Change of L-Ascorbic Acid Content in Synchronized Cultures of *Euglena gracilis*

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The content of total L-ascorbic acid in light–dark synchronized *Euglena gracilis* Z increased rapidly with illumination to reach a maximum after 7 h in the light and then decreased to reach its original level after 18 h in the dark. Total L-ascorbic acid formation was strongly dependent on illumination and was inhibited by cycloheximide, but not by chloramphenicol or streptomycin. Inhibitors of respiration and photosynthesis markedly inhibited L-ascorbic acid formation, indicating that the change of the L-ascorbic acid content may be related to the metabolic activities of mitochondria and chloroplasts.

INTRODUCTION

When grown photoautotrophically with an appropriate light–dark cycle, *Euglena* cells readily divide synchronously. Under these conditions, the cell number doubles in each dark period but remains constant in the light period (Cook & James, 1960; Cook & Hess, 1964). RNA and protein are synthesized in both light and dark periods, while large amounts of paramylon (storage carbohydrate) are accumulated in the light period and utilized as carbon and energy sources for RNA and protein synthesis in the dark (Cook, 1971). DNA synthesis increases in a step-wise manner, starting around 6 h after the onset of the light period (Edmunds, 1964).

In the present paper, we describe changes in the cellular content of total L-ascorbic acid during the cell cycle of *Euglena gracilis* Z and also discuss the physiological function of L-ascorbic acid in relation to photosynthesis and respiration.

METHODS

Organism and culture conditions. *Euglena gracilis* Z was precultured photoautotrophically in the pH 6.8 salt medium of Cramer & Myers (1952). Batch cultures (2 l) were magnetically stirred and aerated with sterile air (8 l h⁻¹). The entire culture apparatus was housed in a thermostatic chamber at 26 °C, and illumination from cool white fluorescent tubes gave an incident light intensity of 9000 lx at the surface of the vessel. The vessel was inoculated to give an initial concentration of about 10⁴ cells ml⁻¹. As previously (Cook, 1971), the cells were synchronized by 14 h : 10 h light–dark cycles. Cell divisions were confined to the dark periods when a doubling of cell number occurred between cell densities of 10⁴ and 8 × 10⁵ cells ml⁻¹. In order to minimize deviations in cell metabolism, all experiments were done with synchronized cultures of the same concentration of 2.0–4.0 × 10⁵ cells ml⁻¹. Cell number was determined with a haemocytometer.

Determination of L-ascorbic acid. The cells harvested at regular intervals throughout the cell cycle were disrupted with an ultrasonic oscillator (10 kHz, 2 min) in 5% (w/v) metaphosphoric acid and the homogenate was centrifuged at 24000 g for 10 min. Total L-ascorbic acid was determined colorimetrically by the 2,4-dinitrophenylhydrazine (DNPH) method was described previously (Shigeoka *et al.*, 1979a). To 1 ml of the

Abbreviations: CV, coefficient of variation; DNPH, 2,4-dinitrophenylhydrazine; DCIP, 2,6-dichlorophenol-indophenol; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea.

supernatant was added 0.2 ml 6 mM-2,6-dichlorophenolindophenol (DCIP) for the quantitative conversion of L-ascorbic acid (reduced form) to dehydroascorbic acid (oxidized form). After standing for 20 min, 0.5 ml 2% (w/v) thiourea in 2% (w/v) metaphosphoric acid was added to reduce excess DCIP. The mixture was incubated with 0.25 ml 2% (w/v) DNPH in 25% (v/v) H_2SO_4 for 1 h at 50 °C for the formation of the hydrazone from dehydroascorbic acid. After the addition of 1.25 ml 85% (v/v) H_2SO_4 on cooling, the hydrazone formed was assayed colorimetrically at 520 nm. Dehydroascorbic acid was determined by this method without the addition of DCIP.

RESULTS

L-Ascorbic acid content during the cell cycle

Fig. 1 shows a typical pattern of the content of total L-ascorbic acid during the cell cycle of *E. gracilis* grown on a 14 h:10 h light-dark cycle. The total L-ascorbic acid content increased rapidly until it reached a maximum (4×10^{-15} mol per cell) after 7 h in the light. It then decreased gradually throughout the rest of the light period followed by a rapid fall to the original level in the dark. The content of total L-ascorbic acid depended on light intensity, since cells synchronized at 9000 lx contained 1.3 times more than those at 5500 lx. The content of dehydroascorbic acid after 7 h in the light was about 20% of that of total L-ascorbic acid and during the cell cycle the ratio of the oxidized form to total L-ascorbic acid was constant.

Transfer of the cells from dark to light resulted in a rapid augmentation of the content of total L-ascorbic acid. When the synchronized cells were transferred to the dark after the start of the light period, the content of total L-ascorbic acid did not change (Fig. 1). However, darkening the cells at several stages in the light period for 3 h caused total L-ascorbic acid content to decrease to the level at the beginning of the light period. When the darkened cells were reilluminated for 2 h, the content of total L-ascorbic acid again increased rapidly (Fig. 1).

Effect of antibiotics on L-ascorbic acid formation

Effects of some antibiotics on the content of total L-ascorbic acid in *E. gracilis* after 7 h in the light are shown in Table 1. Cycloheximide, an inhibitor of protein synthesis on the 87S cytoplasmic ribosomes (Bovarnick *et al.*, 1974*a, b*), completely inhibited the increase in total L-ascorbic acid content, whereas chloramphenicol and streptomycin, specific inhibitors of protein synthesis on the 68S plastid ribosomes (Bovarnick *et al.*, 1974*a, b*), had little effect. When cycloheximide was added to the medium at different stages during the cell cycle, total L-ascorbic acid formation either promptly stopped or slowed down (Fig. 2). Furthermore, the increase in total L-ascorbic acid content was completely inhibited by cycloheximide when the cells in the dark period were illuminated.

Fig. 1. Effect of illumination on total L-ascorbic acid content of *E. gracilis* Z synchronized on a 14 h:10 h light-dark cycle. The white bar at the top indicates the light period of the cycle and the black bar, the dark period. ○, Cells under illumination; ●, cells in the dark. The solid line shows values for cells in the light-dark cycle and broken lines represent cells illuminated for shorter periods at the intervals shown. Each experimental point represents the mean of four assays (CV% $\leq$ 5).

Fig. 2. Effect of cycloheximide and DCMU on total L-ascorbic acid content of *E. gracilis* Z synchronized on a light-dark cycle. ●, Control (no compounds added); △, cycloheximide (3.6×10^{-5} M) added after 3 h, 7 h, 10 h and 18 h of the cell cycle; □, DCMU (5×10^{-5} M) added after 3 h, 7 h and 10 h of the light period; ○, DCMU + D-glucose (10^{-3} M) added after 3 h. Each value shows the mean of three assays ($CV\% \leq 6$).

Table 1. *Effects of some antibiotics on L-ascorbic acid formation by E. gracilis* Z synchronized on a light-dark cycle

Antibiotics were added to the medium 1 h before the start of the light period to the final concentrations shown, which did not affect cell viability. Effects are shown as percentage inhibition after 7 h as compared with the increase of the cellular content of total L-ascorbic acid in the control to which no antibiotic was added. Each value represents the mean of four assays $\pm$ SD.

Antibiotic	Concn (M)	L-Ascorbic acid content (fmol per cell)	Inhibition (%)
None			
After 0 h	—	1.29 ± 0.06	—
After 7 h	—	3.90 ± 0.10	0
Cycloheximide	3.6×10^{-5}	1.26 ± 0.04	100
Chloramphenicol	3.0×10^{-3}	3.90 ± 0.09	0
Streptomycin	1.0×10^{-5}	3.60 ± 0.09	11

Effect of respiratory and photosynthetic inhibitors on L-ascorbic acid formation

When 3-(3', 4'-dichlorophenyl)-1,1-dimethylurea (DCMU), a specific inhibitor of photosynthesis, was added to the medium after 3 h and 7 h in the light, the increase in total L-ascorbic acid content was inhibited (Fig. 2). However, the addition of DCMU at other stages of the cell cycle did not affect the total L-ascorbic acid content. When D-glucose (1 mM) was added in the presence of DCMU after 3 h in the light, the total L-ascorbic acid content after 4 h was restored to about 80% of the level in the control culture (Fig. 2).

Dinitrophenol and valinomycin markedly inhibited the increase of total L-ascorbic acid content when added to the culture medium 1 h before the start of the light period. Rotenone and azide showed lesser effects (Table 2).

DISCUSSION

The maximum content of total L-ascorbic acid was observed after 7 h in the light and then declined to its original level in 18 h during one cell cycle of synchronized cultures in *E. gracilis* (Fig. 1). The total L-ascorbic acid content depended heavily on the light intensity. The pattern of the light-dependent changes of total L-ascorbic acid content of *E. gracilis* was similar to results

Table 2. *Effects of respiratory and photosynthetic inhibitors on L-ascorbic acid formation by synchronized E. gracilis Z*

Inhibitors were added to the medium to the final concentrations shown 1 h before the beginning of the light period. Values are given as percentage inhibition after 7 h as compared with the increase of the cellular content of total L-ascorbic acid in the control. Each value represents the mean of four assays $\pm$ SD.

Inhibitor	Concn (M)	L-Ascorbic acid content (fmol per cell)	Inhibition (%)
None			
After 0 h	—	1.30 $\pm$ 0.05	—
After 7 h	—	3.87 $\pm$ 0.12	0
Rotenone	5 $\times$ 10 ⁻⁵	2.59 $\pm$ 0.07	50
Sodium azide	1 $\times$ 10 ⁻³	2.79 $\pm$ 0.10	42
Dinitrophenol	1 $\times$ 10 ⁻⁴	1.99 $\pm$ 0.06	73
Valinomycin	1 $\times$ 10 ⁻⁶	1.86 $\pm$ 0.08	78
DCMU	5 $\times$ 10 ⁻⁵	1.56 $\pm$ 0.06	90

obtained with synchronized *Chlorella pyrenoidosa* (Gerhardt, 1964). About 20% of total L-ascorbic acid was present as the oxidized form throughout the cell cycle, suggesting that the constant ratio of oxidized form to reduced form is maintained by the biosynthesis, metabolism and oxidation-reduction cycle of L-ascorbic acid in *E. gracilis* cells (Shigeoka *et al.*, 1979*b*, 1980).

The results indicate that L-ascorbic acid formation was affected by the light. We have reported previously that illumination of dark-grown *E. gracilis* caused a significant increase in the cellular content of total L-ascorbic acid. Since the cells were light-saturated at a light intensity of 2000 lx, and DCMU caused no inhibition of total L-ascorbic acid formation, it was suggested that the light-dependent increase of L-ascorbic acid in *E. gracilis* is not primarily associated with photosynthesis (Shigeoka *et al.*, 1979*a*). However, since the content of total L-ascorbic acid was higher in synchronized cells at 9000 lx than in cells at 5500 lx, and the addition of DCMU before total L-ascorbic acid content reached its maximum level prevented further increase, L-ascorbic acid formation is clearly related, directly or indirectly, to photosynthesis in autotrophically grown cells. D-Glucose is a major starting material for L-ascorbic acid biosynthesis in *E. gracilis* (Shigeoka *et al.*, 1979*b*). The addition of 1 mM-glucose, concurrently with DCMU, allowed about an 80% restoration of total L-ascorbic acid content, indicating that the inhibition of L-ascorbic acid formation by DCMU is due to inhibition of the supply of glucose (paramylon), a product of photosynthesis. Following a peak of total L-ascorbic acid after 7 h in the light, DCMU showed no effect, suggesting that, at this stage *E. gracilis* contains sufficient paramylon to synthesize L-ascorbic acid. Paramylon was synthesized continuously in the light period and then consumed in the following dark period under the present experimental conditions (data not shown), as reported by Cook (1971).

Cycloheximide inhibited the increase in total L-ascorbic acid content, showing that the increase is attributable to a photoinduction of *de novo* protein synthesis on cytoplasmic ribosomes. The increase in the total L-ascorbic acid content was also prevented by various respiratory inhibitors and uncouplers, indicating that the light-dependent formation of L-ascorbic acid is also closely related to respiration. Photosynthetic capacity varies in a cyclic manner during the cell cycle of *E. gracilis*, reaching a peak 2 h before the beginning of darkness (Walther & Edmunds, 1973). Giant mitochondria are temporarily formed in *E. gracilis* at an intermediate stage in the light period of the cell cycle, accompanied by a marked decrease in the oxygen uptake activity of the cells (Osafune *et al.*, 1975). Consequently, L-ascorbic acid may function during the cell cycle to maintain the metabolic activities in mitochondria and chloroplasts.

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