GRANA FORMATION AND SYNTHESIS OF CHLOROPLASTIC PROTEINS INDUCED BY LIGHT IN PORTIONS OF ETIOLATED LEAVES

by

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INTRODUCTION

It is well known that most of the higher plants do not synthesize chlorophyll when they are grown in the dark. They become etiolated and the cells of their leaves contain yellowish plastids known as leucoplasts. On exposure to light, the leaves of such plants become green.

On the basis of data from several publications[1,2], it seems that etiolated leaves are poor in chloroplastic proteins, although their cytoplasmic proteins appear to be normal. Hence, it could be expected that the greening of etiolated leaves on exposure to light might be accompanied by the synthesis of chloroplastic proteins; in other words, that we have a means for inducing the transformation of leucoplasts into chloroplasts with specific synthesis of chloroplastic proteins, under more or less simple conditions.

It is the purpose of this communication to present data bearing on transformations induced by light in the plastids of etiolated leaves, under conditions simplified as far as possible.

MATERIALS AND METHODS

The etiolated leaves used in the course of this study are the buds of *Cichorium Intybus* which are produced commercially as a food under the name of chicory in the following way: the green leaves are cut off the roots, one cm above the top of the tap-root. These, with their abundant storage products, are put in soil and kept in the dark in a warm and wet environment. After a period of 15 to 20 days, the roots produce a bud of etiolated leaves, 10 to 15 cm long.

Etiolated leaves were excised from the plant and cut into two symmetrical halves, the thick median vein being discarded. The half leaves so obtained were divided into two equivalent samples constituted by taking alternatively left and right halves. Hence, pairs of samples of leaf portions are obtained with the same weight and in the same physiological state, one sample of one pair being taken as a blank (etiolated sample), the other sample of the same pair being exposed to light.

For fractionation experiments, 100 etiolated leaves were utilized, the wet weight of which was approximately 70 grams.

Light exposure. The leaf portions were placed on cotton wool moistened with tap water, in plates covered with glass. They were illuminated with four 40 W fluorescent tubes placed at 20 cm intervals, 25 cm above the plates, in a dark thermostat room maintained at 20° C $\pm$ 0.5.

Microscopy. The leuco- and chloroplasts were isolated by the method of GRANICK[3], grinding the leaves in cold 0.5 *M* sucrose solution in a precooled Waring blendor for ½ minute and centrifuging the suspension at 410 *g* for 5 minutes.

A Philips electron microscope was used. Shadow casting was performed with a gold-manganin mixture (1/1).

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Fractionation by differential centrifugation was carried out after grinding the leaves in cold 0.5 *M* sucrose solution, in a precooled Waring blendor for ½ minute. The homogenate was then filtered through cheese cloth and centrifuged at 2° C.

Nitrogen determination was made by the method of Kjeldahl, with MARKHAM's apparatus[4], after ashing dry tissue powders freed from lipids by SCHNEIDER's method[5].

Free amino acids and related $-NH_2$ compounds were extracted by the method of AWAPARA[6] and determined by the colorimetric ninhydrin method of MOORE AND STEIN[7].

RESULTS

Electron microscopic observation of a morphological differentiation from leucoplasts to chloroplasts

A comparison between plastids from etiolated and green leaves with the electron microscope (EM) shows a radically different structure in these two types of particles.

While, in the chloroplasts, we recognized the classical figures[8–11] of the grana embedded in the thick membrane of the plastid, we did not observe any similar structure in the leucoplasts. The leucoplasts (Figs. 1 and 2) present a finely granular structure; they do not contain any granum. They are much thinner than the chloroplasts, more transparent to electrons. They are more easily destroyed and their isolation is much more delicate. The size of individual leucoplasts varies very considerably: some are very small, while others approach the size of chloroplasts; but the majority are of intermediate size.

By simply exposing to light portions of etiolated leaves, the transformation of leucoplasts into normal characteristic chloroplasts can be induced within a few hours. The appearance of grana seems to occur simultaneously in all the plastids, in a progressive, but very rapid, way: the formation of grana can be seen in all the mass of each particle.

Fig. 3 shows a chloroplast from a leaf fragment which has reached the maximum of greening, a dark green colour. The grana are well-formed, as in normal green leaves.

If isolated plastids are placed into distilled water for one night, they burst due to the action of osmotic pressure. In the case of chloroplasts, empty membranes were found in the preparation, confirming the observation of ALGERA *et al.*[9]. On the other hand, it seems that grana are also enveloped within a well-defined membrane. This can be seen in Fig. 4. Beside membranes with the size of chloroplasts, empty membranes can be observed with smaller sizes, like those of the grana. These formations do not correspond to small chloroplasts, since the isolation procedure by differential centrifugation in 0.5 *M* sucrose solution had previously excluded particles of such dimensions. Thus, only the action of distilled water can explain their liberation. Fig. 4 also shows grana partially emptied of their contents; their membranes can be discerned.

These observations agree with the fact pointed out by NEISH[12] that "grana hydrate and vacuolate in distilled water".

Leucoplasts, on the contrary, do not present any of these membranous structures. But membranes appear on exposure of the etiolated leaves to light.

Light microscopic observation also shows that most of the leucoplasts are very small as compared with the chloroplasts. They are spherical in shape, while the chloroplasts are "saucer shaped". The cells of etiolated leaves contain fewer plastids than normal cells. On greening, the plastids increase in size and show the typical shape of chloroplasts. Somewhat later, the number of plastids per cell begins to increase.

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Synthesis of chloroplastic proteins during the greening phenomenon

The data obtained by electron microscopic observation seemed to indicate that a synthesis of chloroplastic proteins may take place during the greening of etiolated leaves. In particular, the question arose as to whether the formation grana consists of the transformation of an existing protein or of the net synthesis of new proteins.

We have followed the behaviour of several cell constituents separated by differential centrifugation in the course of the greening process.

1. The first experiment described was performed *4 hours* after the beginning of a constant light exposure. The leaves had become very pale green. The results are recorded in Table I. They show an important increase of the plastid proteins, and also of the proteins of the microsome fraction.

TABLE I

FRACTIONATION EXPERIMENT AFTER 4 HOURS OF LIGHT EXPOSURE

Fractions	*Total dry weight of lipoid-free powder*				*Total protein nitrogen content*			
	etiolated leaves (in mg)	*after light exposure (in mg)*	*increase (in mg)*	*increase in % of the existing quantity*	*etiolated leaves (in mg)*	*after light exposure (in mg)*	*increase (in mg)*	*increase in % of the existing quantity*
1. pellet 5 min, 410 *g* (intact plastids)	12.0	14	2	17	0.78	1.15	0.37	47
2. pellet 10 min, 1450 *g*	17	20	3	17	2.04	2.10	0.06	3.3
3. pellet 10 min, 4700 *g* (grana)	23.5	30	6.5	25	2.34	3.27	0.94	40
4. pellet 10 min, 50,000 *g* (grana fragments)	35	42	7	28	3.30	3.95	0.65	20
5. pellet 30 min, 105,000 *g* (microsomes)	19	22	3	16	1.87	2.44	0.66	35
6. supernatant	90.5	94	3.5	3.9	8.62	8.41	—0.21	—2.4
Total	197	222	25	12	18.97	21.35	2.30	12

Indicated centrifugal forces = average no. times gravity

2. A second fractionation experiment was performed after *47 hours* of light exposure. The results are summarized in Table II. They show that the chloroplast fraction undergoes the greatest transformation (% increase and enrichment in protein content). At this stage, however, all the other fractions have increased, an indication of total cell growth.

3. Nitrogen source for the protein synthesis:

Etiolated leaves are more than ten times richer in nitrogen compounds soluble in ethanol than are green leaves or the same excised leaves after exposure to light, followed

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by greening. Amino compounds determined by MOORE AND STEIN'S[7] method seem to be utilized during protein synthesis, as shown in the example given in Table III.

TABLE II

FRACTIONATION EXPERIMENT AFTER 47 HOURS OF LIGHT EXPOSURE

Fractions	*Total dry weight of lipoid-free powder*				*Total protein nitrogen content*				
	etiolated leaves (in mg)	*after light exposure (in mg)*	*increase (in mg)*	*increase in % of the existing quantity*	*etiolated leaves (in mg)*	*after light exposure (in mg)*	*increase (in mg)*	*increase in % of the existing quantity*	
1. pellet 5 min, 410 *g* (intact plastids)	15.2	30.5	15.3	100	0.91	2.84	1.93	210	
2. pellet 10 min, 1450 *g*	17.5	25	7.5	42	1.31	2.45	1.14	80	
3. pellet 10 min, 4700 *g* (grana)	21	41	20	95	2.12	2.54	0.42	20	
4. pellet 10 min, 50,000 *g* (grana fragments)	28.8	41.3	12.5	43	2.64	4.13	1.49	50	52
5. pellet 30 min, 105,000 *g* (microsomes)	19.8	28.9	9.1	45	2.31	2.50	0.19	10	
6. supernatant	77	132.5	55.5	72	8.43	14.04	5.61	60	
Total	179.3	299.2	119.9	67	17.72	28.50	10.79	61	

TABLE III

	Soluble $-NH_2$ content (in amino acids equivalents): µM % mg dry weight	*Soluble $-NH_2$ content in mg amino acids equivalents % mg dry weight*	*Protein synthesis in mg % mg dry weight during the same time*
Etiolated leaves	580	75.4	0
After light exposure for 48 hours (dark green leaves)	50	6.5	62

DISCUSSION AND CONCLUSION

From the EM observations, it appears that the greening of portions of etiolated *Cichorium* leaves is accompanied by the differentiation of finely granular leucoplasts into typical chloroplasts with well-defined grana. A structure appears within an amorphous mass.

A related phenomenon was recently pointed out by HEITZ AND MALY[13] who have

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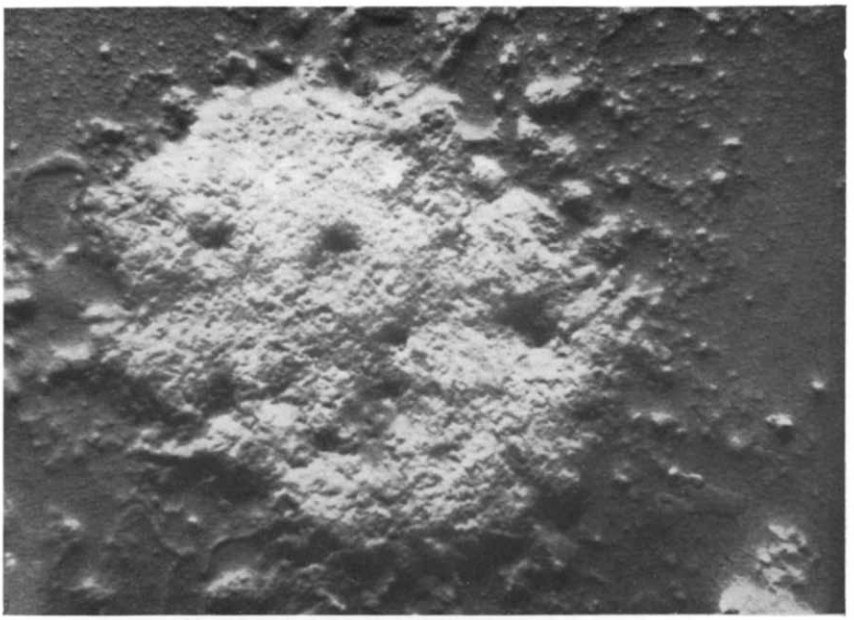

Fig. 1. Leucoplast isolated from etiolated Cichorium leaf, showing a finely granular structure. 17,000 ×.

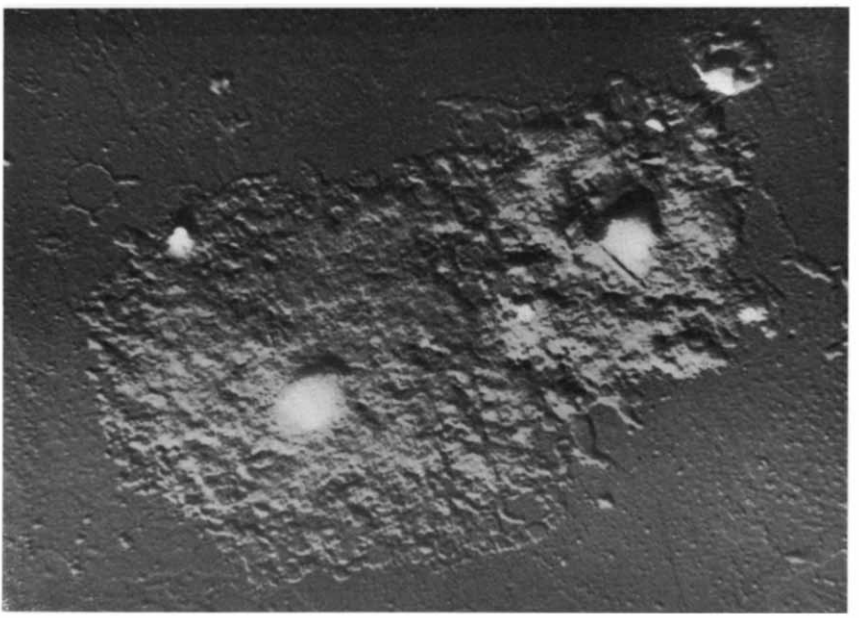

Fig. 2. Two disrupted leucoplasts of different sizes, showing the central starch grain. 10,600 ×.

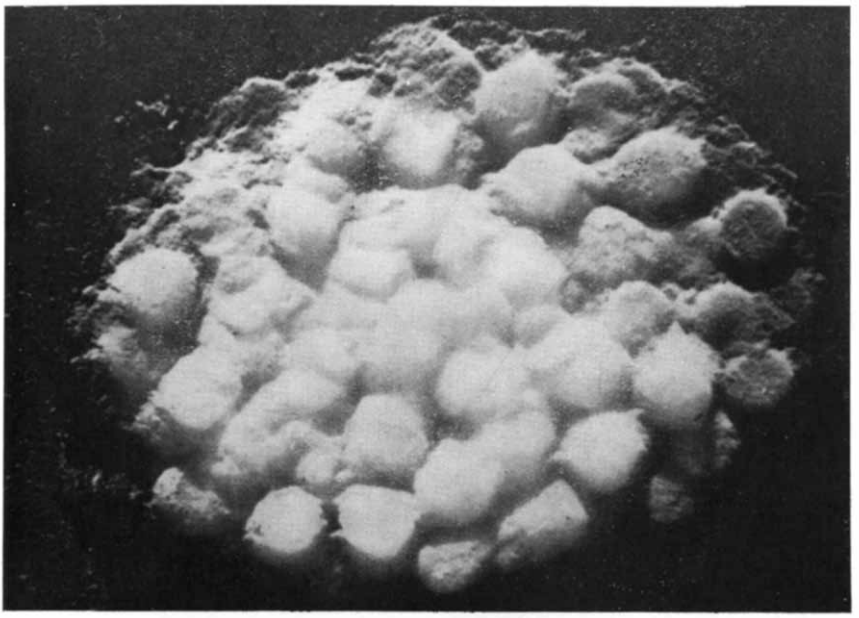

Fig. 3. Chloroplast from leaf fragment which has reached the maximum of greening: a dark green colour. The grana are well formed. 22,000 ×.

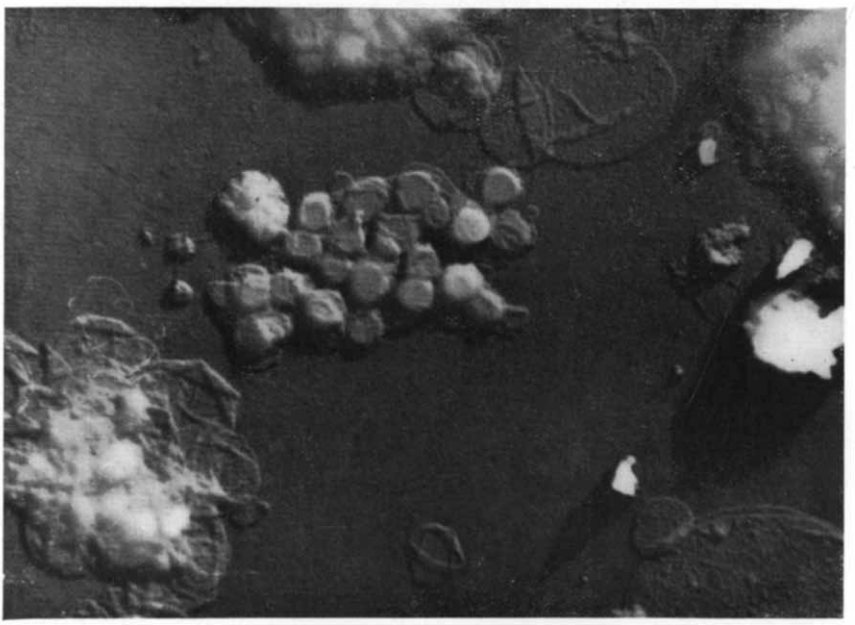

Fig. 4. Grana membranes and grana partially emptied of their contents around which one can see the membranes. 11,000 ×.

observed, by means of the fluorescence microscope, the appearance of grana in young plastids during the differentiation of embryonic cells. Hence, it seems well established that the granum does not represent a factor of chloroplastic heredity; it has no genetic continuity, since it may appear de novo in cells which are devoid of it, as is the case in two known instances. The autoduplicating particle is another constituent of the plastid or of its precursor, the proplastid.

These results do not agree with the conclusions of STRUGGER[21], according to which each proplastid would contain (besides a starch grain) a primary granum, the origin of all the other grana and factor of their genetic continuity. Indeed, the leucoplasts do not contain any primary granum (Figs. 1, 2). Fig. 2 is particularly clear in this respect: it shows that each leucoplast contains only one single grain and this has been identified as starch. The gradual appearance of grana in the observations of STRUGGER results probably from the progressive edification of grana during growth of the whole cel, rather than from the autoduplication of a plastidogene.

Furthermore, darkness appears to block specifically the development of plastids; but the continuation of this development may be induced by light.

The observed transformation is not only the organization of an existing protein mass, but protein synthesis. The net synthesis of proteins by excised leaves is an exceptional phenomenon (except when regeneration occurs, with cell multiplication and formation of roots, as in special cases). In general, leaves, when excised from the plant, undergo a rapid loss of proteins, whatever the conditions under which they are placed: light or darkness, petioles in water or nutrient solutions (see general review of this question in "Plant Biochemistry", BONNER[14]). It seems, however, that when the excised leaves are taken from plants grown on a low nitrogen medium, they are able to synthesize proteins when supplied with available nitrogen (PHILLIS AND MASON[15]).

We could demonstrate that a net synthesis of proteins is induced in etiolated leaves on exposure to light. This phenomenon occurs in several steps and seems to have its first seat in the plastids. From the first fractionation experiment, it appears:

1. that a synthesis of chloroplastic proteins does actually occur when etiolated leaf fragments are exposed to light, since the cell fractions corresponding to intact plastids or grana liberated from destroyed chloroplasts increase appreciably.

2. that this synthesis seems to be restricted to the plastid constituents during this first stage of the phenomenon. It must be noted that the microsome fraction also shows a net increase in protein content, which, however, might be related with the formation of cytoplasmic proteins and bigger particles (BRACHET[16,17]), plastids in this case.

During the time the plastids are turning green and enlarging, the ratio protein nitrogen/total dry weight increases. Hence, while the dry weight of the intact plastids fraction increases by 17%, the protein content increases by 47% (*cf.* Table I): the plastids become richer in proteins.

Later, the cells begin to grow as a whole. A few cells in division can be observed; but growth remains disharmonic, the plastid fraction being favoured, so that progressively the characteristic particle equilibrium of green leaves is restored (*cf.* Table II).

These results seem to agree with the observations of ANDREEVA AND PLYSHEVSKAYA[18] (of which we only were able to read an abstract), who have found that plants previously deprived of nitrogen supply and then supplied with $(^{15}NH_4)_2SO_4$ incorporate ^{15}N into both plastid and plasma proteins when they are able to perform photosynthesis; in the dark, on the contrary, they incorporate ^{15}N only into plasma proteins. Although

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there is here no demonstration of any net synthesis of proteins, it seems that these experiments could be interpreted in the same manner as ours: it is possible that those plants depleted of proteins begin to synthesize proteins actively when supplied with available nitrogen, chloroplastic proteins being formed only under conditions where chloroplasts are active.

We may suppose that the net synthesis of proteins observed in our experiments would correspond to the synthesis of a number of enzymes playing a role in photosynthesis, the formation of which could be induced directly by light or under the influence of new substrates. TOLBERT AND COHAN[19] have recently demonstrated that light induces the appearance of glycolic acid oxidase in etiolated leaves. The glycolic acid produced as one of the first steps of the photosynthesis process was shown to be responsible for this phenomenon. We must note, however, that we have here no evidence regarding either an eventual net synthesis of the enzyme or any indication as to whether the enzyme is located in the plastids or in the cytoplasm of the cell. Nevertheless, it is a demonstration that adaptative enzyme formation may be indirectly induced by light in higher plants.

Furthermore, etiolated leaves seem to represent a material similar to *E. coli* cells which have been subjected to a "pretreatment induction" in the presence of chloromycetin as in the experiments of PORTER *et al.*[20]. These authors have shown two distinct phases in the induction process of β-galactosidase by lactose: a first "presynthetic" phase in which the cells assimilate inductor, nitrogen, carbon and energy in the presence of chloromycetin (which completely inhibits enzyme formation) and a second phase in which active enzyme is formed in a linear way after chloromycetin has been removed. Dark seems to play, in our experiments, the same role as chloromycetin in those of PORTER *et al.*: synthesis of chloroplastic proteins is stopped, but amino precursors are still formed and accumulate in etiolated cells. Light induces the synthesis of chloroplastic proteins and the utilization of accumulated precursors.

The quantity of free amino acids or equivalent $-NH_2$ compounds used during the greening corresponds approximately to the quantity of proteins synthesized during the same time (Table III). Hence, it appears that the net protein synthesis observed occurs at the expense of free $-NH_2$ compounds or, at least, of the nitrogen they contain.

The nature of the free $-NH_2$ compounds has not so far been further determined. In fact, the important factor here is the total nitrogen quantity available for protein synthesis in the closed system (as far as nitrogen supply is concerned) represented by etiolated leaf fragments in water.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor R. JEENER, for his helpful interest. I am greatly indebted to Professor J. M. WIAME, "Service de Recherches du Centre d'Enseignement et de Recherches pour l'Industrie et l'Agriculture (CERIA)", who put the electron microscope of this centre at my disposal, and to Mrs. BOUILLON-NYSSEN who made the electron micrographs.

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SUMMARY

A comparison between plastids from etiolated and green leaves with the electron microscope shows a radically different structure in these two types of particles. The leucoplasts do not contain grana; they show a finely granular structure. They are smaller than chloroplasts. By simply exposing to light fragments of etiolated leaves, the transformation of leucoplasts into normal characteristic chloroplasts is induced within a few hours. This transformation is accompanied by a significant net synthesis of chloroplastic proteins that occur at the expense of free amino compounds of which etiolated leaves contain a considerable reserve.

RÉSUMÉ

La comparaison des plastides de feuilles étiolées et de feuilles vertes de Cichorium à l'aide du microscope électronique révèle une structure radicalement différente dans les leucoplastes et dans les chloroplastes. Les leucoplastes ne contiennent pas de grana, mais présentent une structure finement granulaire; ils sont plus petits que les chloroplastes.

Le simple fait d'exposer à la lumière des fragments de feuilles étiolées induit en quelques heures la transformation des leucoplastes en chloroplastes caractéristiques.

Cette transformation est accompagnée d'une importante synthèse nette de protéines chloroplastiques qui semble être réalisée aux dépens d'une réserve de composés aminés contenue dans les feuilles étiolées.

ZUSAMMENFASSUNG

Aus dem Vergleich von Plastiden etiolierter und grüner Blätter mit Hilfe des Elektronenmikroskops geht hervor, dass die Leuco- und die Chloroplasten eine gänzlich verschiedene Struktur aufweisen. Die Leukoplasten enthalten keine Granen, aber sie zeigen einen fein granularen Aufbau auf; sie sind kleiner als die Chloroplasten.

Die einfache Beleuchtung der Bruchteile etiolierter Blätter verursacht, binnen einiger Stunden, die Transformation der Leukoplasten in charakteristiche Chloroplasten.

Die Transformation wird von einer wichtigen Netto-Synthese chloroplastischer Proteine begleitet, welche auf Kosten einer in den etiolierten Blättern enthaltenen Reserve von Aminostoffen ausgeführt wird.

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Received December 22nd, 1953