The Relation of Oxygen Evolution to Chlorophyll and Protein Synthesis in a Mutant Strain of Chlamydomonas reinhardi [1, 2]

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The preceding paper (2) has described some of the changes that occur in the photosynthetic activity of the mutant strain *y-2* of the unicellular green alga *Chlamydomonas reinhardi* accompanying changes in chlorophyll content. These results indicated that photosynthetic activity might be regulated by a mechanism which is related to either a critical chlorophyll content or structural configuration within the chloroplast. In this paper, the postulated regulatory mechanism will be given further consideration by studying the effects of chloramphenicol on chlorophyll synthesis, protein synthesis, and photosynthetic O_2 evolution. Electron micrographs of cells of regreening cultures were prepared to determine the relationship between the rate of photosynthesis and the structural configuration of the chloroplast.

Materials and Methods

Organism and Growth Conditions. Cultures of the *y-2* mutant strain of *C. reinhardi* were grown in the dark as described by Hudock and Levine (2). After 96 hours of growth in the dark, the cells were harvested by centrifugation (2 minutes at 4,080 × *g*, 0°), washed in 0.068 M K phosphate buffer at pH 6.8, and resuspended in fresh high salt medium (4) supplemented with 0.20 % sodium acetate (Ac medium) and permitted to regreen.

Chlorophyll Synthesis, Protein Synthesis and Oxygen Evolution. Chlorophyll concentration was measured as previously described (2).

The rate of protein synthesis was measured by a study of the kinetics of incorporation of C^{14}-phenylalanine into trichloroacetic acid (10 % at 90°) insoluble material. Cultures of *y-2* were grown in the dark and returned to the light in fresh Ac medium supplemented with C^{14}-phenylalanine. Aliquots were taken at zero time and at hourly intervals during regreening. The cells were collected on millipore filters in a Tracerlab Precipitation Apparatus and were washed once with phosphate buffer and, then, twice with trichloracetic acid. The filter discs were pasted to planchets and the incorporated radioactivity was determined with a gas flow counter.

Photosynthetic O_2 evolution was measured polarographically using a Clark electrode (3).

Enhancement of photosynthesis was calculated as the ratio of the rate of oxygen evolution detected when a culture of *y-2* was simultaneously illuminated with lights of equal energies at 650 and 690 mμ (table I) to the sum of the rates of oxygen evolution detected upon illumination with lights of 650 and 690 mμ separately.

Chloramphenicol was provided by Park, Davis and Company.

Preparation of Electron Micrographs. Cells were harvested by centrifugation and suspended for 1 hour in a mixture of equal volumes of a 4 % solution of oxmium tetroxide and 0.10 M veronal-acetate buffer, pH 8.1. After fixation, the cells were harvested and dehydrated by successive suspension in 50, 70, 95 and 100 % acetone. The cells were then imbedded in an araldite (1) mixture as follows: 30 minutes in a mixture of 3.0 parts araldite, 7.0 parts acetone; 30

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Table I. *Effect of Chloramphenicol (20μg/ml) on Chlorophyll Synthesis, Oxygen Evolution, and Photosynthetic Enhancement*

Lights of equal energies at 650 and 690 mμ were obtained with Bausch and Lomb interference filters, half-band width of 5 to 7 mμ, backed by Corning glass cut off filters.

Hours of exposure to light before addition of chloramphenicol	μg chlorophyll/10^6 cells at 4.5 hours	Oxygen evolution rel. units	Enhancement value
0	1.35	39	1.0
2	1.29	52	1.0
3	1.30	79	1.7
4	1.30	86	1.7
Untreated	1.25	87	1.7

minutes in a mixture of 1.0 parts araldite, 1.0 parts acetone; 30 minutes in a mixture of 7.0 parts araldite, 3.0 parts acetone; and finally, 3 separate 4-hour treatments in 100 % araldite. The material was then stored overnight in accelerated araldite (1) and finally transferred to gelatin capsules for storage at 60° for 5 days.

Thin sections were cut with glass knives on a Porter-microtome and collected on 200 mesh copper grids coated with a film of 0.25 % collodion in butyl acetate. Staining was accomplished by floating the grids on a saturated solution of uranyl acetate for 90 minutes. Electron micrographs were prepared with an RCA EMU-3D electron microscope.

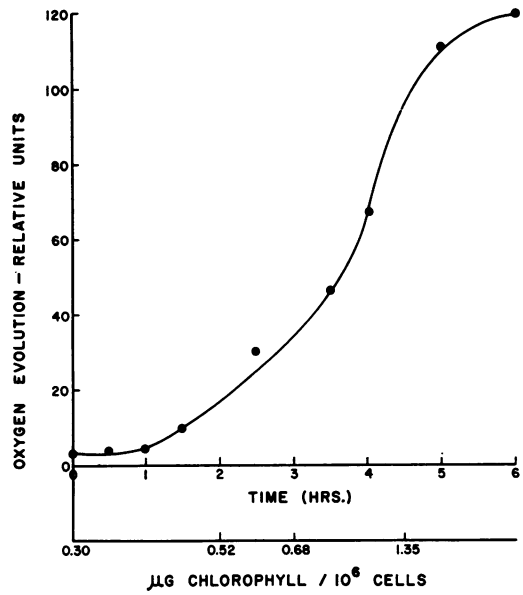
Results

Development of Oxygen Evolution. As shown in figure 1, the change in the rate of oxygen evolution was not directly proportional to increase in chlorophyll content during the early stages of regreening (3). The rate increased slowly until a chlorophyll content of about 0.50 μg/10^6 cells was attained, and a rapid increase in the rate of O_2 evolution was then observed. A maximum rate of O_2 evolution was detected when the chlorophyll content of the culture approached 1.50 to 2.0 μg/10^6 cells.

Effects of Chloramphenicol on Protein Synthesis and the Development of Photosynthetic Activity. The effect of chloramphenicol on chlorophyll synthesis was measured by permitting dark-grown cultures of *y-2* to regreen in the presence of the antibiotic in concentrations ranging from zero to 500 μg/ml. The rate of chlorophyll synthesis was exponential in all cases, and the percent inhibition of synthesis (fig 2) was calculated from the rate constants characterizing chlorophyll synthesis at the various chloramphenicol concentrations. Concentrations of chloramphenicol below 20 μg/ml had no effect on the rate of chlorophyll synthesis. A maximum inhibition of about 50 % was obtained at a concentration of 200 μg/ml and higher concentrations produced no further effect.

The incorporation of C^{14}-phenylalanine into trichloroacetic acid insoluble material was also exponential at all chloramphenicol concentrations. A 50 % inhibition was observed at a concentration of 20 μg/ml and higher concentrations produced a maximum inhibition of about 65 % (fig 2).

Chloramphenicol (20 μg/ml) was added to regreening cultures of *y-2* before exposure to light and to others after 2, 3 or 4 hours of exposure. After 4.5 hours of exposure to light, the cells were harvested, washed, and resuspended in fresh Ac medium. The rate of oxygen evolution and enhancement of photosynthesis were then measured polarographically. Addition of chloramphenicol before exposure to light resulted in a 55 % inhibition of oxygen evolution (table I). Addition of chloramphenicol after 2 hours of exposure to light was only slightly less inhibitory. Addition of the antibiotic after 3 or 4 hours of exposure to light had little or no effect on the rate of oxygen evolution. Enhancement of photosynthesis was also inhibited if chloramphenicol was added either before exposure to light or after 2 hours of exposure. After 3 hours of exposure, the addition of chloramphenicol to the medium had no effect on enhancement.



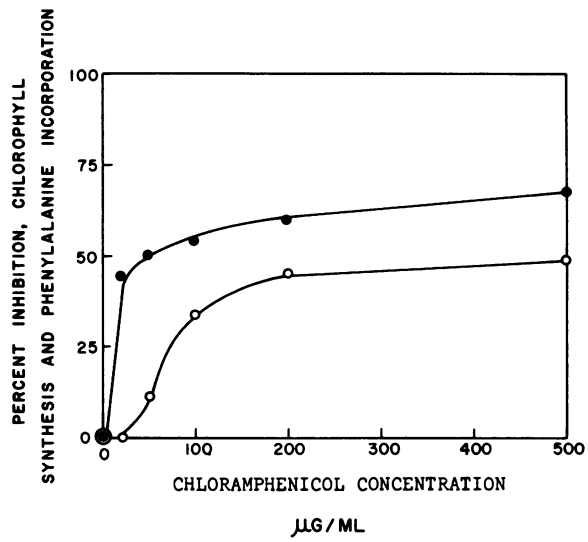


FIG. 1. Development of oxygen evolution in a regreening culture of *y-2*. Oxygen evolution in relative units is plotted as a function of both hours of regreening and chlorophyll content/10^6 cells. Oxygen evolution at 25° was measured in white light from tungsten reflector flood lights at an intensity of 8,500 lux.

FIG. 2. Percent inhibition of chlorophyll synthesis and of C^{14}-phenylalanine incorporation (1.2×10^{-4} M, specific activity 1.65μc/μmole) in regreening cultures of *y-2* by various concentrations of chloramphenicol. ○ = chlorophyll synthesis; ● = phenylalanine incorporation.

Electron Micrographs. In electron micrographs of cells of a dark-grown culture of *y-2* (fig 3), the chloroplast envelope was evident but little or no lamellar structure could be detected. Following 2 hours of exposure to light (fig 4), isolated chloroplast lamellae were apparent but an organized lamellar structure had not developed. After 3 hours of exposure to light (fig 5), a well-developed, lamellar chloroplast was seen. Large increases in the activities of both photosynthetic pyridine nucleotide reductase (PPNR) and ribulose 1,5-diphosphate (RuDP) carboxylase (2) and a large increase in the rate of oxygen evolution also were observed following a similar period of regreening. When a regreening culture of *y-2* was permitted to regreen for 3 hours in the presence of chloramphenicol (fig 6), the formation of chloroplast lamellae was suppressed.

Discussion

Hudock and Levine (2) found that the activities of PPNR and RuDP carboxylase increased rapidly only after a total chlorophyll content of 0.50 to 0.70 $\mu g/10^6$ cells was attained in regreening cultures of the mutant strain *y-2* of *C. reinhardi*. It has been shown here that the rate of oxygen evolution increased markedly at a similar chlorophyll content of

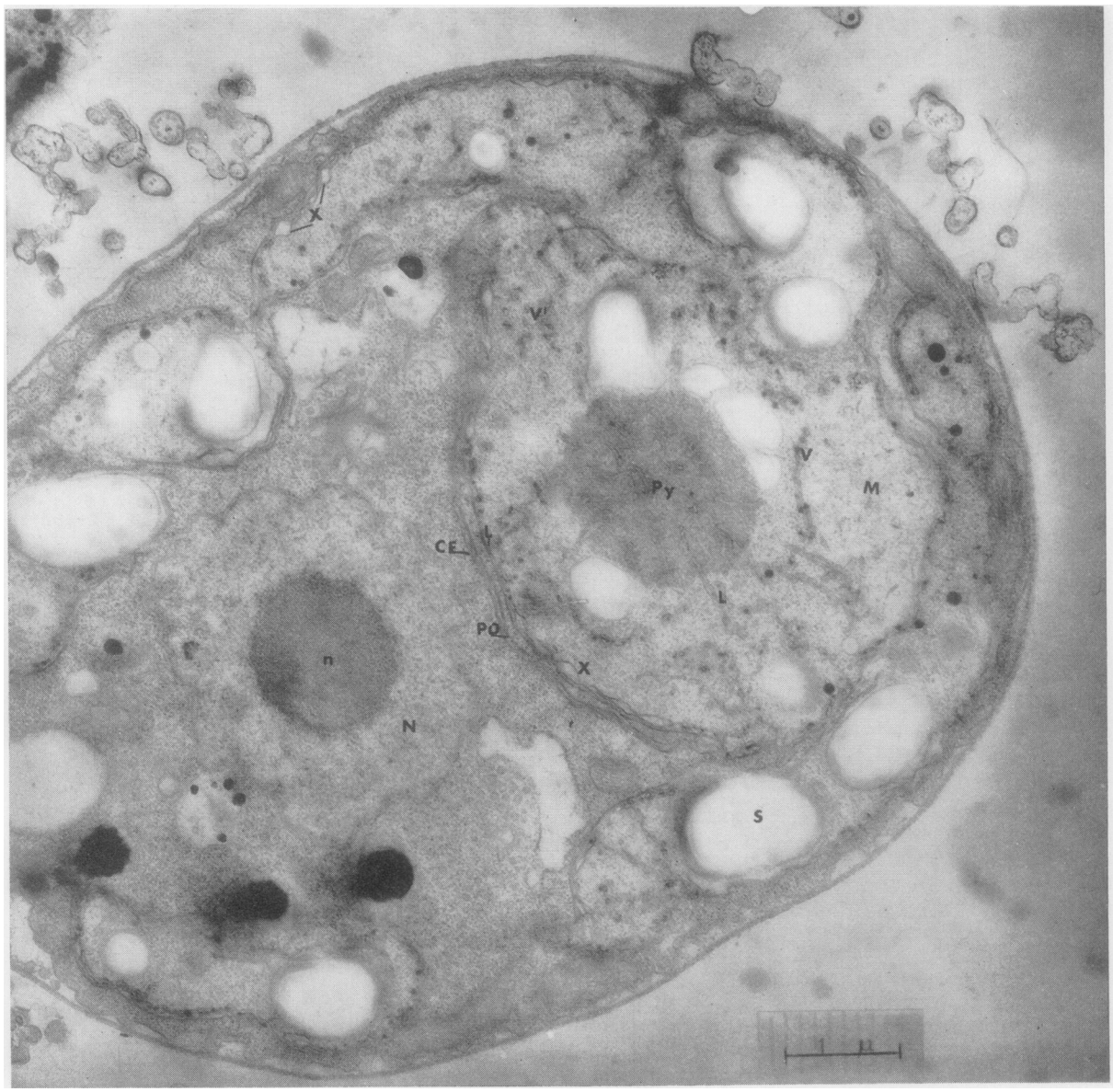


FIG. 3. Electron micrograph of a cell from a 96 hour dark-grown culture of *y-2*. C = chloroplast, CE = chloroplast envelope, L = lamella, M = chloroplast matrix, N = nucleus, n = nucleolus, PD = peripheral disc, PB = prolamellar body, Py = pyrenoid, S = starch grain, V = vesicle.

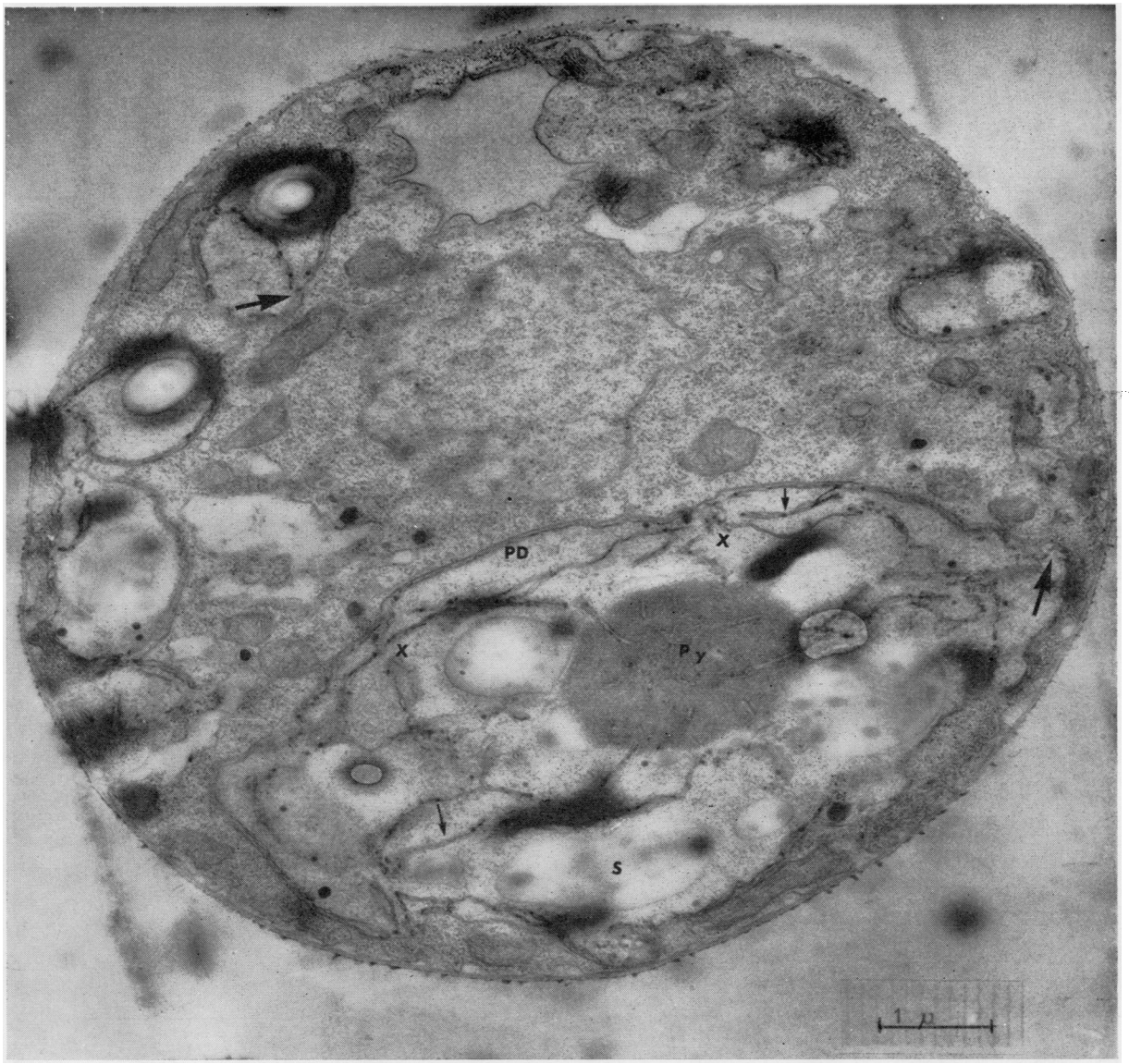


FIG. 4. Electron micrograph of a cell from a regreening culture of *y-2* following 2 hours of exposure to light. Symbols as in figure 3.

the cells. Treatment with chloramphenicol (20 μg/ml) during the first 3 hours of regreening inhibited the development of O_2 evolution and the incorporation of phenylalanine into protein but did not affect the rate of chlorophyll synthesis. These results provide tentative evidence for the conclusion that a factor directly related to chlorophyll synthesis or content is not responsible for the sudden increase in the rates of several photosynthetic reactions observed. However, these experiments do not eliminate the possibility that a factor directly related to chlorophyll synthesis acts as an inducer for these reactions. Chloramphenicol might inhibit protein synthesis in the presence of an inducer which is directly related to chlorophyll synthesis.

If it is true that the sudden and rapid increase observed in the rates of several photosynthetic reactions in regreening *y-2* is not correlated with a factor directly related to chlorophyll synthesis or content, it may be suggested that the structural development of the chloroplast to a certain stage is critical in triggering such activity. Addition of chloramphenicol before a structurally active chloroplast develops might result in an altered proportion of chloroplast components and interfere with the development of specific photosynthetic reactions. Addition of the antibiotic after such development would have less effect on the potential activity of the photosynthetic apparatus. On the basis of this hypothesis and the results of the present study, it would be predicted that

a major change in the structural configuration of the chloroplast would be detected between the second and third hours of exposure of a dark-grown culture of *y-2* to the light. The electron micrographs presented (fig 3–6) demonstrate that this is the case.

An analysis of the effect of chloramphenicol on chlorophyll synthesis, protein synthesis, the development of photosynthetic oxygen evolution, and the development of lamellar structure within the chloroplast permits certain conclusions concerning the mechanism regulating the development of photosynthetic activity in regreening *y-2*. Rapid increases in the activities of PPNR and RuDP carboxylase and in the rate of oxygen evolution occur simultaneously with the development of a well-organized, lamellar chloroplast. Since treatment with chloramphenicol inhibited the development of a lamellar chloroplast and of several photosynthetic reactions but had no effect on the rate of chlorophyll synthesis at low concentrations, it seems reasonable to suggest that the structural development of the chloroplast rather than simple chlorophyll content is critical in triggering the development of photosynthetic activity. While chlorophyll may be necessary for such development, it clearly is not sufficient.

Summary

The *y-2* mutant strain of *Chlamydomonas reinhardi* can be made to undergo bleaching and regreening under rigidly defined and controllable conditions.

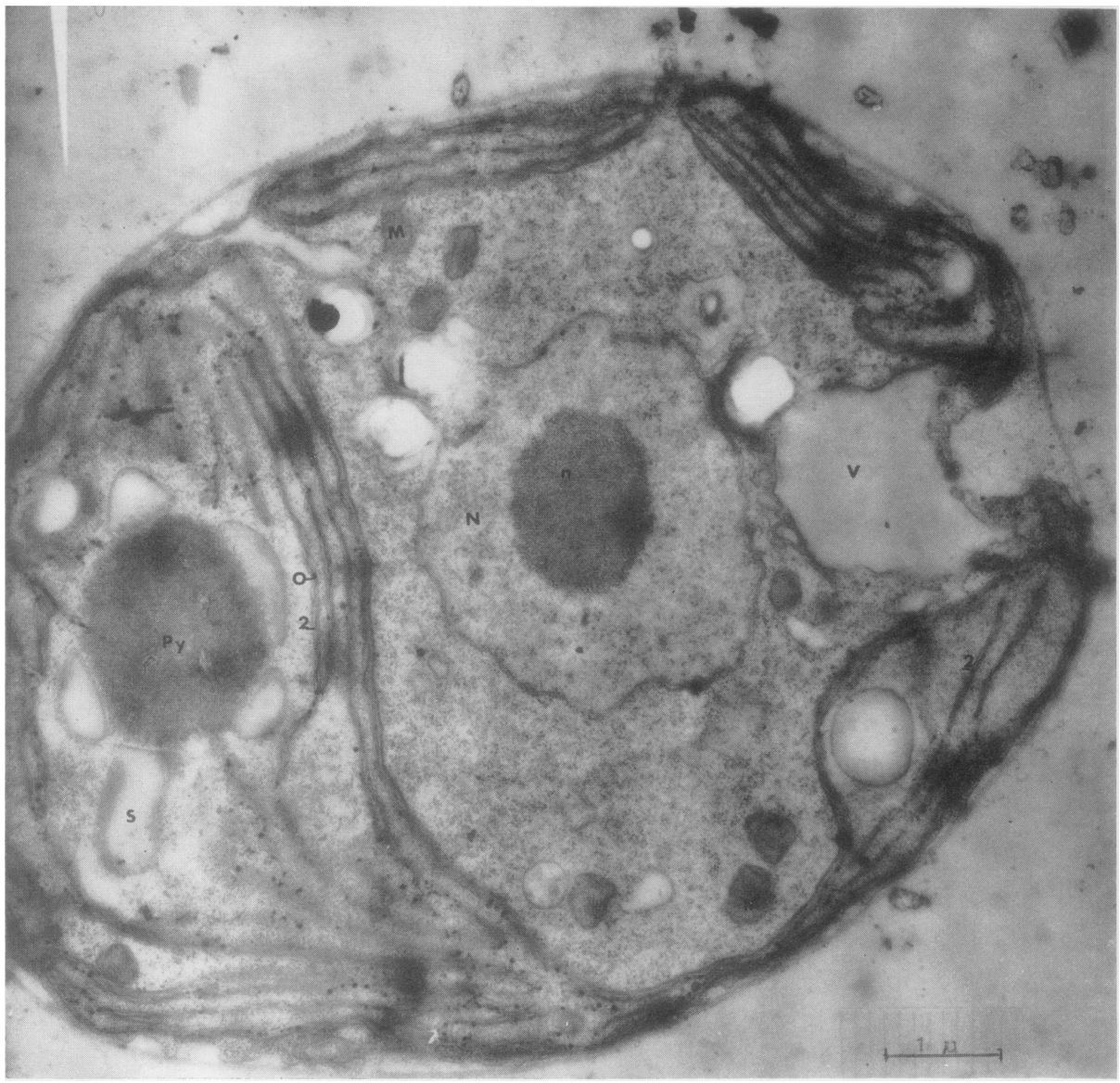


FIG. 5. Electron micrograph of a cell from a regreening culture of *y-2* following 3 hours of exposure to light. Symbols as in figure 3.

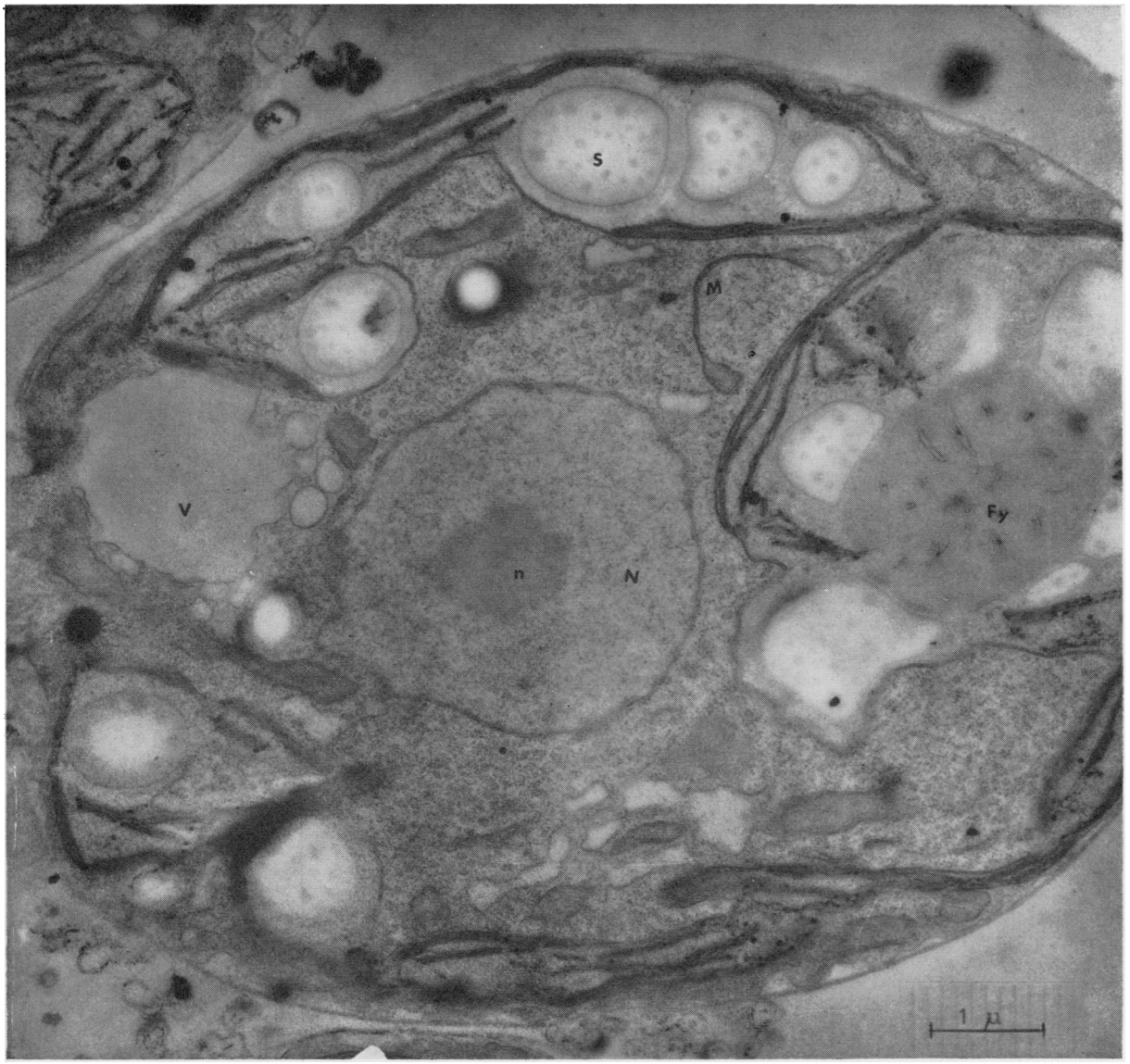


FIG. 6. Electron micrograph of a cell from a regreening culture of *y-2* following 3 hours of exposure to light in the presence of chloramphenicol (20μg/ml). Symbols as in figure 3.

The development of several photosynthetic reactions during the regreening process was studied. The rates of several reactions remained relatively constant during the first 2 to 3 hours of regreening and then increased rapidly. It is suggested that this rapid increase in photosynthetic activity is triggered by the development of a properly structured photosynthetic apparatus. Studies on the effects of chloramphenicol indicate that protein synthesis during the early stages of regreening is essential if a well-organized chloroplast and a high rate of photosynthesis are to develop.

Acknowledgments

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