LIGHT-INDUCED GREENING IN LIQUID CULTURES OF STREPTOMYCIN-BLEACHED MUTANTS OF *CHLAMYDOMONAS REINHARDI*

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1. Introduction

Streptomycin-resistant mutants of *Chlamydomonas reinhardi* first described by Sager [1], represent an appropriate tool for the investigation of the biogenesis of thylakoid membranes. Strain sr_3 [2] with a chromosomal inheritance of streptomycin-resistance forms yellow cells in the dark in the presence of sublethal amounts of streptomycin. In this type of cells the typical lamellar structure of chloroplasts is absent. On illumination, however, cells turn green with a simultaneous formation of thylakoid membranes.

Until recently, streptomycin-bleached cells of this strain could be cultivated exclusively on solid media, giving poor yields and inhomogeneous cell populations. Since, for studying the biochemical events during greening, amounts of yellow cells sufficient for chemical analysis of membrane components in different stages of differentiation were needed, a cultivation procedure capable of giving higher yields of homogeneous cells had to be developed.

In this contribution, a procedure is reported for the cultivation of yellow cells in liquid media. This is shown to be possible only under selected physiological conditions. Chlorophyll content and fatty acid pattern as a representative index of biochemical changes during greening are described.

Investigations on streptomycin-bleached cells of *Chlamydomonas* can contribute substantially to the knowledge of thylakoid formation. Until now this has been studied with y-1 mutants of *Chlamydomonas* [3] and with chlorotic green plants [4].

2. Materials and methods

Strain sr_3 [5] of *Chlamydomonas reinhardi* was cultivated in Erlenmeyer flasks in 35 ml of Medium I according to Sager and Granick [6], containing 100 mg arginine hydrochloride and 2 g Na-acetate · 2 H_2O per liter of medium. Cultivation was carried out under fluorescent light at 27°C for 3 days. Cultures of yellow cells were grown in 1200 ml of 1.5-fold concentrated Medium I with arginine and acetate added and containing 20 μg streptomycin sulfate per ml. Cylindrical glass tubes (7 × 48 cm) with spherical bottoms were used as cultivation vessels and were inoculated with 2.5×10^7 cells. The cotton-pluged vessels were aerated (2 liters/min) trough a glass tube leading to the bottom. During incubation for 12–14 days in the dark, the cell number increased about 10 times to a final value of 2×10^5 cells/ml. Cells were harvested by centrifugation for 15 min at 2000 *g* in autoclaved steel beakers equipped with aluminum covers (Heraeus-Christ Cryofuge 6-6). The pellets were washed with and suspended in 'greening medium', which contained only 1/5 of the acetate, 1/5 of the phosphate and 1/10 of the nitrogen of Medium I. Reduction of the mineral salt content reduces cell division, while reduction of acetate promotes the adaptation of cells to phototrophic growth. To compensate for the change in ionic strength, 0.3 g KCl and 1.23 g $NaHCO_3$ were added per liter of medium. The pH of the solution was 7.9.

Greening of cells occured in 35 ml of 'greening medium' in Erlenmeyer flasks which were shaken

under 900 lux of fluorescent light at 27°C. Cell number and chlorophyll [7] were determined periodically. Lipids were extracted with chloroform–methanol (2:1) from aliquots of cell suspensions by heating the mixture to 70°C for 20 sec. Fatty acid methyl esters were prepared from the extract with Na-methoxide [8] and chromatographed on Chromosorb W loaded with 5% diethylene glycol succinate (DEGS), 1/8 in., 6 ft., 146°C, 12 ml N_2/min using a flame ionization detector. Relative amounts of single components were calculated from the peak areas without regard for individual response factors. The identity of fatty acids was established by gas-liquid chromatography and mass spectrometry. The analytical data on the lipids of *Chlamydomonas reinhardi* will be published separately. Samples of greening cells were also prepared for electron microscopy [2].

3. Results and discussion

In order to investigate the de novo synthesis of thylakoid membranes of streptomycin-bleached *Chlamydomonas* cells, a procedure for cultivation and greening of yellow cells in a liquid medium was developed. The method produces thylakoid-free, microscopically uniform cells. For the greening process, experimental conditions were chosen, which promote the production of chlorophyll and other membrane components but inhibit cell division.

Under greening conditions, the cells turn pale green, as observed in the light microscope. The initial cell concentration remains at 4×10^6 cells/ml for the first 70 hr, whereas chlorophyll per ml of cell suspension increases 7-fold, as shown in fig.1, indicating a marked increase of chlorophyll per cell. Corresponding changes in the fine structure of the cells could be seen in electron micrographs taken before and during greening. Yellow cells contain large amounts of starch, but no visible thylakoid structure. Within 22 hr, the starch grains disappear and lamellar structures are formed. Young cells newly formed by cell division are not seen in the light microscope, nor in the electron micrographs. No significant bacterial contamination could be observed during the first 46 hr of illumination.

From these observations we conclude, that the

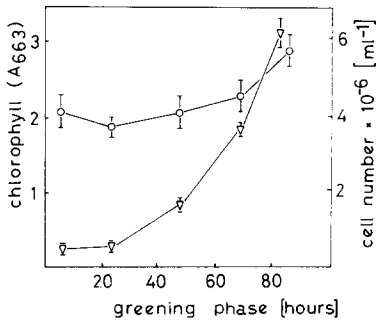

Fig.1. Greening of streptomycin-bleached cultures of *Chlamydomonas reinhardi* sr_3 in liquid media. Cell number (o), chlorophyll content (▽).

green color developed during the first 46 hr of greening is due entirely to the synthesis of chlorophyll and a simultaneous formation of thylakoids in formerly yellow cells. Since the cell concentration in greening sr_3 cultures starts increasing only after 70 hr, cell division in this strain is apparently inhibited for a longer period of time than in the y-1 mutant, where cell division starts after about 6–8 hr of illumination [9,10].

Greening is accompagnied also by a typical change in the fatty acid pattern, as shown in fig.2. The relative amounts of 16:4, 18:3ω3, 18:3ω6, and 18:2 acids increase, whereas that of 16:0 acid decreases significantly. The percentage of 16:1 and 18:1 acids keep nearly the same level. Since poly-unsaturated fatty acids of the C_{16} and C_{18} series constitute an

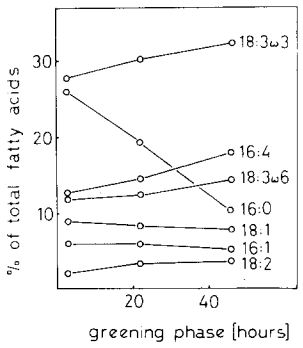

Fig.2. Changes in the fatty acid pattern during greening of streptomycin-bleached cultures of *Chlamydomonas reinhardi* sr_3. 18:3ω3 = α-linolenic acid. 18:3ω6 = γ linolenic acid.

integrating part of chloroplast membranes [11], these results correlate with the formation of lamellar structures detected on micrographs. In greening y-1 mutant cells, an increase of 18:3 and 18:2 acids has been reported [12]. Significant differences, however, in the relative amounts of 16:4 and 16:0 acids have not been mentioned by these authors.

Basing on the described procedure, the formation of individual proteins and lipids in greening cells is under further investigation.

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References

[1] Sager, R. (1954) Proc. Natl. Acad. Sci. U.S. 40, 356–363.
[2] Boschetti, A. and Walz, A. (1973) Arch. Mikrobiol. 89, 1–14.
[3] Eytan, G., Jennings, R. C., Forti, G. and Ohad, I. (1974) J. Biol. Chem. 249, 738–744.
[4] Machold, O. (1971) Biochim. Biophys. Acta 238, 324–331.
[5] Boschetti, A. and Bogdanov, S. (1973) Eur. J. Biochem. 35, 482–488.
[6] Sager, R. and Granick, S. (1954) J. Gen. Physiol. 37, 729–742.
[7] Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576–578.
[8] Thies, W. (1971) Z.Pflanzenzüchtung 65, 181–202.
[9] Ohad, I. Siekevitz, P. and Palade, G. E. (1967) J. Cell Biol. 35, 521–551.
[10] Ohad, I., Siekevitz, P. and Palade, G. E. (1967) J. Cell Biol. 35, 553–584.
[11] Erwin, J. A. (1973) in: Lipids and Biomembranes of Eucaryotic Microorganisms (Erwin, J. A., ed.), pp. 41–144, Academic Press, New York.
[12] Goldberg, I. and Ohad, I. (1970) J. Cell Biol. 44, 563–571.