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Chloroplasts in growing bean leaf

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Many intracellular structures are formed *de novo* or are enlarged in the growing leaf. The formation of these structures is under nuclear genetic control. It should appear that the chloroplasts are an exception because DNA and RNA have been discovered in them (Kirk 1966 and literature cited there). There is also some evidence for continuity of chloroplasts (see Granick 1955).

However some observations indicate that these organelles are to certain extent under the influence of intracellular environment e.g. the growth of chloroplasts is related to the cell expansion (Green, 1964; Holowinsky *et al.* 1965); there exists also a certain physiological relationship between chloroplasts and mitochondria (Dolzmann an Ullrich, 1966) and a genetic one between chloroplasts and the nucleus (Kirk 1966).

Proplastids occur in the meristems and in etiolated plants. In suitable conditions they are converted into chloroplasts. The ultrastructure of the developing chloroplasts has been investigated under three circumstances: 1. after illumination of etiolated plants (e.g. v. Wettstein 1959), 2. in the meristems during their normal development (e.g. Manton 1962), 3. in the leaves of different morphological ages (e.g. Mikulska 1963). It seems to be proved that most of the intermediate steps of the lamellar system development is similar in all three circumstances.

In etiolated plants the tubes are formed in the stroma of proplastids. These tubes are converted into a prolamellar body (Hodge *et al.* 1956; v. Wettstein 1959 and others); prolamellar body is formed only in etiolated plants. After illumination of etiolated plants the tubes are transformed into a lamellar system. v. Wettstein and his co-workers (v. Wettstein, 1959; Ericson *et al.* 1961; Virgin *et al.* 1963) discovered three light dependent steps in the lamellar system formation. In the first step the tubes of a prolamellar body transform into vesicles. Concomitantly protochlorophyll is reduced to chlorophyll *a*. In the second step the vesicles are arranged in primary layers and in the third step fusion of the vesicles into discs and their aggregation into grana takes place.

Studies on size of chloroplasts and their ultrastructure in various phases of leaf growth were the aim of the experiments described in this paper.

MATERIAL AND METHODS

Studies were carried out on primary bean leaves (*Phaseolus vulgaris* var. 'Bronowicka'). The plants were cultivated in continuous illumination under constant conditions (for details see Więckowski, 1959).

Counting of chloroplasts. Chloroplasts in the palisade and spongy mesophyll cells were counted after the separation of cells from each other according to the Brown and Rickless' method (Brown and Rickless 1949). Small pieces of a leaf blade were immersed into 5% chromic acid for 6 hours. These pieces were subsequently put on a slide glass, the cells were mechanically dispersed and chloroplasts were counted under a light microscope. The counts were made in about 100 palisade cells and about 100 spongy cells. Mean values and standard deviations were than calculated.

Measurement of chloroplast diameter. Diameters of chloroplasts were measured in a leaf homogenate prepared in a solution of 0.5 M sucrose +0.05 M phosphate buffer at pH 7. Preliminary experiments showed that in this solution chloroplasts do not change their dimension during experimentation. A drop of this homogenate was put on a slide glass and the longest diameters of unbroken chloroplasts were measured under a microscope (Lumipan, 15×60, immersion) by using a calibrated ocular micrometer (1 division = 1,74 μ). Measurement with accuracy

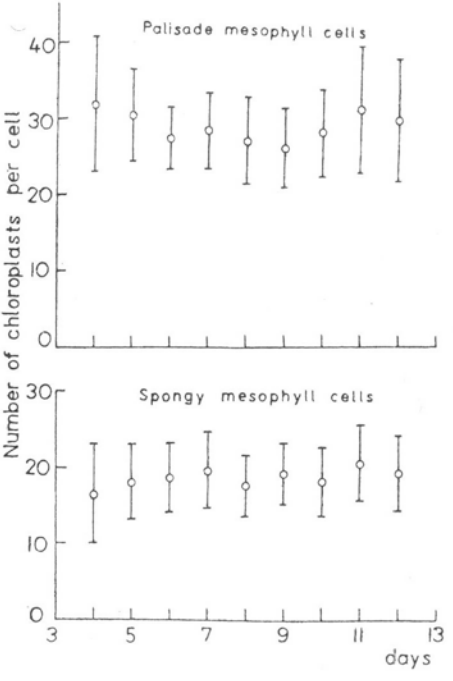

Fig. 1. Chloroplast numbers in various phases of leaf growth

(Time was counted form the day of placing seeds for germination; seeds were soaked one day earlier – see Więckowski. 1959).

to 0,5 division did not render any difficulty. About 100 chloroplasts were measured and mean values and standard deviations were subsequently calculated.

Studies under an electron microscope. Small pieces of a leaf blade were fixed in OsO_4 buffered to 7.2 pH (phosphate buffer) and dehydrated with graded alcohols. Epon 812 was used as the embedding medium (Luft 1961). Ultrathin sections were obtained on a Porter — Blum Ultramicrotome equiped with a diamond knife. Electromicrographs were taken by JEM-5Y electron microscope.

RESULTS

A. Changes in chloroplasts number

The number of chloroplasts per one palisade and spongy mesophyll cell varies markedly at any stage of leaf development (Fig. 1). In all investigated phases 10 to 45 chloroplasts per one palisade cell and 10 to 35 chloroplasts per one spongy cell were found; about 28 and 17 chloroplasts were the mean values per one palisade and spongy cell, respectively. It may be concluded from these results that the average number of chloroplasts per one cell does not change very notably during the investigated stages of leaf development. Divisions of chloroplasts which took place during the first 6 days of leaf development were rather rare and did not modify the average number of chloroplasts per cell.

B. Size of chloroplasts

The diameters of chloroplasts increase concomitantly with the growth of the leaf lamina (Fig. 2). A comparison of the data presented in Fig. 2 with earlier results (Więckowski 1959 Fig. 4, and 1960, Figs 1, 2) indicates that there are correlation between the growth of chloroplasts and the growth of the leaf lamina. The increase of the chloroplast diameter is very intensive during the first 4 days; the rate of chloroplasts expansion gradually decreases during the following two days and stops at about the seventh day.

C. Changes in the chloroplasts ultrastructure

Ultrastructure of chloroplasts was investigated at four phases of the differentiation (I, II, III, IV — as marked in Fig. 2). Typical electromicrographs are presented in Figs. 4—8.

Fig. 4 shows the section of chloroplasts at the first examined stage of their development. Many chloroplasts are ameboidal in shape. Their double lamellae with an electron transparent layer in the middle part are well formed. The arrangement of vesicles into oriented primary layers was the first stage of a lamellar system formation which could be established (Figs 4, 5 A, B). In many cases the vesicles are separated from each other and they become gradually longer; the distance

between two vesicles is gradually reduced and presumably many of them fuse into a greater unit. Sometimes only one or two vesicles lies along the longer axis of the chloroplast (Fig. 5A). Five to seven primary layers may be visible in a cross-section of a chloroplast. The formation of granum initial also take place at this stage of development. It seems that the granum initial are formed as the results of growth of a vesicle (thylakoid)* membrane. If the primary layers are built

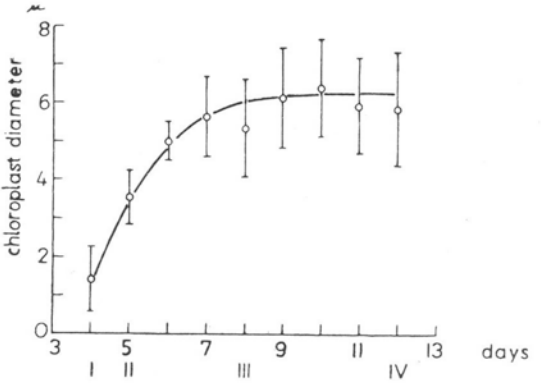

Fig. 2

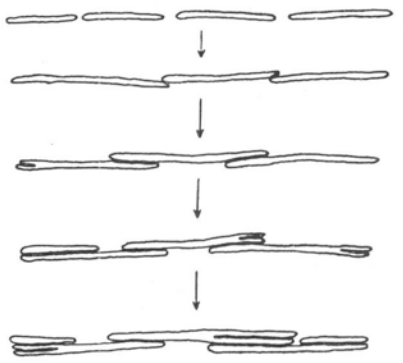

Fig. 3

Fig. 2. Changes of chloroplast diameters during the period of leaf growth (I, II, III, IV — phases at which the ultrastructure of chloroplasts was investigated).
Fig. 3. Scheme of structural changes of a primary layer during the initial steps of lamellar system formation.

of several vesicles two neighbouring vesicles may creep over one another and a double lamellae is formed between them (Fig. 5 C, 3). New thylakoids are also formed by invagination of parent thylakoids membranes. This is presumably connected with unequal growth of the membrane. During the formation of the first grana initial it is possible to distinguish a primary grana lamellation and a primary stroma lamellation (Fig. 4). These two lamellations are formed simultaneously. In some cases the granum region is longer at this stage of chloroplasts structure differentiation than in the fully developed chloroplasts (Figs. 4, 8). About six granum initials are formed in every primary layer. There are more separated vesicles at the ends of every primary layer. It is supposed that in this region new thylakoids are also formed from the inner chloroplasts membrane. Sometimes two neighbouring thylakoids in a granum do not cement closely to each other.

* We regard after Menke (1960) that a „thylakoid" is a closed system of double parallel membranes. However, we postulate that in the later stages of lamellar system formation every thylakad consists of a granum region (disc) and an intergrana region (stroma lamella). Majority of closed discs would hawe connection with stroma lamellae if the section of the same granum were made in another plane.

Plate I

Fig. 4. Cross-section of chloroplasts at the first investigated stage (the stages were marked in Fig. 2). *PGL* – primary granum lamellae; *PSL* – primary stroma lamellae; *OG* – osmophilic globules; *FV* – free vesicles X 28800.

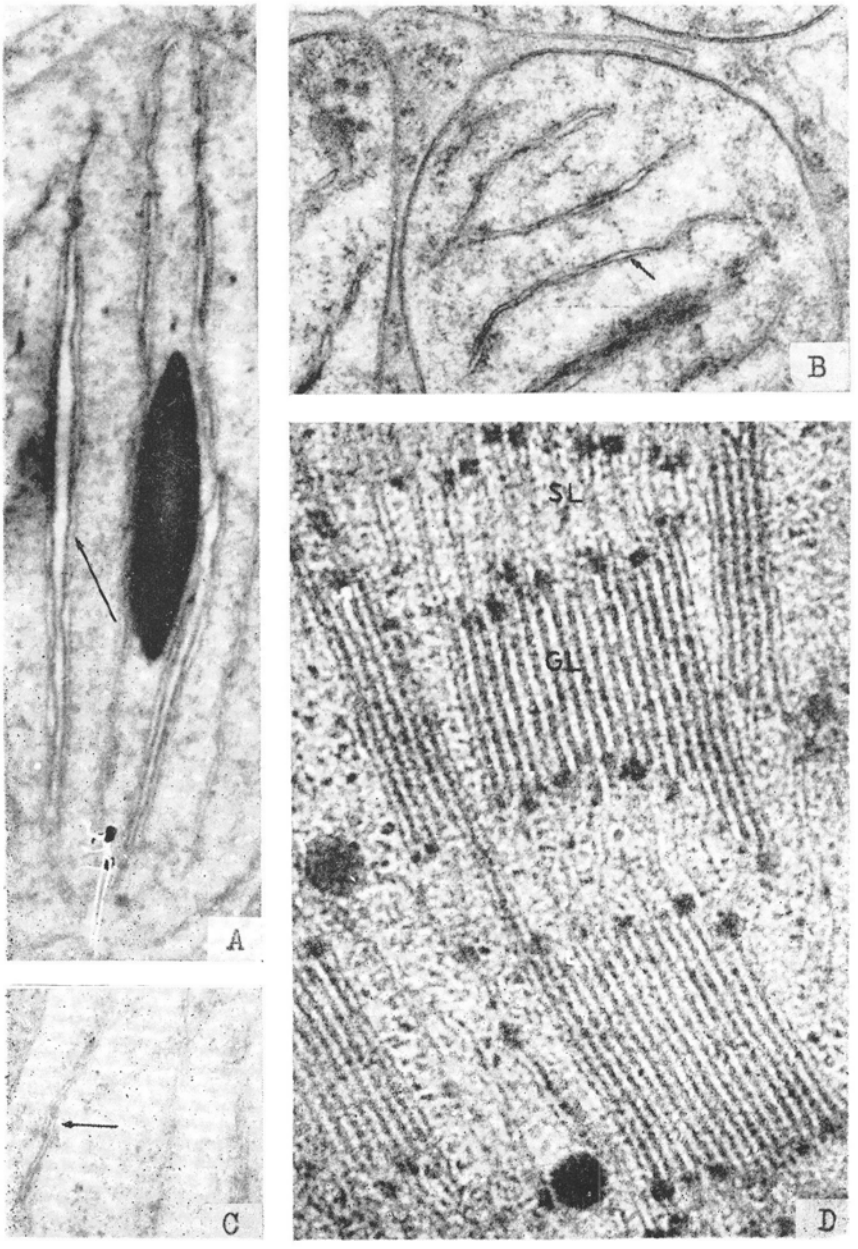

Fig. 5. Cross-section of chloroplasts for the demonstration of some details of lamellar system for mation and structures.

A – elongated vesicles, x 30000. *B* – primary layers with the initial stage of invagination. x 30000. *C* – creeping over of two vesicles. x 30000. *D* – structure of lamellar system in mature chloroplast x 80300.

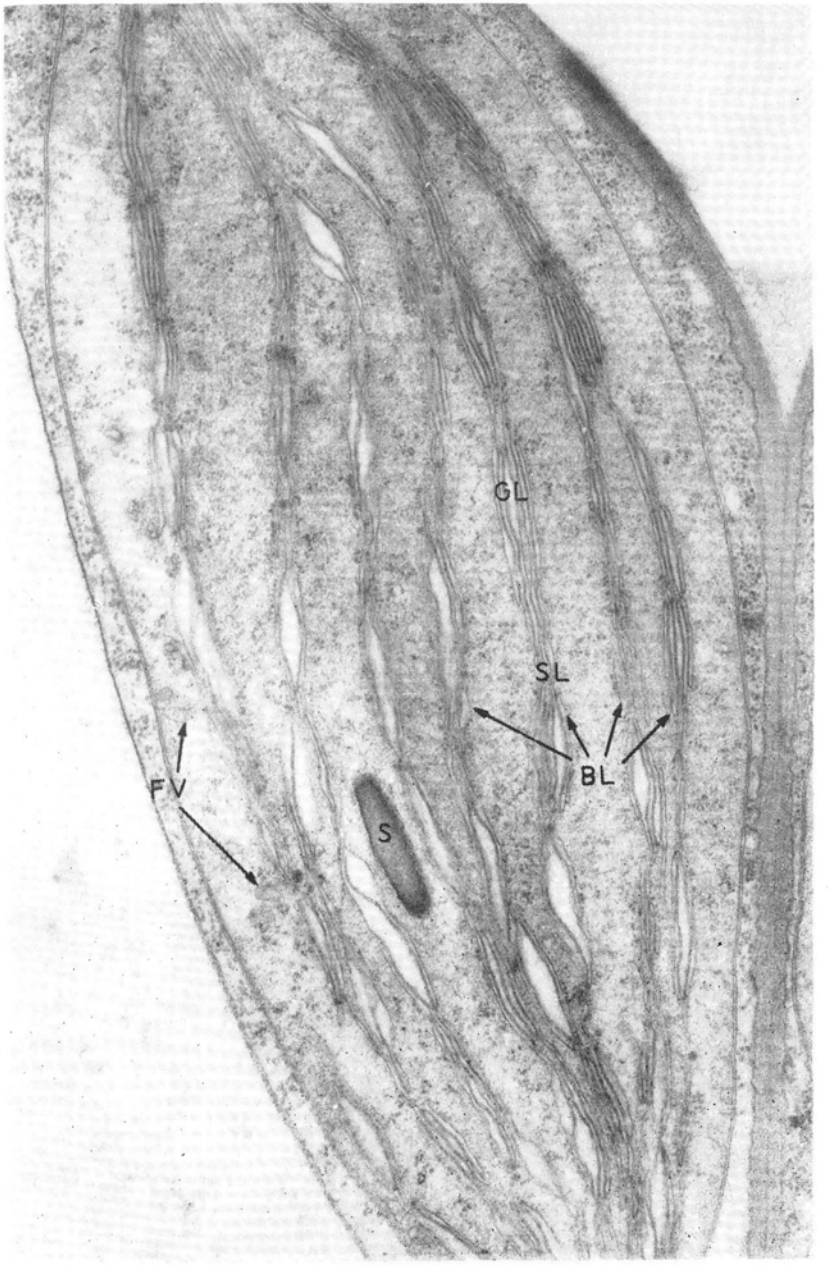

Fig. 6. Cross-section of a chloroplast at the second investigated stage.
GL — grana lamellae; *SL* — stroma lamellae; *BL* — bundles of lamellae; free vesicles. x 25000.

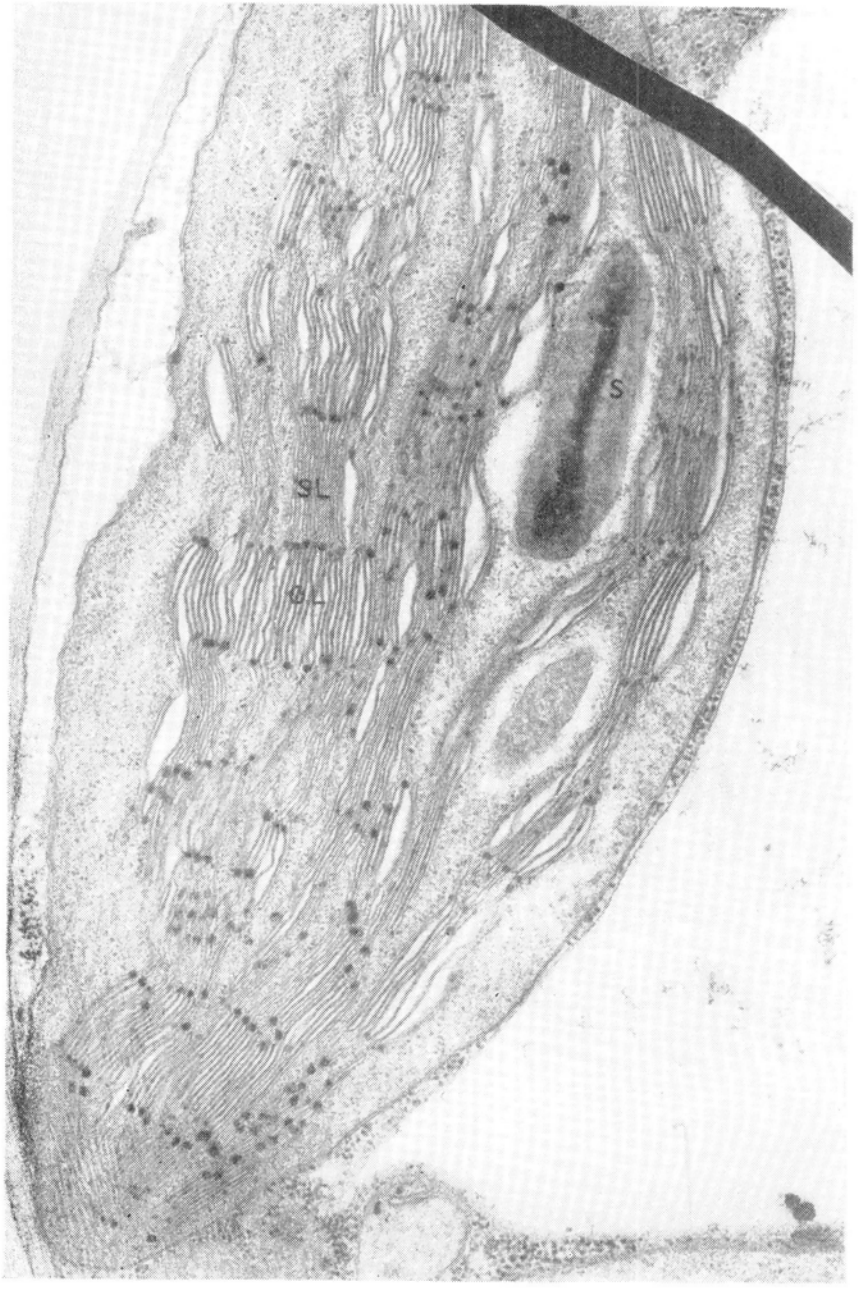

Fig. 7. Cross-section of a chloroplast at the third investigated stage.
G — granum; *SL* — stroma lamellae; *S* — starch; *OG* — osmophilic globules. x 30000.

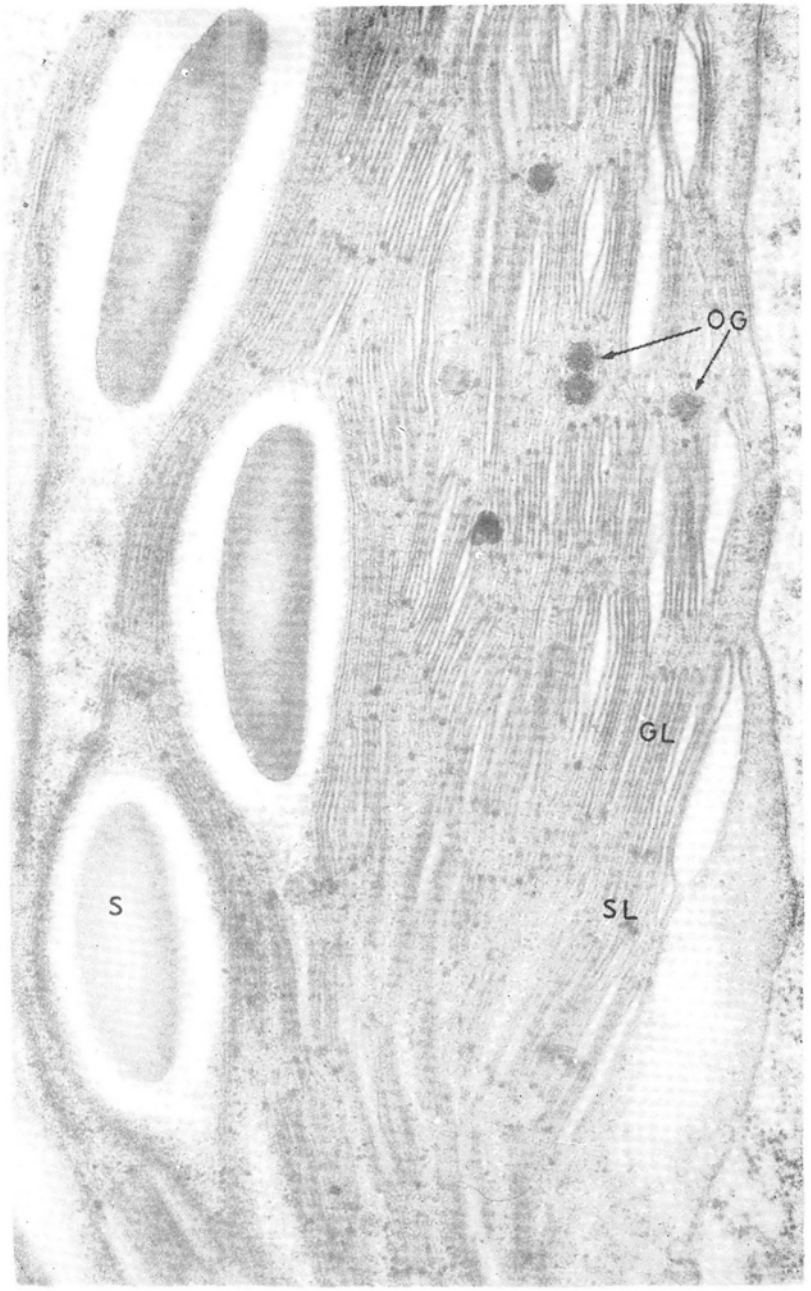

Fig. 8. Cross-section of a chloroplast at the fourth investigated stage. Details as in Fig. 7. $\times$ 38000

Starch and osmophilic globules occur in the stroma of many chloroplasts.

Figure 6 shows the cross-section of chloroplasts in the second stage of lamellar system formation. The chloroplasts are bigger and ellipsoidal in shape. The primary layers are seen as bundles of lamellae situated in parallel to the longer axis of chloroplasts. Some of these bundles may branch. It is possible to distinguish about 15 grana in each bundle and maximum about 6 thylakoids in each granum. Very often neighbouring grana lay close to each other and therefore the area of stroma lamellae is very limited. Processes connected with formation of new thylakoids still exist. All kinds of discs described in the literature (opend or closed at one or both sides) occur in the grana. There is a small osmophilic globula at the ends of many discs.

A comparison of Fig. 4 with Fig. 6 indicates that the number of grana increases in every bundle of lamellae. We belive that it results from the fact that new grana are formed at the ends of every lamellae bundle and a certain kind of „division" of thylakoids take place (see discussion).

Starch grains are not often found in this stage of chloroplasts development. It is possible to distinguish osmophilic globules in stroma.

In the third phase the chloroplasts are at a more advanced stage of their development (Fig. 7). In every bundle the number of lamellae increases very markedly and borders between them are sometimes not very distinguishable. If the granum of one bundle lies under the granum of another bundle the new formed granum is high and regular, otherwise the new granum is irregular in shape. It is possible that new thylakoids are formed also at this stage of chloroplast development. The distances between the grana increase i.e. the area of stroma lamellae increases.

Starch and osmophilic globules occur very often. The structure is generally similar to that of a mature chloroplast.

The last picture shows the chloroplast from the phase when leaf has finished its growth. The borders between the bundles of lamellae which were formed from primary layers are difficult to be seen. A great part of each chloroplast is filled by starch. These starch grains cause some deformation in the regularity of the lamellar system. Each starch grain is surrounded by the electron transparent space. Many osmophilic globules occur in the stroma. A small osmophilic globule occurs at the ends of many discs. The grana are usually irregular in shape.

The analysis of mature chloroplasts (Fig 5D) and of their developmental stages indicates that thick lamellae are built of two thin lamellae which belong to two neighbouring thylakoids, and a cementing layer is found between them. Thylakoid membrane remain thin at the ends contacting with stroma lamellae, and at the ends of every granum.

DISCUSSION

The number of chloroplasts per one palisade or spongy mesophyll cell in bean leaves is constant at the moment when the leaves appear from cotyledons and seed coats. It is possible to find chloroplasts in the division stages during the whole pe-

riod of leaf growth. This process, however, is relatively rare and variation in the chloroplast content in cells is rather great and therefore the division does not modify markedly the increase of the average number of chloroplasts per cell. A comparison of the present results with those of other authors indicates that the rate of chloroplast division ceases at various stages of cell growth in various species of plants: in bean plants it presumably ceases at the proplastid stage or at the earliest stage of chloroplast development and e.g. in *Nitella* cells the chloroplasts number per cell increases very markedly during the whole period of cell elongation (Green 1964). In bean plants the increase of chloroplasts size is correlated with the increase of leaf lamina. These two processes are stoped nearly at the same time. These results are in agreement with the results of Holowinsky *et al.* (1964) who showed that the size of chloroplasts in leaves of bean and *Xanthium pensylvanicum* are related to the size of leaf lamina and to the morphological age of leaf (leaf plastochron index), respectively. According to Green (1964) the rate and direction of chloroplast growth in *Nitella* depend on the cell elongation. Mechanical changes of the rate or direction of cell expansion modifies the direction of chloroplast growth. In *Funaria hygrometrica* (Heitz 1922) and *Agapanthus umbellatus* (Fasse-Franzisket 1955) it was also found that the rate of chloroplasts growth is correlated to the rate of cells expansion.

Opinions on the final ultrastructure of mature chloroplasts of higher plants are not in agreement (see v. Wettstein 1959; Weier 1961; Wehrmayer and Perner 1962; Heslop-Harrison 1963; Wehrmayer 1964; Menke 1966; Mühlenthaler 1966 and others). Among others these differences concern mutual relationship between stroma lamellae and grana lamellae. According to Menke (1960) thylakoid is the basic unit of every lamellar system. Weier and Benson (1966) postulated partition (double membrane of two neighbouring thylakoids) as the basic functional unit of this system.

Our electromicrographs of mature bean chloroplasts are very similar to those of Weier (1961), Thomson (1965) and Manton (1962). The description of the structure of lamellar system given by those authors agree in general with ours although Weier's terminology was not applied here. Many stroma lamellae appear to be a continuation of grana lamellae. Only in the grana region two neighbouring thylakoids are cemented to each other and in the stroma region the lamellae are presumably stretched, not cemented (see further discussion).

Development of chloroplast structure take place during the whole period of leaf growth. The scheme of this development in bean leaf is given in Fig. 3.

Our scheme of bean chloroplast structure development is different in details from that of v. Wettstein and his co-workers (v. Wettstein 1959; Ericsson et al. 1961). It is more similar to that proposed by Menke (1964). These differences may be partly ascribed to different plant species used for observations.

The observed changes in chloroplast development suggest that grana and stroma amellae are formed simultaneously. They are formed as the result of the growth

of vesicles (thylakoids) membranes. Invagination or creeping over each other of two vesicles is a results of this growth. In certain regions two neighbouring thylakoids are cemented to each other (these regions are granum initials); stroma lamellae initials are between them.

It is generally accepted that the first vesicles in proplastids originate directly or indirectly from the inner chloroplast membrane (see Manton 1962; Menke 1966). Some investigators postulated that at further chloroplast development stages new thylakoids are formed also from the inner chloroplasts membrane (Hodge et al. 1956; Manton 1962, and others). Other authors indicated that invagination and envagination of parent thylakoids (Menke 1960; and others) or longitudinal division of lamellae (v. Wettstein 1959) lead to the formation of new thylakoids. Our observations indicate that in bean chloroplasts new thylakoids are formed by the invagination of parent thylakoids and from the inner membrane of chloroplasts. Small vesicles in the stroma near the chloroplast membrane were found in earlier stages of chloroplast development; some of them were in connection with this membrane (Figs. 4, 6, 7). The process of new thylakoids formation take place during the whole period of leaf growth. Thick lamellae are uniform only in mature chloroplasts whereas in ealier investigated stages an electron transparent layer is often found in them.

An increase of grana number was found in every bundle of lamellae during the period of leaf development; the distances between two neighbouring grana also increase. It is suggested that new grana are formed at the ends of each bundle and by "division" of older thylakoids. The "division" is possible if we accept Heslop-Harrison's (1962, 1963) or Wehrmayer's (1964) three dimensional arrangement of a granum. If the distance between two areas of elongated thylakoid increases the structures between these areas undergoes a gradual stretching. During the chloroplast growth the area of intergrana lamellae also increase between two grana which do not divide. It is in agreement with Mühlenthaler's opinion (1966) who assumed that the particles on the stroma lamellae "become more and more separated during the extensive growth of the lamellae".

Chloroplasts in the earliest examined stage of their development are able to a very active CO_2 assimilation (Więckowski 1966). This indicates that there are ready photosynthetic centers at that stage of lamellar system formation. The activity of photosynthetic centers is presumably connected with the formation of a double membrane (Kyle and Gross 1966; Weier and Benson 1966). The rate of pigments and some of photosynthetic enzymes synthesis should coincide with the rate of the thick lamellae formation. It is still an open question why the rate of photosynthesis per mg of chlorophyll (Więckowski 1966) and presumably per one photosynthetic center is higher at the first examined stages of chloroplast structure development. Presumably many factors are responsible for it (Więckowski 1966). It is also possible that more CO_2 molecules reach photosynthetic centres at the stage of a poorly developed double lamellae system.

SUMMARY

A study was made on the changes in the chloroplasts number per cell, the increase of chloroplast diameters and the development of lamellar system in the growing bean leaf.

It was established that the mean number of chloroplasts per one palisade and spongy mesophyll cell do not change markedly during the period of leaf growth. It was also established that the chloroplast diameters and the lamellar system formation take place during the whole period of leaf blade expansion. At that time new thylakoids are formed and the distances between two neighbouring grana increases.

A scheme of structural changes of primary layer in bean leaf is proposed.

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REFERENCES

Brown R. and Rickless P., 1949, A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and of nutrients, Proc. Roy. Soc. London, 136B:110—125.

Dolzmann P. und Ullrich H., 1966, Einige Beobachtungen über Beziehungen zwischen Chloroplasten und Mitochondrien im Palisadeparenchym von *Phaseolus vulgaris*, Z. Pflanzenphysiol. 55:165—180.

Eriksson G., Kahn A., Walles B. und Wettstein D. von, 1961, Zur makromolekularen Physiologie der Chloroplasten III, Ber. dtsch. bot. Ges. 74:221—232.

Fasse-Franzisket U., 1955, Die Teilung der Proplastiden und Chloroplasten bei *Agapanthus umbellatus* l'Herit, Protoplasma 45:194—227.

Granick S., 1955, Plastid structure, development and inheritance. In "Encyclopedia of Plant Physiology" W. Ruhland, ed., vol. I pp. 507—564, Springer — Verlag, Berlin.

Green P. B., 1964, Cinematic observations on the growth and division of chloroplasts in *Nitella*, Am. J. Bot. 51:334—342.

Heitz E., 1922, Untersuchungen über die Teilung der Chloroplasten, J. H. Ed. Heitz, Strassburg.

Helsop-Harrison J., 1962, Evanescent and persistent modifications of chloroplast ultrastructure induced by unnatural pyrimidine, Planta 58:237—256.

Helsop-Harrison J., 1963, Structure and morphogenesis of lamellar systems in grana-containing chloroplasts. I. Membrane structure and lamellar architecture, Planta 60:243—260.

Hodge A. J., McLean J. D. and Mercer F. V., 1956, A possible mechanism for the morphogenesis of lamellar system in plant cells, J. Biophys. Biochem. Cytolog. 2:597—608.

Holowinsky A. W., Moore P. B. and Torrey J. G., 1965, Regulatory aspects of chloroplast growth in leaves of *Xanthium pensylvanicum* and etiolated red kidney bean seedling leaves, Protoplasma 60:94—110.

Kirk J. T. O., 1966, Nature and function of chloroplasts DNA. In: "Biochemistry of chloroplasts" T. W. Goodwin, ed. vol. I, pp. 319—340. Academic Press, London and New York.

Kyle J. L. and Gross J. A., 1966, Correlative studies of structure and function in lamellar subunits of chloroplasts, Plant Physiol. 41, X (suppl.).

Luft J., 1961, Improvement in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9, 409.

Manton J., 1962, Observations on plastid development in the meristem of *Antheoceros*, Jour Exp. Bot. 13:325—333.
Menke W., 1960, Das allgemeine Bauprincip des Lamellarsystem der Chloroplasten, Experientia 16:537—538.
Menke W., 1964, Feinbau und Entwicklung der Plastiden. Ber. dtsch. bot. Ges. 77:340—354.
Menke W., 1966, The structure of the chloroplasts. In "Biochemistry of Chloroplasts" T. W. Goodwin, ed. vol. I, pp. 3—18, Academic Press, London and New York.
Mikulska E., 1964, Badania nad ultrastrukturą komórek liści *Clivia miniata* i *Bilbergia* sp. ze szczególnym uwzględnieniem chloroplastów, (Thesis for docent degree), University of Łódź.
Mühlenthaler K., 1966, The ultrastructure of the plastid lamellae. "Biochemistry of Chloroplasts" T. W. Goodwin, ed. vol. I, pp. 49—64. Academic Press, London and New York.
Thomson W. W., 1965, The ultrastructure of *Phaseolus vulgaris* chloroplasts, Jour. Exp. Bot. 16:167—176.
Virgin H., Kahn A. and Wettstein D. von, 1963, The physiology of chlorophyll formation in relation to structural changes in chloroplasts, Photochem. Photobiol. 2:83—98.
Wehrmeyer W., 1964, Zur Klärung der strukturellen variabilität der Chloroplastengrana des Spinats in Profil und Aufsicht, Planta 62:272—293.
Wehrmeyer W. und Perner E. 1962. Der submikroskopische Bau der Grana in den Chloroplasten von *Spinacia oleracea*, Protoplasma 54:573—593.
Weier T. E., 1961, The ultrastructure of starch-free chloroplasts of fully expanded leaves of *Nicotiana rustica*, Am. J. Bot. 48:615—629.
Weier T. E. and Benson A. A., 1966, The molecular nature of chloroplast membranes, In: "Biochemistry of Chloroplasts", T. W. Goodwin, ed. vol. I pp. 91—113, Academic Press, London and New York.
Wettstein D. von, 1959, Developmental changes in chloroplasts and their genetic control. In: "Developmental Cytology", D. Rudnick, ed., pp. 123—160, The Ronald Press Company, New York.
Więckowski S., 1959, The relation between the growth of leaves and the synthesis of chlorophyll, Acta Biol. Crac. ser. Bot. 2:1—11.
Więckowski S., 1960, Relation between the increase of dry and fresh weights and chlorophyll formation in the leaf, Bull. Acad. Polon. Sci. Ser. sci. biol. 8:357—362.
Więckowski S., 1966, Photosynthesis in growing leaf of *Phaseolus vulgaris*, Acta Soc. Bot. Polon. 35:437—443

Chloroplasty w rosnącym liściu fasoli

Streszczenie

W pracy tej opisano liczbę, wielkości i ultrastrukturę chloroplastów w zależności od fazy wzrostu liścia.

Stwierdzono, że średnia liczba chloroplastów w komórce miękiszu palisadowego i gąbczastego nie ulega istotnym zmianom w okresie wzrostu blaszki liściowej. Stwierdzono również, że chloroplasty powiększają swoją średnicę oraz wykształcają system lamellarny w ciągu całego okresu wzrostu liścia. Zmiany w ultrastrukturze polegają na zwiększaniu się liczby tylakoidów w każdym granie i przestrzeni pomiędzy dwoma sąsiadującymi granami.

Na podstawie otrzymanych wyników zaproponowano schemat wykształcania się systemu lamellarnego w liściu fasoli.