and 700 g/l of sucrose. Centrifugation was for 30 min at 124,000 *g*. Mature bean chloroplasts were isolated from plants grown in a glasshouse (Anderson and Boardman 1964).

(c) *Microscopy*

The structure of the plastids was studied by phase-contrast and fluorescence microscopy using a Zeiss research model microscope equipped with Neofluar phase objectives or, in fluorescence microscopy, with Planachromat objectives. For phase-contrast, illumination was either from a tungsten source or from a high-pressure mercury vapour lamp (Osram HBO 200) screened with a neutral density filter to reduce the light intensity. For fluorescence, the source was the high-pressure mercury vapour lamp used in conjunction with a purple filter, BG.12. Observations under phase-contrast were made at magnifications of 1250 while fluorescence was observed at magnifications of 800 or 1250.

A Zeiss camera attachment with basic body II was used for photography. With phase-contrast, Kodak Panatomic X film was used in conjunction with the Zeiss electronic flash attachment. Fluorescence was photographed either on a high-speed panachromatic film, Ilford HPS, or in colour on Kodak high-speed Ektachrome. Fluorescence observations made at a magnification of 1250 could not be photographed satisfactorily because of the small amount of light reaching the film.

III. Results

(a) *Purification of Plastids*

As observed previously with proplastids from dark-grown leaves (Boardman and Wildman 1962) sedimentation, in an aqueous sucrose column, of the plastids obtained from illuminated plants produced two sharp bands. Microscopic examination showed that the upper band contained both intact plastids and fragments while the lower band contained intact plastids. Although the lower band appeared

to be free of mitochondria, measurements of respiratory activity showed that it was unlikely that this was the case (Anderson and Boardman, unpublished data).

The developing plastids withstood the high osmotic conditions of the sucrose column remarkably well in distinct contrast to mature bean chloroplasts. The plastids as obtained from the tube after centrifugation were in a medium containing 1·5M sucrose. The high osmotic pressure caused a shrinkage of the plastids, giving them the appearance of compact spherical particles which were optically dense under phase-contrast microscopy. This was in contrast to their appearance in 0·4M sucrose before purification in the aqueous sucrose column. However, the plastids in 1·5M sucrose readily regained their original appearance when returned to 0·4M sucrose.

(*b*) *Microscopic Observations*

Under phase-contrast microscopy, proplastids isolated from dark-grown plants were seen as transparent particles, 3–5 μ in diameter (Plate 1, Fig. 1), but located within the particles were small discrete, optically dense bodies, 0·7–1·3 μ in diameter and which we have named "1 μ centres". In some of the proplastids even smaller bodies were seen. Fluorescence microscopy (Plate 2, Fig. 1) indicated that the protochlorophyll of the proplastid was confined to the 1 μ centres; no fluorescence was detected in the smaller bodies. Although up to four or five fluorescing centres have been observed in a single proplastid, it was more usual to find one or two such centres. No detailed structure was observed within the 1 μ centres of the proplastid.

The high intensity of illumination used to activate the fluorescence observable in the microscope resulted in the rapid conversion of 75–80% of the protochlorophyll of the isolated proplastid to chlorophyll *a* (Anderson and Boardman, unpublished data). Prolonged illumination caused a bleaching of the red fluorescence. It was observed that the fluorescence of the developing plastid was more susceptible to photobleaching than that of mature chloroplasts.

Illumination of dark-grown plants for periods of up to 3 hr did not result in any observable change in proplastid structure (Plate 1, Fig. 2). After 3 hr, the intensity of fluorescence within the 1 μ centres appeared to increase and a red fluorescence was beginning to be visible in the remainder of the plastid. After 4–5 hr some structure was seen in those parts of the plastid which were originally transparent and the intensity of fluorescence in these regions was enhanced slightly (Plate 2, Fig. 2).

Many of the plastids isolated from plants which had received 6 hr of illumination (Plate 1, Fig. 3) showed the indistinct grana-like structures observable with mature bean chloroplasts. The grana were seen first in the regions which were originally transparent and then throughout the plastid after the 1 μ centres disappeared. However, at 6 hr of illumination, about 80% of the plastids still contained 1 μ centres. Observations at the microscope suggested that the 1 μ centres of plastids obtained from plants illuminated for 6 hr were more strongly fluorescent than the 1 μ centres of proplastids, indicating that new chlorophyll was probably being deposited within these regions as well as the other regions of the plastid. There was a 10–12 fold increase in the average chlorophyll content of the plastid (Anderson and

Boardman 1964) before all the 1 μ centres finally disappeared. It is interesting to speculate that the 1 μ centres might be the sites of chlorophyll synthesis during the early stages of greening. A few isolated observations have been made of the appearance of grana-like structures within the 1 μ centres of plastids obtained from plants illuminated for 6 hr, but generally no detailed structure was observed within the 1 μ centres. Fluorescence microscopy showed also the presence of the indistinct grana in the originally transparent regions although these are not visible in the photographs. Generally, structure observed by fluorescence microscopy was more clearly visible in the microscope than on the film.

Most of the plastids isolated from plants which had received 10–12 hr of illumination were devoid of 1 μ centres (Plate 1, Fig. 4). They showed the grana-like structures of mature bean chloroplast although the plastids were much smaller than mature chloroplasts, being very little larger than proplastids. In plastids lacking 1 μ centres, the intensity of fluorescence was fairly uniform over the whole plastid (Plate 2, Fig. 4). A few plastids only showed fluorescing centres in agreement with the observations under phase-contrast.

Plastids obtained from plants illuminated for 24 hr were similar to the 10 hr plastids lacking 1 μ centres (Plate 1, Fig. 5). Observations under phase-contrast showed that the grana structures were somewhat disrupted, in many instances being replaced by lined structures. The fluorescence micrographs of these plastids (Plate 2, Fig. 5) resembled those of mature bean chloroplasts; the grana were difficult to see.

As may be seen from Plate 2, Figure 6, the grana of chloroplasts isolated from mature bean plants were less distinct than those of spinach chloroplasts, and channels were often seen within the bean chloroplasts. In one of the chloroplasts shown in Plate 1, Figure 6 the grana-structure appears to be partly disrupted, being replaced by a lined structure similar to that observed in one of the 24-hr plastids. Fluorescence microscopy (Plate 2, Fig. 6) showed also that the grana of isolated bean chloroplasts were less distinct than the grana of spinach chloroplasts.

IV. Discussion

Electron microscopy of mature chloroplasts from many higher plants (Thomas 1960) indicates that they consist of a lamellated system embedded in a stroma and surrounded by a membrane. At certain regions there is a considerable increase in the lamella density and it is considered that these regions correspond to the grana regions observed by phase-contrast and fluorescence microscopy. Fluorescence microscopy (P. Metzner 1937; Strugger 1950; Düvel and Mevius 1952) as well as the light-induced reduction of silver nitrate to metallic silver (H. Metzner 1952; Thomas, Post, and Vertregt 1954) indicate that chlorophyll is localized mainly in the grana regions of the mature chloroplast.

The lamellae of the mature chloroplast are absent from the proplastids of dark-grown or meristematic tissue. The 1 μ centres observed in bean proplastids by phase-contrast and fluorescence microscopy correspond in size and number with

the structures, termed prolamellar bodies, which have been widely observed in the electron microscope at the proplastid stage of chloroplast development (Leyon 1954; Hodge, McLean, and Mercer 1956; von Wettstein 1958; Mühlethaler and Frey-Wyssling 1959). The vesicles of the young plastids are aggregated into one or more of these prolamellar bodies which sometimes appear crystalline.

Of particular relevance to the work reported in this paper are the electron microscope studies of von Wettstein and Kahn (1960) on the fine structure of developing bean chloroplasts. The proplastids of 6-day-old etiolated bean plants showed vesicles forming by invaginations of the inner plastid membrane. At 8–12 days the vesicles were partly aligned into primary layers and partly accumulated in prolamellar bodies, while from 10 to 16 days the vesicles in the primary layers were transformed into relatively long tubes. Crystallization of the prolamellar bodies took place between 14 and 18 days. Von Wettstein and Kahn (1960) reported that a short exposure of 16-day dark-grown leaves to light of high intensity caused a rapid reorganization of the plastid structures. All tubes and many prolamellar bodies disappeared during as little as 2 min of illumination and they were replaced by vesicles arranged in concentric layers. With continuous illumination of the dark-grown leaves, von Wettstein and Kahn (1960) found no further structural changes during the first 2 hr. Grana formation was in progress after 3 hr of illumination. Eilam and Klein (1962) found similar vesicular ring structures in etiolated bean leaves which had been exposed to low light intensity (2 f.c.) for 24 hr.

von Wettstein and Kahn (1960) and Eilam and Klein (1962) interpreted formation of the concentric layers as a photorearrangement of units already present in proplastids. Klein (1962) has suggested that the rearrangement may be connected with the photoconversion of protochlorophyllide to chlorophyllide *a*, which occurs rapidly, but not with the phytylation to chlorophyll *a*, which is a much slower process.

From the observations reported in this paper it is difficult to interpret the formation of concentric layers as being due to a photorearrangement of the prolamellar bodies or fluorescing centres. Phase-contrast and fluorescence microscopy showed that there was little change in the structure of bean plastids during the first 3 hr of illumination and certainly there was no visible redistribution of the chlorophyll in that time. Since there was no new chlorophyll formation during the first 3 hr of illumination (Anderson and Boardman 1964) it seems extremely unlikely that chlorophyll or chlorophyllide was associated with the vesicles arranged in concentric layers, which appeared within minutes of illumination (von Wettstein and Kahn 1960). Moreover, the observations reported in this paper of the presence of both 1 μ fluorescing centres and grana-like structures within the same plastid also would tend to argue against a rapid rearrangement of the structural units already existing in the proplastids.

However, the pattern of development of bean chloroplasts from proplastids, as obtained from phase-contrast and fluorescence microscopy, agrees with the changes in fine structure observed by a number of workers for chloroplasts of a number of higher plants developing from meristematic or etiolated tissue. Thus the development of chloroplasts was studied in corn by Hodge, McLean, and Mercer (1956) and von Wettstein (1958), in barley by von Wettstein (1958), in *Aspidistra* by Leyon (1954),

and in *Elodea*, *Lilium*, and *Begonia* by Mühlethaler and Frey-Wyssling (1959). In all these studies it was observed that the lamellae of the developing plastid appeared to grow out from the prolamellar bodies. Hodge, McLean, and Mercer (1956) and Mühlethaler and Frey-Wyssling (1959) imagined vesicles emerging from the prolamellar bodies and fusing together to form membranes which evolved into stroma or grana lamellae. This hypothesis would support our idea that the prolamellar bodies might be sites of chlorophyll synthesis during the early stages of greening.

V. Acknowledgments

We wish to thank Dr. D. Spencer for valuable discussions during the course of this work and Mrs. S. Sapiets for technical assistance.

VI. References

Anderson, J. M., and Boardman, N. K. (1964).—*Aust. J. Biol. Sci.* **17**: 93–101.

Boardman, N. K., and Wildman, S. G. (1962).—*Biochim. Biophys. Acta* **59**: 222–4.

Düvel, D., and Mevius, W. Jr. (1952).—*Naturwissenschaften* **39**: 23.

Eilam, Y., and Klein, S. (1962).—*J. Cell Biol.* **14**: 169–82.

Granick, S. (1955).—In "Encyclopedia of Plant Physiology". Vol. 1. pp. 507–64. (Ed. W. Ruhland.) (Springer-Verlag: Berlin.)

Hodge, A. J., McLean, J. D., and Mercer, F. V. (1956).—*J. Biophys. Biochem. Cytol.* **2**: 597–607.

Klein, S. (1962).—*Nature* **196**: 992–3.

Leyon, H. (1954).—*Exp. Cell Res.* **7**: 609–11.

Metzner, H. (1952).—*Protoplasma* **41**: 129–67.

Metzner, P. (1937).—*Ber. dtsch. bot. Ges.* **55**: (16).

Mühlethaler, K., and Frey-Wyssling, A. (1959).—*J. Biophys. Biochem. Cytol.* **6**: 507–12.

Strugger, S. (1950).—*Naturwissenschaften* **37**: 166.

Thomas, J. B. (1960).—In "Encyclopedia of Plant Physiology". Vol. 5. Pt. 1. pp. 511–65. (Ed. W. Ruhland.) (Springer-Verlag: Berlin.)

Thomas, J. B., Post, L. C., and Vertregt, N. (1954).—*Biochim. Biophys. Acta* **13**: 20–30.

Wettstein, D. von (1958).—"The Photochemical Apparatus." Brookhaven Symposia in Biology No. 11. pp. 138–59.

Wettstein, D. von, and Kahn, A. (1960).—Proc. European Regional Conf. Electron Microscopy, Delft. Vol. 2. pp. 1051–4.

Explanation of Plates 1 and 2

Plate 1

Photomicrographs of isolated bean plastids taken under phase-contrast. ×1700

Fig. 1.—Proplastids from dark-grown bean plants.

Figs. 2–5.—Plastids from dark-grown bean plants which were illuminated for 3, 6–8, 10–12, and 24 hr, respectively.

Fig. 6.—Chloroplasts from bean plants grown in a glasshouse. *S*, chloroplast isolated from a spinach leaf.

PLATE 2

Fluorescence-micrographs of isolated bean plastids. ×1600

Fig. 1.—Proplastids from dark-grown bean plants.

Figs. 2–5.—Plastids from dark-grown bean plants which were illuminated for 4, 7, 10, and 24 hr, respectively.

Fig. 6.—Chloroplasts from bean plants grown in a glasshouse. *S*, chloroplast isolated from a spinach leaf.

GREENING OF DARK-GROWN BEAN PLANTS. I

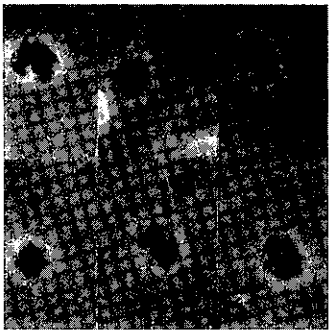

Fig. 1

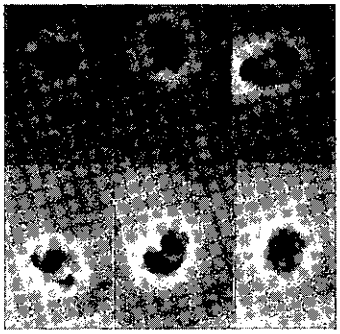

Fig. 2

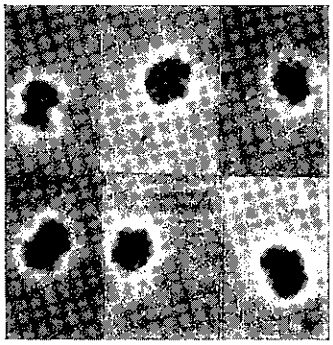

Fig. 3

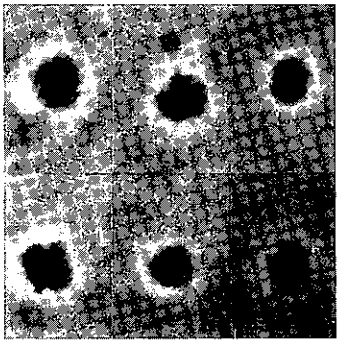

Fig. 4

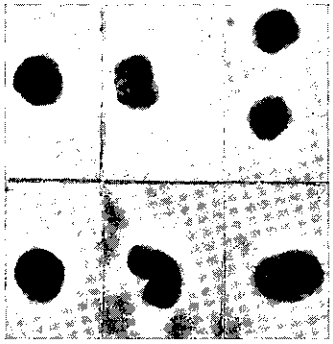

Fig. 5

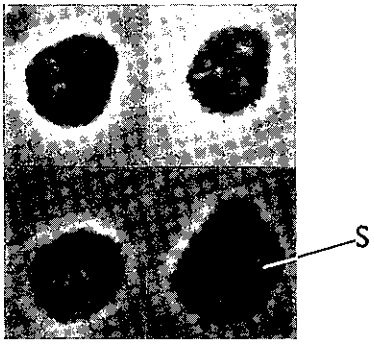

Fig. 6

GREENING OF DARK-GROWN BEAN PLANTS. I

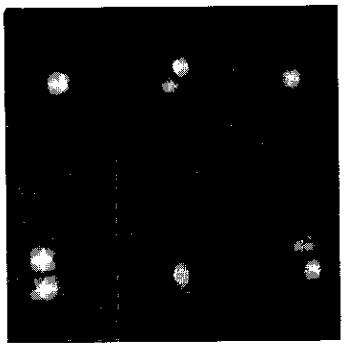

Fig. 1

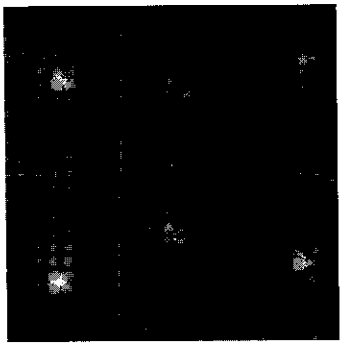

Fig. 2

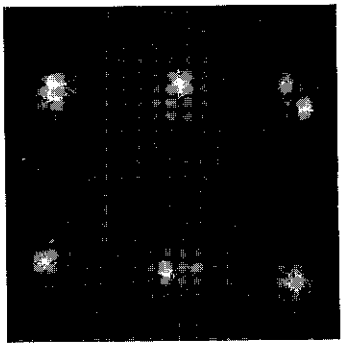

Fig. 3

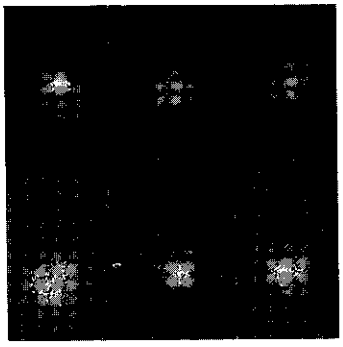

Fig. 4

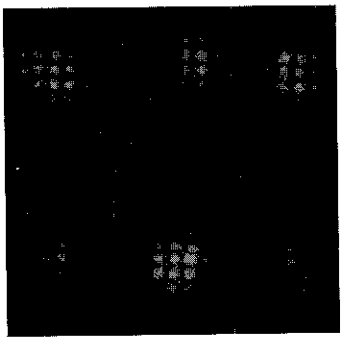

Fig. 5

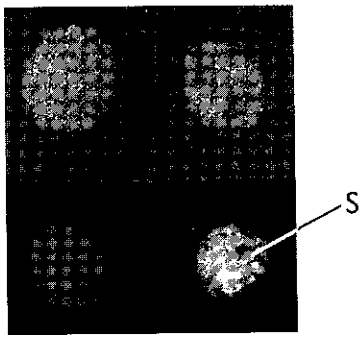

Fig. 6