Light intensities of 400 to 700 ft-c inhibit development of photosynthetic capacity in Euglena.

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Studies of Chloroplast Development in Euglena VII. Fine Structure of the Developing Plastid [1, 2]

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Dark-grown Euglena contain proplastids 1 μ in diameter (1, 2) having double membranes and lacking any appreciable inner structure. When such cells are exposed to the optimal intensity of light, a number of events occur leading to the formation of fully mature chloroplasts. The fine structure of these fully mature chloroplasts has been described by Gibbs (4). We have previously inferred from both fluorescence and electron microscopy that the development of the proplastid into the mature chloroplast takes place by internal formation of lamellae and fusion of proplastids (1, 2). The sequence found by electron microscopy indicated that the appearance of lamellae is linear with time. It was also shown that the development of photosynthetic competence also exhibits linear kinetics. This paper describes the development of the ultrastructure of the chloroplast and provides further substantiation for several aspects of our previous model (1, 2).

Materials and Methods

Euglena gracilis var. bacillaris Pringsheim was cultured in Hutner's pH 3.5 medium (6) or in resting medium as described previously (16). All details concerned with the conditions for chloroplast development were the same as described previously (16, 17).

Samples were prepared for electron microscopy as follows. Cells were centrifuged lightly and the pellets fixed in 1.0% OsO_4 in 0.28 M veronal acetate buffer at pH 7.3 to 7.6 for 6 to 24 hours at 4°. After fixation, the cells were washed 2 to 3 times with buffer and were dehydrated by successive 15 minute ex-

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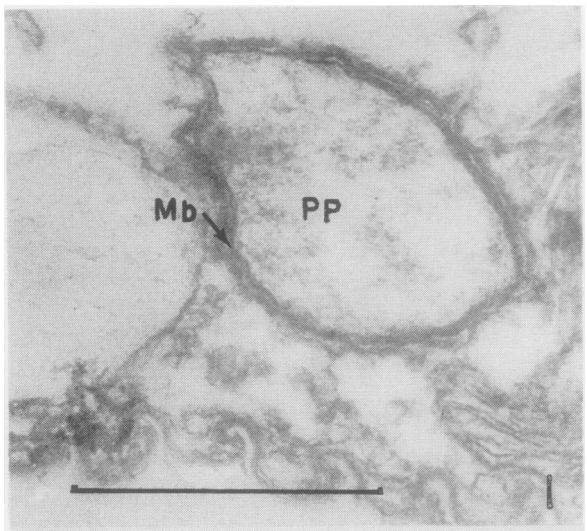
posures to 25%, 50%, and 75% (v/v) acetone. They were then transferred successively to 96% (v/v) acetone for 30 minutes, 2 changes of 100% acetone for 30 minutes each followed by 100% acetone to which about 20% Vestopal (enough to cover the pellet) was added. This stood overnight at room temperature to permit evaporation of the acetone. The residue was placed in gelatin capsules which received more Vestopal until filled. The contents were hardened at 60° for 2 to 3 days before being sectioned by glass or diamond knives on a Porter-Blum microtome. The sections were transferred to coated grids by the usual procedures. In most cases, these grids then were stained with 1 to 2% $KMnO_4$ for 10 minutes according to Lawn's method (9). They were then examined with an R.C.A. EMU-3C electron microscope.



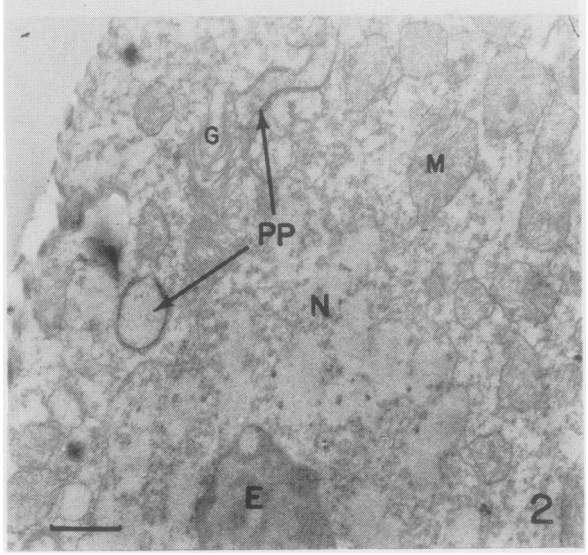


FIG. 1. Nondividing cell at zero time of development (dark-grown cell), showing detail of proplastid. Abbreviations used in figures: D, disc; E, endosome; G, golgi apparatus; L, lamella; M, mitochrondion; Mb, membrane; N, nucleus; P, pyrenoid; Pm, paramylum; PP, proplastid; V, vacuole. Marker indicates 1 μ.

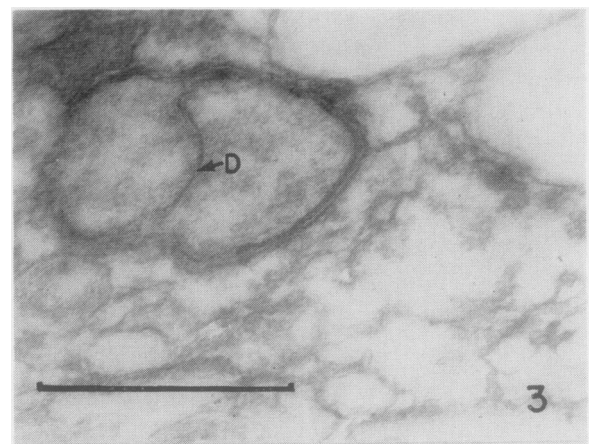
FIG. 2. Nondividing cell at zero time of development (dark-grown cell). (Key to abbreviations in legend of figure 1.).

Results and Discussion

Formation of Discs[4] *and Lamellae during the Normal Sequence of Chloroplast Development at 100 ft-c in Nondividing Cells.* In the following discussion, dark-grown cells are taken as zero time of development and all developmental times are measured from the time at which these cells are placed in the light.

In Figures 1 and 2 are shown the double-mem-

[4] Throughout this paper we follow the nomenclature of Gibbs (4). The basic internal chloroplast structure is the disc, a membrane 40 to 60 A in thickness. In the mature chloroplast, each lamella consists of several discs (usually 4) which are fused together along their entire length to form the elongated thickened lamella.



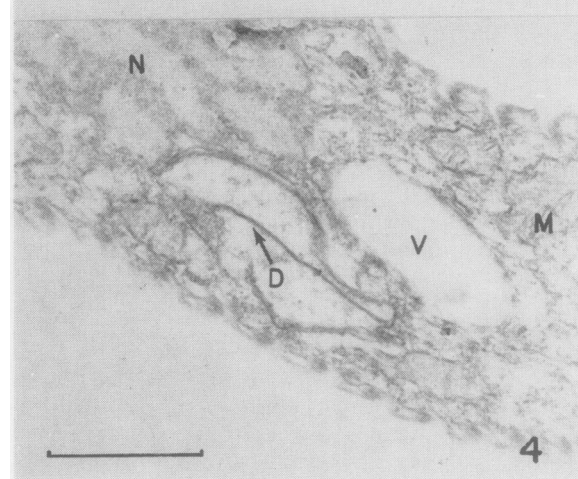


FIG. 3. Nondividing cell, plastid after 2 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

FIG. 4. Nondividing cell, plastid after 2 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

braned proplastids about 1 μ in diameter which are found in dark-grown cells. No prolamellar body as described for higher plants (7, 10, 13, 18) has been observed although on rare occasions small clumps of particles resmbling ribosomes or other small vesicles are seen. The thickness of the double membrane is about 100 to 150 A and the number of proplastids per section was 2 to 6.

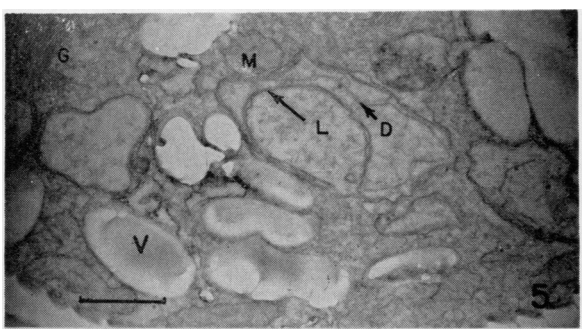
At 2 hours the developing plastids contain at least 1 disc (figs 3, 4). One end of the developing disc is frequently seen to be associated with a clump of ribosome-like particles (fig 4).

After 4 to 6 hours, the plastids generally contain 1 to 3 individual discs and 1 lamella usually containing 2 discs (fig 5). The plastids are further elongated with practically no change in width.

After 7 to 10 hours, the typical plastid has 2 to 3 individual discs and 1 to 2 lamellae each containing 2 discs (figs 6, 7). Sometimes the lamellae at this stage contain 3 discs each, 40 to 60 A in thickness (fig 8). The plastids continue to elongate.

After 12 to 24 hours, there are many changes in the developing plastids (fig 9). The number of individual discs has risen to about 6 and the number of lamellae is 5 to 6. The number of discs per lamella has increased to about 2 to 4. The lamellae containing 4 fused discs appear as 2 thin outer membranes enclosing an inner membrane double in thickness as described by Gibbs for mature chloroplasts (4). At this time, the pyrenoid appears as a region of dense material containing lamellae. The length of the plastid is now about 2 to 3 times the length that it was after 7 to 10 hours of development.

From 24 to 96 hours of development, the number of lamellae increases to about 13, containing 4 to 6 discs each, but the number of unfused discs decreases



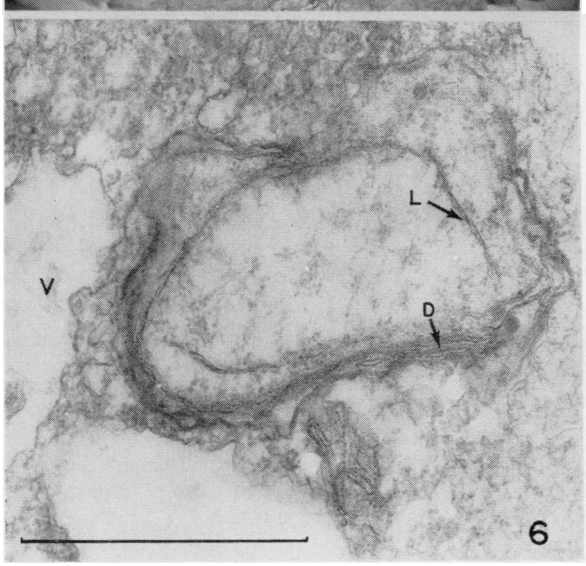
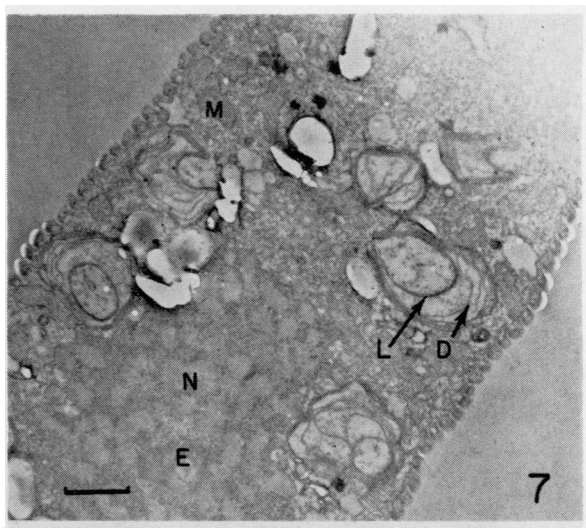


FIG. 5. Nondividing cell, plastid after 6 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

FIG. 6. Nondividing cell, plastid after 8 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).



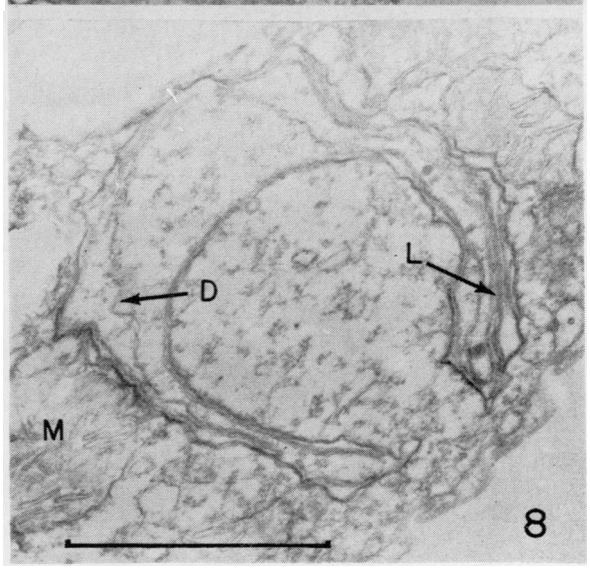


FIG. 7. Nondividing cell, plastid after 10 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

FIG. 8. Nondividing cell, exceptional plastid after 18 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

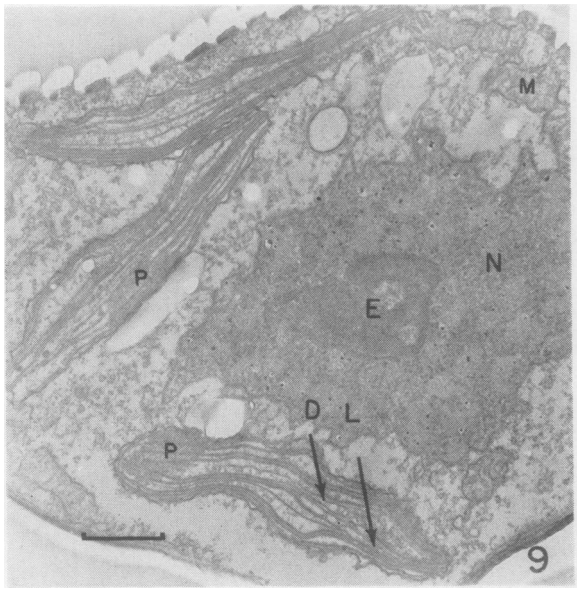


FIG. 9. Nondividing cell, plastid after 24 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

to zero, eventually (figs 10, 11, 12, 13). There is little change in the length of the plastid during this period.

Parallel studies following chloroplast development on cells kept continuously in the logarithmic phase of growth show a similar sequence of developmental events to the ones described above for nondividing cells. This is in agreement with our previously published observations on chloroplast development in growing cells (1, 2).

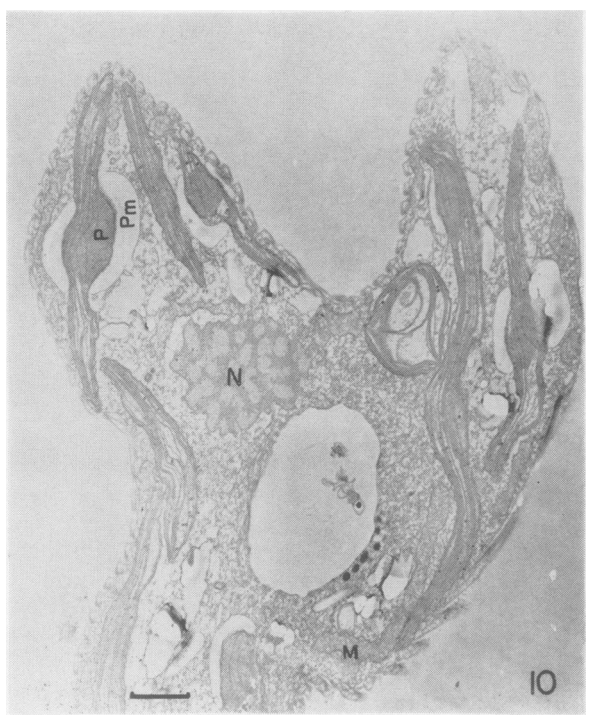


FIG. 10. Nondividing cell, pastid after 36 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

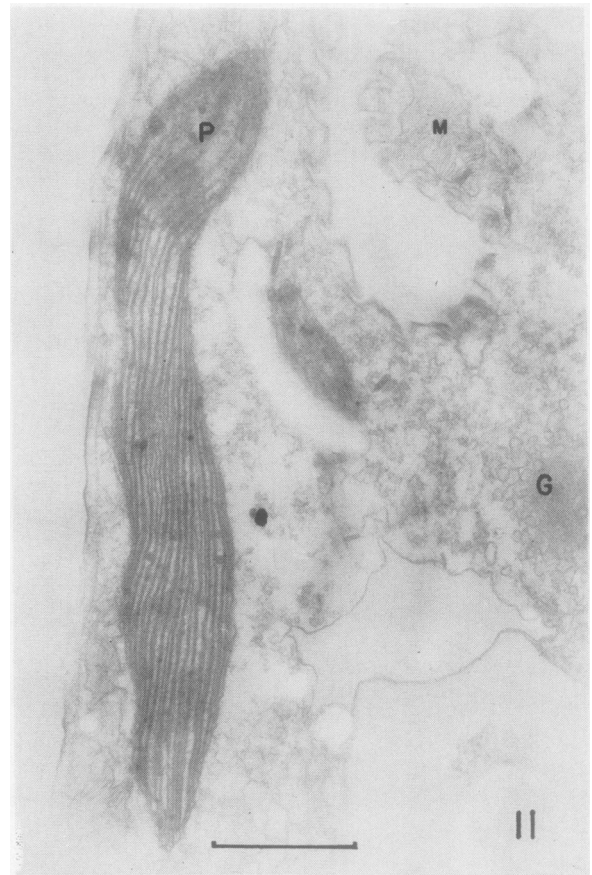


FIG. 11. Nondividing cell, plastid after 48 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

Kinetics of Appearance of Discs and Lamellae during the Normal Sequence of Chloroplast Development at 100 ft-c in Nondividing Cells. Figure 14 summarizes the number of discs and lamellae at various developmental times. The number of free discs is at a maximum after 12 to 24 hours and subsequently declines to zero. Lamella formation, however, continues and is essentially linear from 14 hours onwards until 12 to 13 lamellae are formed characteristic of the mature chloroplast in this species under these conditions. The simplest interpretation of these results is that discs are formed up to a certain number after which their formation slows down and then ceases completely. As discs are forming, however, they become fused into lamellae, eventually leading to the consumption of all the available discs as they become incorporated into completed lamellae.

Another interesting inference which can be drawn from these data concerns the dramatic increase in numbers of free discs and lamellae between 10 to 14

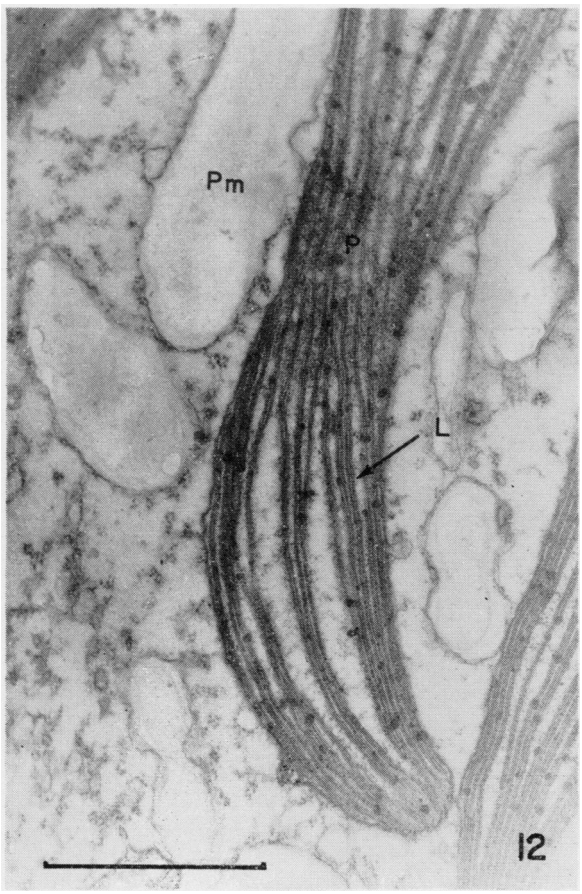


FIG. 12. Nondividing cell, plastid after 72 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

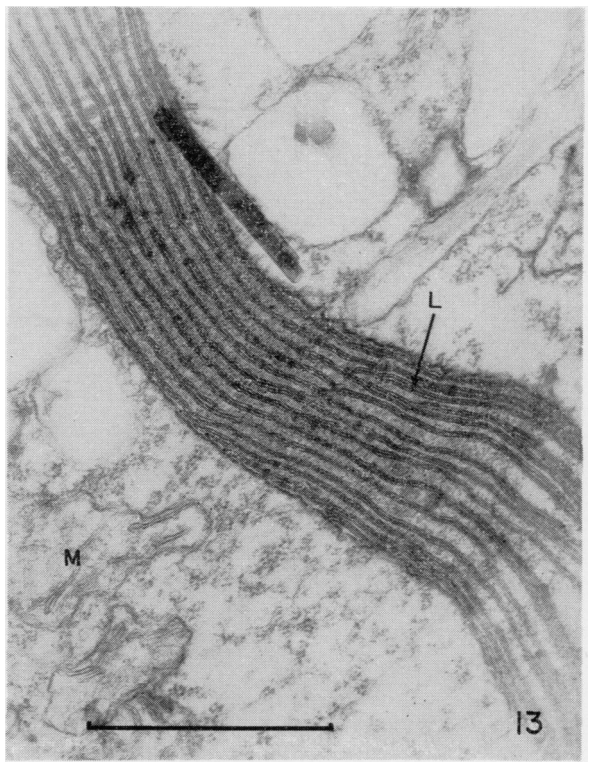


FIG. 13. Nondividing cell, plastid after 95 hours of development at 100 ft-c. (Key to abbreviations in legend of figure 1).

hours of development. Our previous work (11) suggested that there are approximately 30 proplastids in a dark-grown cell which fuse at some time during development in groups of 3 to form the approximately 10 chloroplasts characteristic of light-grown cells. It is interesting, therefore, that the number of free discs per plastid roughly triples between 8 and 14 hours. If the factor of increase is assumed to be an integer, it is best considered to be a tripling as can be seen from the statistical analysis in table II.

In the case of the increase of lamellae between 8 and 14 hours, quadrupling gives the best fit. Since discs undoubtedly continue to be formed even during the fusion process, one would expect the chance of lamella formation to be enhanced leading to a somewhat greater increment during fusion.

Size of the Chloroplast during Development. Figure 14 also shows that there is a tripling of the length of the chloroplast at the same time as the tripling of the number of discs and this is again substantiated statistically in table II. The width of the chloroplast remains relatively constant during development except for a statistically significant (table II) transient change at about 10 hours. These data for plastid length and numbers of lamellae and discs again suggest the fusion of 3 developing proplastids to make 1 chloroplast. This would lead to the conclusion that fusion occurs linearly, i.e. the 3 proplastids become fused in a linear sequence which increases the length 3 times without markedly affecting the eventual width of the plastid. Occasionally, pictures of developing chloroplasts show a tripartite arrangement within the plastid (fig 15). Whether this is the direct result of the fusion process is not known.

A Model for the Formation of Discs and Lamellae. Figure 16 diagrams our interpretation of the formation of discs and lamellae inferred from the electron micrographs presented above. We start with the proplastid (fig 16A and 1). The discs are invaginated successively from the inner proplastid membrane (ref 2, fig 13). After the first disc is formed (fig 16B), the second disc begins to invaginate (fig 16C) and disc formation continues in this manner. As more discs form, some begin to fuse (fig 16D, 7) to form lamellae. We do not know whether the fusion of discs is a random process or whether it proceeds in some orderly manner. This fusion would later continue until all the discs which had been formed are fused in groups of 2 to 6 to form completed lamellae.

Development at Low Light Intensity (7 ft-c) in Nondividing Cells. Cells which develop at 7 ft-c

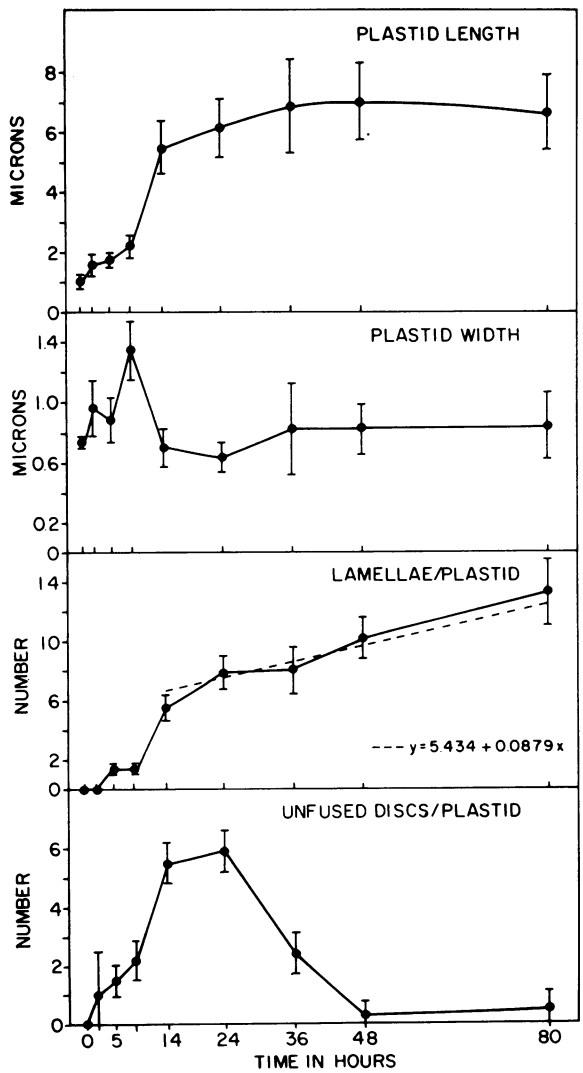


FIG. 14. Kinetics of plastid parameter during plastid development showing length of plastid, width of plastid, number of lamellae and number of unfused discs. Each point is a mean of several observations, the bar representing the 95% confidence interval of the mean (see table I). The broken line shows the least squares fit to lamellar data from 14 to 80 hours.

of light contain less chlorophyll and have lower photosynthetic capacities (17). It was of interest, therefore, to study the fine structure of plastids which had developed at this low intensity.

Dark-grown cells, exposed to 7 ft-c of light, show initially a developmental pattern similar to that described above for cells at 100 ft-c. After 10 to 15 hours, however, abnormalities begin to appear. At 15 hours (fig 17), the size of the plastid does not exceed 3 μ compared with 5.5 μ found for plastids developing at 100 ft-c. The number of lamellae is less than normal (2–5) and there are few unfused discs. Between 45 and 95 hours, the plastids are definitely abnormal. Either the lamellar system is incomplete (fig 18, plastid A) or many discs have not fused to form lamellae and are concentrated in the interior of the plastid (fig 18, plastid B). Occasionally, the plastids seem normal in internal structure but are

Table I

Summary of Measurements of Developing Plastids

Time*	Range**	N***	$\bar{x} \pm SE_{95}$†
	Length of plastid (μ)		
0 hours	0.78–1.21	9	1.04 ± 0.260
2	0.87–2.08	9	1.57 ± 0.329
4–6	1.3–2.2	10	1.75 ± 0.178
7–10	1.4–2.65	10	2.21 ± 0.342
12–16	3.50–8.50	11	5.45 ± 0.893
24	4.3–8.5	8	6.13 ± 1.11
36	5.0–9.4	7	6.85 ± 1.53
48	5.0–11.1	10	6.99 ± 1.29
72–95	3.6–8.8	10	6.62 ± 1.27
	Width of plastid (μ)		
0	0.56–0.86	9	0.74 ± 0.04
2	0.63–1.25	9	0.96 ± 0.18
4–6	0.54–1.20	10	0.88 ± 0.15
7–10	0.84–1.50	10	1.35 ± 0.22
12–16	0.50–1.10	11	0.70 ± 0.12
24	0.5–0.7	8	0.64 ± 0.10
36	0.55–1.5	7	0.82 ± 0.30
48	0.54–1.2	10	0.83 ± 0.16
72–95	0.30–1.2	10	0.84 ± 0.22
	Number of Lamellae per plastid		
0 hours	0	9	0 ± 0
2	0	9	0 ± 0
4–6	1–2	10	1.3 ± 0.25
7–10	1–2	10	1.4 ± 0.37
12–16	3–8	11	5.5 ± 1.01
24	6–9	8	7.8 ± 1.01
36	6–11	7	8.1 ± 1.6
48	7–13	10	10.2 ± 1.4
72–95	8–16	10	13.3 ± 2.2
	Number of Unfused Discs per plastid		
0	0	9	0 ± 0
2	0–2	9	1.0 ± 1.5
4–6	1–3	10	1.5 ± 0.51
7–10	1–3	10	2.2 ± 0.66
12–16	3–7	11	5.5 ± 0.69
24	4–8	8	5.9 ± 1.04
36	1–3	7	2.4 ± 0.72
48	0–2	10	0.30 ± 0.48
72–95	0–2	10	0.50 ± 0.61

* Developmental time in hours (dark-grown cells taken at zero time; all times of development represent time dark-grown cells have been in the light).

** Highest and lowest measurement.

*** Number of measurements taken.

† Mean of measurements ($\bar{x}$) plus or minus the standard error of the mean for 95% confidence level (SE_{95}). SE_{95} was computed as follows: the standard deviation of the measurements (s) was computed in the usual way. The standard error of the mean ($s_{\bar{x}}$) was computed from $s_{\bar{x}} = s/\sqrt{N}$ and was multiplied by t_{95} for the appropriate number of degrees of freedom to yield SE_{95}.

Table II

Summary of Computation of the Magnitude of Change in Plastid Parameters between 7 to 10 and 12 to 16 Hours

	Plastid length (μ)	Plastid width (μ)	Number of lamellae	Number of unfused discs
A) Observed (7–10 hrs)	2.21	1.35	1.4	2.2
B) Observed (12–16 hrs)	5.45	0.70	5.5	5.5
C) B/A $\pm$ SE_{95}*	2.48 $\pm$ 0.523	0.522 $\pm$ 0.115	3.93 $\pm$ 1.17	2.50 $\pm$ 0.75

* The standard error of the ratio B/A for 95% confidence was computed using Fieller's theorem.

rounded rather than elongated, and are lacking in pyrenoids. (fig 19).

The conspicuous differences between development at 7 ft-c and normal intensities are: the plastids never exceed about 3 μ in length compared with about 5.5 μ for normal plastids; and disc formation appears unaffected, but the fusion to form normal lamellae does not proceed in most cases. This would suggest that the process which is prevented at low light intensities is the fusion of all 3 proplastids to yield one chloroplast as well as the normal, orderly fusion of discs to make lamellae. Indeed, the 2 fusion processes may be closely interrelated and plastid fusion might precede and be the initiator of the fusion of discs into lamellae. It is very likely that the plastids described by Moriber et al. (12), from cells grown on agar, actually developed at low light intensity, since their pictures resemble ours for chloroplasts developed at 7 ft-c. This is particularly likely since light would not penetrate readily to the innermost cells due to self-absorption.

When cells which have undergone chloroplast development at 7 ft-c are exposed to normal intensities (100 ft-c), pigments and photosynthetic abilities in-

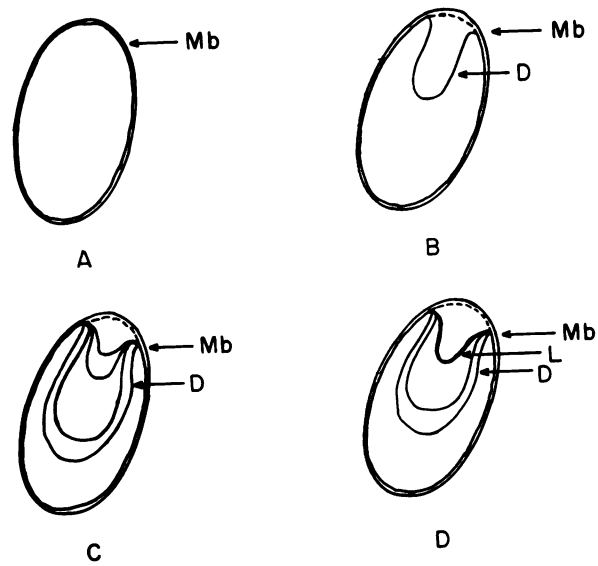


FIG. 16. Model for the early development of the Euglena chloroplast. A) Diagram of proplastid showing double membrane. B) Invagination of first disc from inner membrane. C) Formation of more discs in the same manner. D) Fusion of 2 discs to form a lamella. (Key to abbreviations in legend of figure 1).

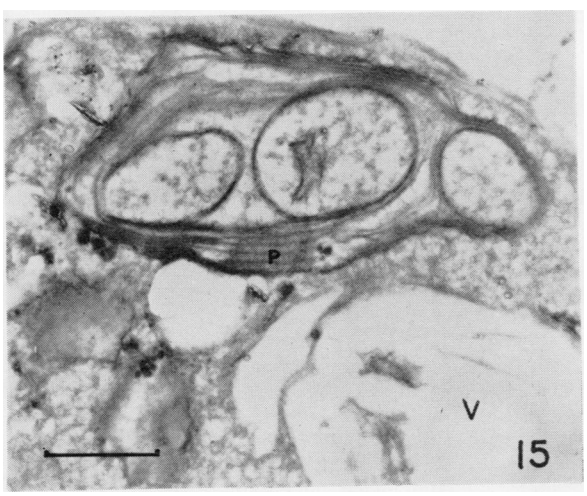


FIG. 15. Nondividing cell, plastid after 24 hours of development at 100 ft-c showing exceptional tripartite internal structure. (Key to abbreviations in legend of figure 1).

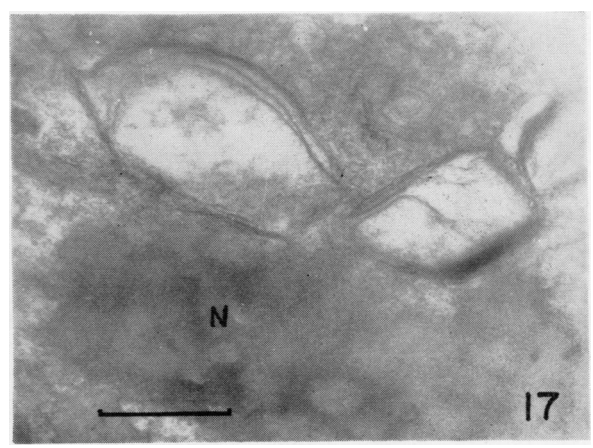


FIG. 17. Nondividing cell, plastid after 15 hours of development at 7 ft-c. (Key to abbreviations in legend of figure 1).

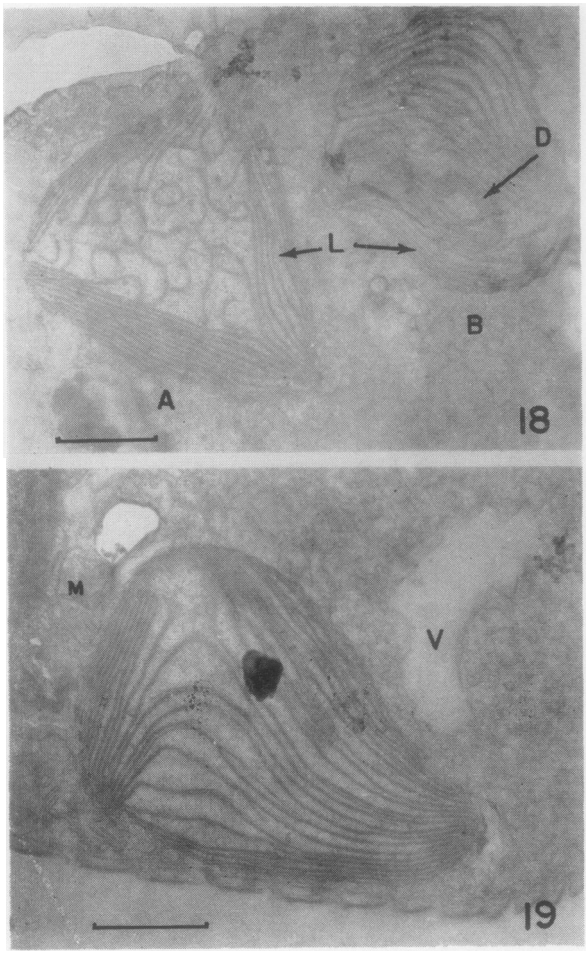


FIG. 18. Nondividing cell, plastid after 72 hours of development at 7 ft-c. (Key to abbreviations in legend of figure 1).

FIG. 19. Nondividing cell, plastid after 95 hours of development at 7 ft-c. (Key to abbreviations in legend of figure 1).

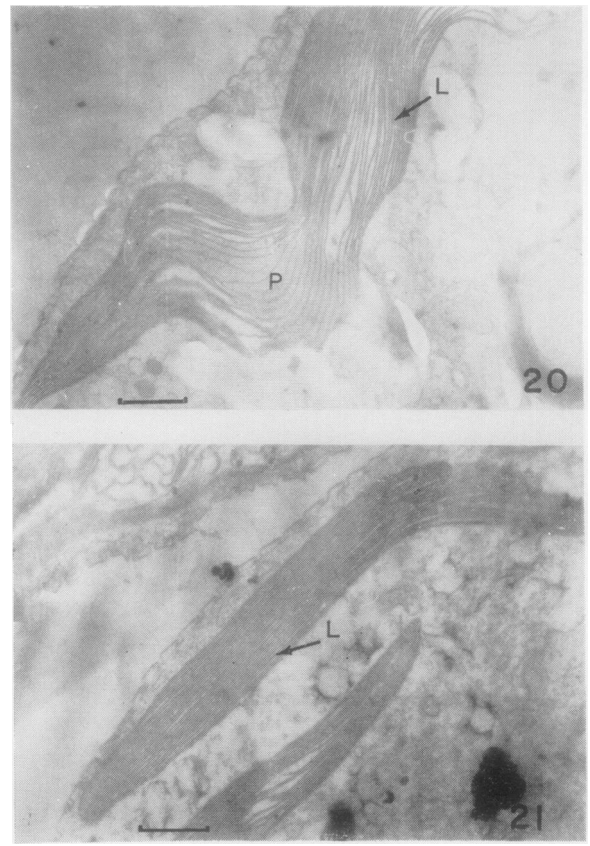


FIG. 20. Nondividing cell, plastid showing hyperdevelopment after 95 hours at 7 ft-c followed by 95 hours at 100 ft-c. (Key to abbreviations in legend of figure 1).

FIG. 21. Nondividing cell, plastid showing hyperdevelopment after 95 hours at 7 ft-c followed by 160 hours at 100 ft-c. (Key to abbreviations in legend of figure 1).

crease and eventually exceed those of cells which have developed entirely at 100 ft-c (17). These hyperdeveloped cells exhibit approximately double the number of lamellae per chloroplast when compared with normally developed cells (figs 20, 21). The lamellae appear to have a normal composition of discs.

Relation of Structure and Function during Chloroplast Development. The normal sequence of development described above can be correlated with the data on pigment formation and photosynthetic abilities obtained under identical conditions (16). During the initial formation of discs, before any lamellae is completed, (1–4 hours of development) there is a small increase in chlorophyll, but carotenoids remain relatively constant. Net photosynthetic O_2 evolution commences at about 4 hours of development but may be present to some extent prior to this time (15). Thus photosynthetic O_2 evolution might be most efficient in completed lamellae but might be associated to some extent with free discs.

Photosynthetic CO_2 fixation is first clearly seen at 6 hours of development when there is at least 1 completed lamella per plastid. The rate of photosynthetic O_2 evolution, CO_2 fixation, and pigment formation increases dramatically at about 10 hours of development and thereafter the increase in these parameters is linear with time until they achieve constant values after about 72 hours of development. From the developmental micrographs presented above, this dramatic inception of linear kinetics in photosynthetic parameters correlates well with the linear rates of lamellar completion following the jump at 12 to 16 hours.

The reduced pigment contents and photosynthetic abilities of cells developing at 7 ft-c of light (17) correlate with the abnormal structure of the plastids under these conditions.

The hyperdevelopment in all photosynthetic parameters (17) which occurs when cells which have undergone chloroplast development at 7 ft-c of light are subsequently exposed to normal intensities (100 ft-c) can be correlated with the electron micrographs shown in figures 20 and 21. As noted above, these hyperdeveloped chloroplasts appear normal in basic

structure but have almost double the number of lamellae when compared with normal chloroplasts.

Comparison of Chloroplast Development in Euglena, Other Algae, and Higher Plants. Many algae are able to form chloroplasts in the dark and, therefore, considerations of chloroplast development do not apply. In a few cases, such as Ochromonas (5) and a mutant of Chlamydemonas (8, 17) attrition of the chloroplast occurs in the dark. The details for Ochromonas suggest that the developmental process is different from that in Euglena.

In higher plants (7, 10, 13, 18) however, the usual situation is the appearance of tubules or vesicles apparently invaginating from the inner membrane of the proplastid on exposure to light. These vesicles arrange themselves in layers and fuse to form discs. These discs eventually organize into grana and stroma lamellae.

It would appear that Euglena shows a very distinctive type of development unlike these other systems, since discs appear to form directly from the inner plastid membrane and grow steadily without necessitating the fusion of tubules. In addition, disc fusion and perhaps plastid fusion appear to play important parts. Finally, the sequential nature of plastid development in Euglena permits a quantitation which has not been possible in other systems.

Summary

Representative electron micrographs of Euglena cells undergoing chloroplast development are presented. Dark-grown cells contain proplastids 1 μ in diameter which have double membranes but little internal structure. The prolamellar body reported for plastids of higher plants appears to be absent. When nondividing dark-grown cells are exposed to optimal light intensities (100 ft-c), chloroplast development begins. During the first 10 hours, numerous discs are produced by invagination of the inner proplastid membrane. After a few discs are formed, some of them fuse into lamellae and this fusion becomes a continuing feature of development. A model for this process is presented. At about 10 hours of development, the number of discs and the length of the plastid triples while the width of the plastid, aside from a small transient change at about this time, remains essentially constant throughout development. These observations give further support to our previously presented model of proplastids fusing by threes to form the basis of the mature chloroplast. The pyrenoid becomes evident in the plastid after about 18 to 20 hours of development. After about 14 hours of development, the formation of lamellae is linear with time but disc formation begins to decrease in rate and eventually ceases. By 72 hours, development is complete and the plastids contain about 13 lamellae and no unfused discs.

Observations of chloroplast development in nondividing cells of Euglena at 7 ft-c reveal a pattern similar to that found at 100 ft-c up to approximately 10 hours of development. After this time, several abnormalities appear. The plastids never triple in length as do their normal counterparts. Discs appear to be formed at a normal rate but often fail to fuse into lamellae or else are observed to form lamellae of abnormal shape and orientation. This suggests that low light intensities may not allow the fusion of proplastids inferred above and also interfere with the normal fusion of discs to form lamellae. No pyrenoids have been observed in plastids developing at 7 ft-c.

The normal developmental sequence at 100 ft-c correlates well with physiological parameters such as chlorophyll and carotenoid formation, photosynthetic O_2 evolution and CO_2 fixation reported previously. The inception of photosynthetic O_2 evolution and CO_2 fixation occur at approximately the same time as the formation of the first lamella (4–5 hours). All parameters become linear between 14 and 72 hours of development as does lamella formation. The abnormal structure of the plastid at 7 ft-c also correlates with the impaired photosynthetic abilities observed for these cells previously. Hyperdevelopment, when cells which have developed at 7 ft-c are exposed to 100 ft-c, is also reflected in changes in chloroplast structure.

Chloroplast development in Euglena appears to differ from that described for higher plants since discs, rather than vesicles, are invaginated from the inner proplastid membrane. Proplastid fusion seems to have no counterpart in higher plants. The sequential nature of development found here appears to be unique to Euglena and permits a quantitation of physiological and developmental events not possible before.

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Water Uptake and Heat Evolution by Germinating Cotton Seed

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If the rate at which water is taken up during the early stages of germination has been studied for the seeds of many plants, the information on the cotton seed is not very abundant in that respect. On the other hand, no parallel studies on the kinetics of water uptake and heat evolution have been made. The purpose of this investigation was to gather information about the kinetics of both processes during the first day of germination of the cotton seed.

Materials and Methods

Cotton seeds (*Gossypium hirsutum*, L. var. 1021-849) from the 1961 harvest at the Lubarika Agricultural Station of the Ruzizi Valley (Congo) were used. They were delinted by treatment with concentrated sulfuric acid for about 5 minutes followed by rinsing with water. During rinsing, the floating seeds were eliminated. After wiping off excess moisture, they were stored at 25° and 40% relative humidity. Water uptake was made to occur in a cylindrical glass vessel with a fritted glass bottom plate through which a stream of water-saturated air was passed. This vessel with a shallow layer of distilled water was kept in a water thermostat, the temperature of which was kept constant within 0.1° from 20° to 47.5°. Water uptake was measured on a 10 seed lot, after wiping with blotting paper, by weighing after a given period of time. The wet weight determination was followed by oven drying at 105°.

Heat evolution was measured in a Calvet Microcalorimeter (2, 3) on groups of 5 seed (about 0.4 g dry matter) to which 0.5 ml water was added. The observation of the heat evolution was made during periods of time ranging from 3 to 24 hours and the water uptake was determined at the completion of each experiment. The initial moisture content of the seeds which had been kept under the conditions indicated above namely 25° and 40% relative humidity varied from 7 to 10% and their viability was about 90%.

Results

When the water uptake during the first 12 hours is plotted against time, fairly smooth sigmoid curves are obtained (fig 1). It should be observed that the variability of the results is fairly low even though each experimental point corresponds to a different 10 seed lot. The sigmoid aspect of the curves sug-

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