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Changes of ribulose 1,5-diphosphate carboxylase level during the processes of degeneration and regeneration of chloroplasts in *Chlorella protothecoides*

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1. RuDP carboxylase was active mainly in chloroplasts and PEP carboxylase active principally outside of chloroplasts in *Chlorella protothecoides*.

2. During the process of chloroplast degeneration in algal cells induced by addition of glucose, the activity of RuDP carboxylase significantly decreased, whereas the activities of PEP-carboxylase and -carboxykinase markedly increased.

3. During the process of chloroplast regeneration in "glucose-bleached" algal cells, which contained no detectable amounts of Fraction I protein and showed only traces of RuDP carboxylase activity, a light-dependent development of RuDP carboxylase proceeded almost in parallel with the light-induced formation of chlorophyll. The activities of PEP-carboxylase and -carboxykinase, which were negligibly low in glucose-bleached cells, developed independently of light.

4. Both chloramphenicol and cycloheximide severely inhibited the development of RuDP carboxylase activity. A relatively low concentration of glucose also caused a significant suppression. Under these conditions, chlorophyll formation was inhibited only slightly by chloramphenicol and very strongly by cycloheximide and glucose.

Earlier papers (*1–3*) dealing with the development of RuDP carboxylase in various plants and autotrophic microorganisms under different environmental conditions, have shown that the development of this enzyme was greatly affected by environmental factors, such as light and the organic source of carbon in the medium.

Chlorella protothecoides displays degeneration and regeneration of chloroplasts depending upon nutritional and light conditions (*4*). When the alga is grown in media rich in glucose or other metabolizable organic carbon compound and poor in nitrogen source, chlorophyll-less cells with profoundly degenerated plastids —called "glucose-bleached" cells—are produced either in the light or dark. On the other hand, when it is cultured in media rich in nitrogen source and poor in organic carbon source, normal green cells with fully-organized chloroplasts are

Abbreviations: RuDP, ribulose 1,5-diphosphate; PEP, phosphoenolpyruvic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl) aminomethane; CH, cyclohexane; CHI, cycloheximide; CP, chloramphenicol.

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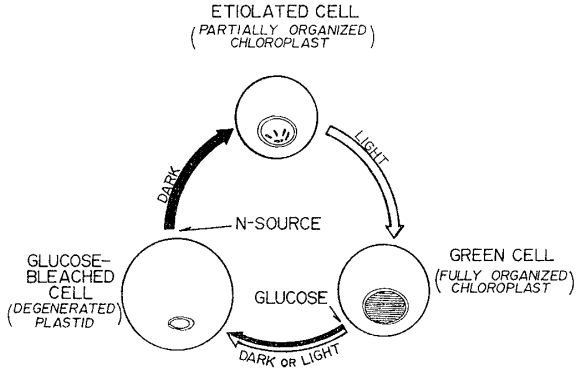

Fig. 1. *A schematic representation of the processes of degeneration and regeneration of chloroplasts in* Chlorella protothecoides.

obtained in the light, while in darkness pale green cells containing only partially organized plastids—called "etiolated" cells—are produced. These three different types of algal cells are transformed into each other as schematically illustrated in Fig. 1. When the green cells are incubated in a medium containing a high concentration of glucose but no nitrogen source, they are strongly bleached, eventually becoming glucose-bleached cells. The glucose-bleached cells are turned into green cells when incubated, in the light, in a medium containing nitrogen source but no organic carbon source, and into etiolated cells with dark incubation. Degeneration and development of photosynthetic CO_2-fixation and related activities have been shown to occur during the processes of degeneration and regeneration of chloroplasts in algal cells (*5*, *6*).

We studied the fate of RuDP carboxylase in the degeneration and regeneration of chloroplasts in *Chlorella protothecoides*. Our results disclosed that the activity of RuDP carboxylase is markedly degraded and actively developed in parallel with degeneration and regeneration of chloroplasts, respectively. Strikingly different modes of changes in activity were observed with PEP-carboxylase and -carboxykinase.

Material and methods

The strain of *Chlorella protothecoides* used was originally supplied from the algal culture collection at University of Indiana, U.S.A. (ACC No. 25), and is maintained at the Algal Culture Collection of our institute.

Methods of preparation of green and glucose-bleached algal cells, which were used as starting material in the "bleaching" and "greening" experiments, respectively, have been described previously (*7*, *8*).

Separation of subcellular fractions in non-aqueous media

The procedures are schematically shown in Fig. 2. They were essentially the same as those used in our previous work (*9*).

Bleaching and greening experiments

Procedures for the bleaching experiment were the same as those described

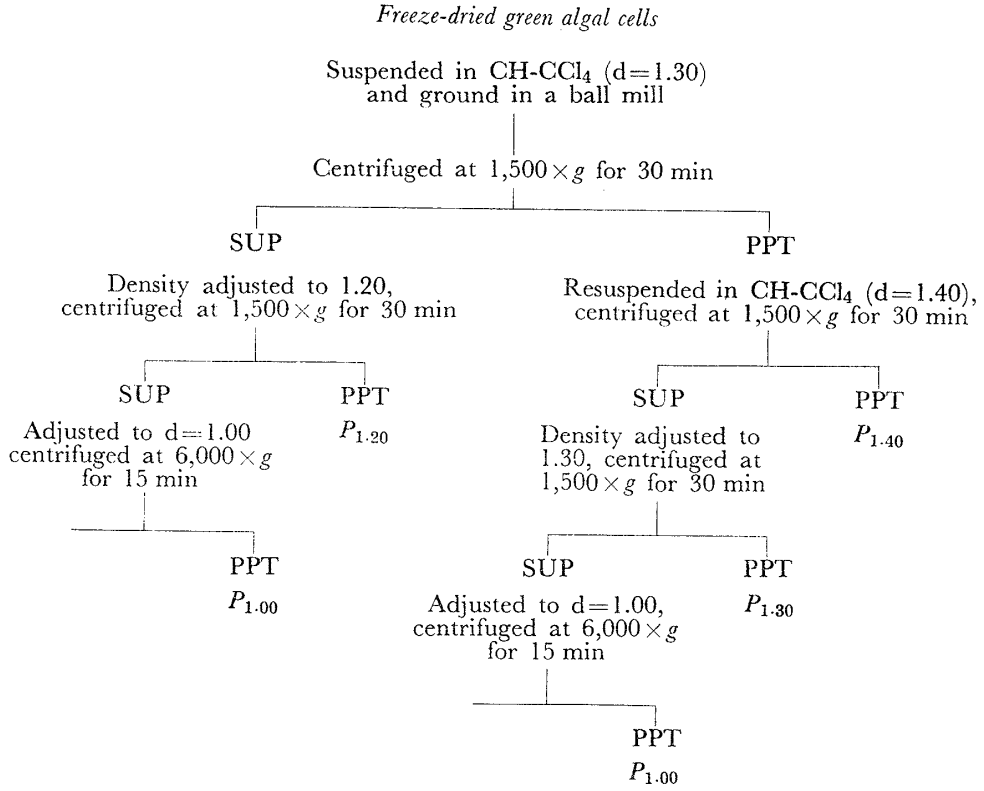

Fig. 2. *Separation of subcellular fractions of* Chlorella protothecoides *by centrifugation in mixtures of cyclohexane (CH) and* CCl_4. d: specific gravity determined at 5°C. SUP: supernatant. PPT: precipitate.

previously (*5*). In the greening experiment, glucose-bleached algal cells were incubated in a medium containing glycine (0.075 or 0.037 M) as the nitrogen source and basal mineral nutrients (*4*). The culture was illuminated with white light fluorescent lamps to give a light intensity of 2,000 lux at the level of culture. Other procedures have been described previously (*7*).

Analytical methods

Determination of chlorophyll content of algal cells was described in our previous paper (*10*). Measurements of photosynthetic CO_2-fixation by algal cells were performed by the method of Oh-hama et al. (*5*). In the assays of enzyme activities and soluble protein content, the harvested cells (2–3 ml in packed-cell volume) were washed with 2 mM K_2SO_4 solution and suspended in 10 ml of a mixture of 0.1 M Tris-HCl buffer, pH 7.5, 5 mM mercaptoethanol and 1 mM EDTA. The suspended cells were sonicated at 10 kHz for 30 min, and the homogenate obtained was centrifuged at 27,000×*g* for 20 min. An aliquot (0.5 ml) of the supernatant was passed through a Sephadex G-25 column (1.8×10 cm) which had been equilibrated with a mixture of 0.01 M Tris-HCl buffer (pH 7.5), 5 mM mercaptoethanol and 1 mM EDTA, and then used as the enzyme preparation. Assay methods for enzyme activities will be described elsewhere (*11*). The protein content of the supernatant obtained above was determined by Lowry's method (*12*) and expressed as soluble protein.

Results

Distribution of RuDP carboxylase and PEP carboxylase among non-aqueously separated subcellular fractions

Table 1 shows the distribution of the two carboxylases among the subcellular fractions separated in non-aqueous media. The data show that RuDP carboxylase was active mainly in chloroplasts, and PEP carboxylase mainly outside; a minor part of the latter activity existed in the chloroplast fraction.

Changes in enzyme activities during chloroplast degeneration

Fig. 3-a and b show that the activity of RuDP carboxylase decreased approximately in parallel with that of photosynthetic CO_2-fixation during chloroplast degeneration. Similar results were obtained when the bleaching of cells was induced by addition of 0.1 M acetate (cf. *8*). (During dark incubation without addition of glucose or acetate, no significant decrease of the enzyme activity was observed.) In contrast, the activities of PEP-carboxylase and -carboxykinase conspicuously increased. Chloramphenicol did not affect the decrease of RuDP carboxylase activity, but caused a slight suppression of the increase in PEP carboxylase activity and a greater suppression of the increase in PEP carboxykinase activity. Whether

Table 1
Distribution of RuDP- and PEP-carboxylase among non-aqueously separated subcellular fractions of Chlorella protothecoides

	Fraction [a]		
	Chloroplast fraction [b]	$P_{1.30}$	$P_{1.40}$
RuDP-carboxylase			
Activity (cpm/10 min)[c] per mg protein	3210	1381	1506
Total activity of each fraction	694×10^3	154×10^3	310×10^3
Ratio (%)	60	13	27
PEP-carboxylase			
Activity (cpm/10 min)[c] per mg protein	16100	17500	36400
Total activity of each fraction	350×10^4	194×10^4	750×10^4
Ratio (%)	27	15	58
Chlorophyll			
in mg	73.0	25.4	22.6
Ratio (%)	60	21	19
Dry weight			
in g [d]	1.3	0.9	2.0
Ratio (%)	31	21	48

[a] See Fig. 2 for the procedures of fractionation procedures.
[b] This fraction is $P_{1.00}$ plus $P_{1.20}$ in Fig. 2, and was composed of intact and fragmented chloroplasts.
[c] cpm of $NaH^{14}CO_3$ fixed in 10 min. See reference (*11*).
[d] The starting algal material used in fractionation was 5.1 g in dry weight, and the recovery was 82%.

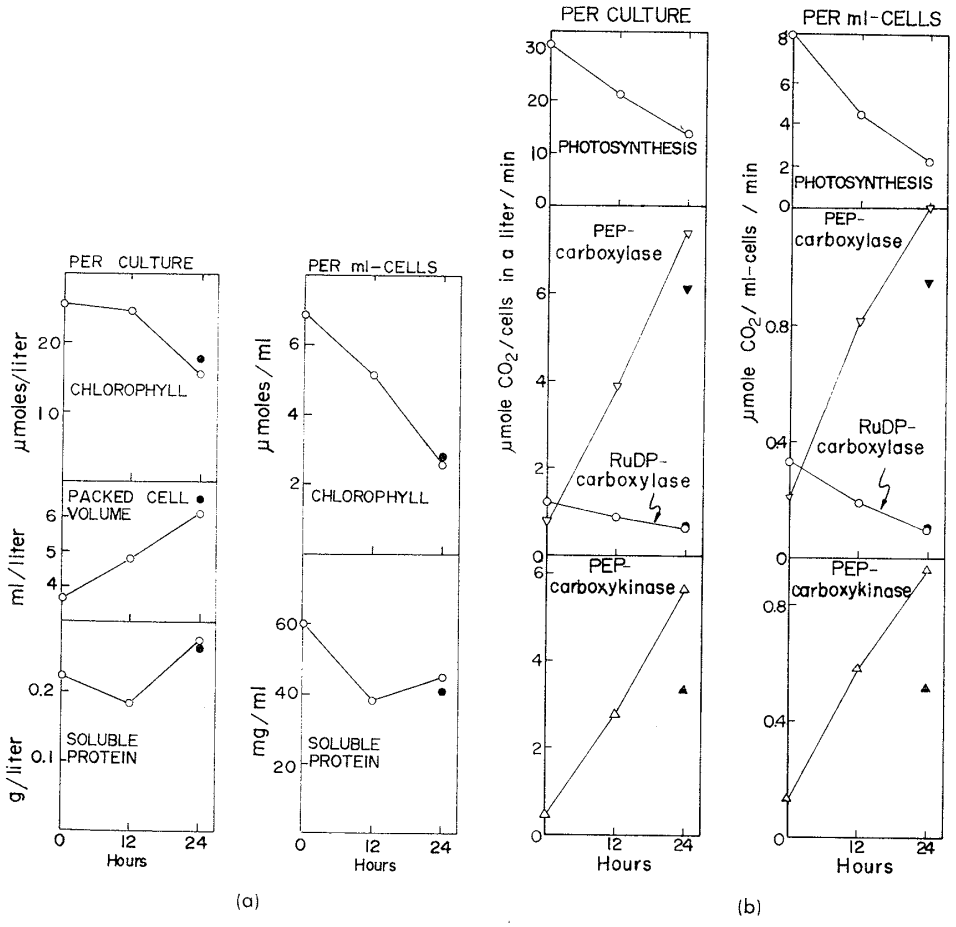

Fig. 3. *Changes in chlorophyll and soluble protein contents of algal cells (a), and in photosynthesis and enzyme activities (b) during chloroplast degeneration.* Solid circles and triangles show data obtained in the presence of chloramphenicol (10^{-2} M).

the decrease of RuDP carboxylase activity is due to changes in the enzyme protein or to a certain inhibitor(s) produced by addition of glucose or acetate, is an interesting problem to be studied in the future. Because the enzyme preparation used for the activity assay was passed through Sephadex G-25, it was probably free of low molecular weight inhibitor(s), if any had been present.

Changes in enzyme activities during chloroplast regeneration

Fig. 4-a and b and Fig. 5 show the modes of development of RuDP carboxylase and PEP carboxylase activities, and Table 2 that of PEP carboxykinase activity, under various conditions. These enzyme activities were negligibly low in glucose-bleached algal cells. When extracts of glucose-bleached cells were subjected to polyacrylamide gel-electrophoresis and to Sephadex G-200 column chromatography, no appreciable amounts of Fraction I protein were detected. This implies that the observed development of RuDP carboxylase activity was the result of net synthesis of the enzyme protein. This is supported by the result that the

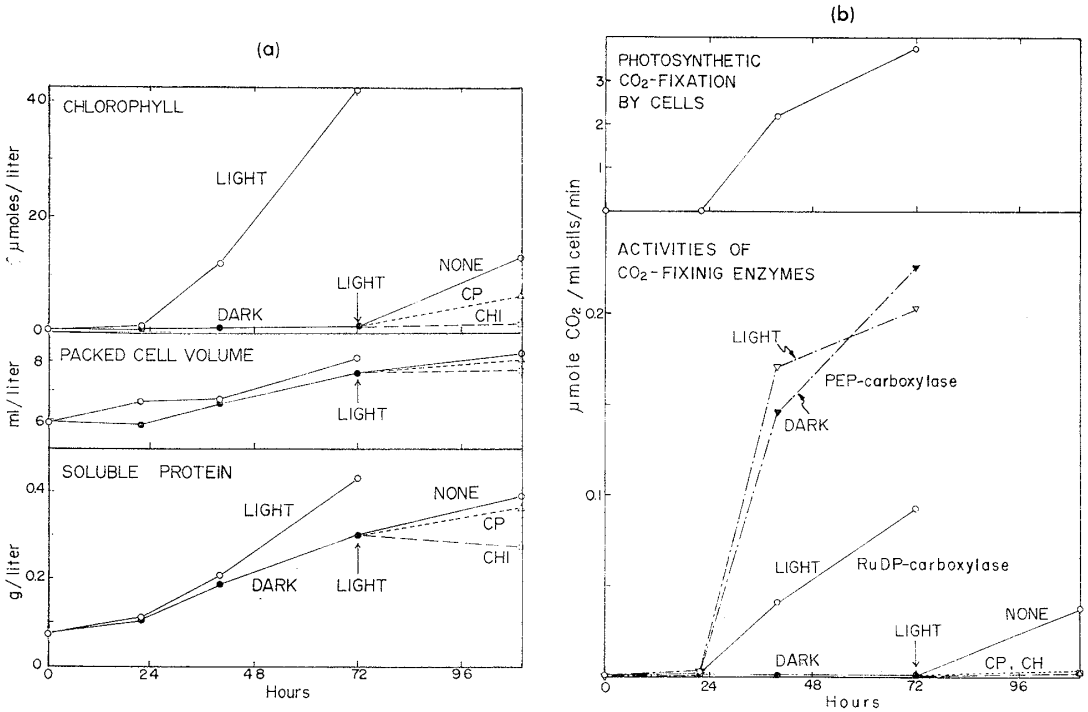

Fig. 4. *Changes in chlorophyll and soluble protein contents of algal cells (a) and in photosynthesis and enzyme activities (b) during chloroplast regeneration in glucose-bleached algal cells.* CP: chloramphenicol 10^{-2} M. CHI: cycloheximide 15 μg/ml.

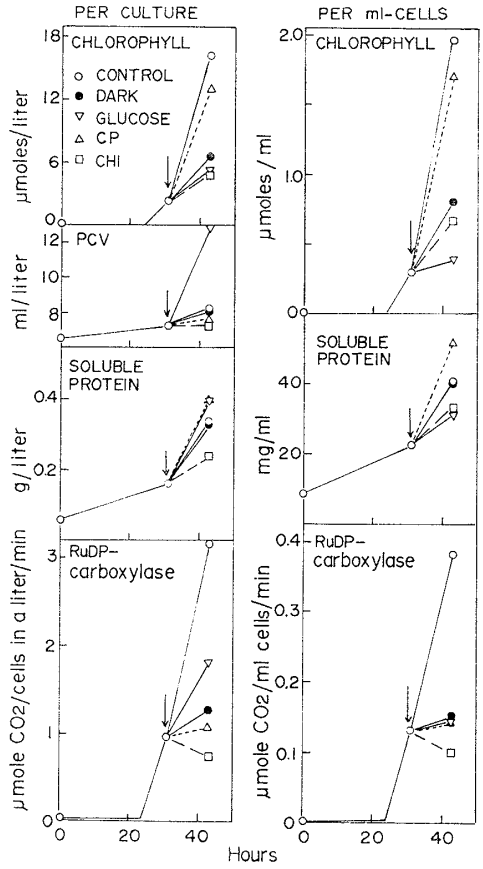

Fig. 5. *Effects of chloramphenicol (CP) (10^{-2} M), cycloheximide (CHI) (15 μg/ml), glucose (initial conc. 0.1%) and transfer to darkness upon the development of RuDP carboxylase activity in illuminated algal cells.* Treatments were started at the time indicated by the arrows. PCV: packed cell volume.

Table 2
Development of PEP carboxykinase activity during the greening process

	Incubation time (hr)			
	0	22	40	72
	(μmole CO_2/ml-cells/min)			
Light	Negligibly small	Negligibly small	7.9×10^{-2}	10.8×10^{-2}
Dark	Negligibly small	Negligibly small	8.1×10^{-2}	14.5×10^{-2}

development of this enzyme was strongly inhibited by chloramphenicol and cycloheximide[2], potent inhibitors of protein synthesis. Fig. 4-b clearly indicates that the development of the activity of this carboxylase was greatly dependent upon light, whereas those of PEP-carboxylase and -carboxykinase (Table 2) were totally independent of light. Fig. 4-a indicates that a larger amount of soluble protein was produced in the light than in darkness. The increase in soluble protein was inhibited only slightly by chloramphenicol and very severely by cycloheximide. Fig. 5 shows that the increase in RuDP carboxylase activity in illuminated algal cells was stopped by turning off the light or by addition of chloramphenicol or cycloheximide in the light. However, the formation of chlorophyll was only slightly suppressed by addition of chloramphenicol. A relatively low concentration of glucose has been shown to severely inhibit chlorophyll formation in algal cells (*13*). Fig. 5 shows that a low glucose concentration also considerably suppressed the development of RuDP carboxylase activity. Fig. 5 also indicates that the increase of soluble protein under these conditions was not inhibited but accelerated by chloramphenicol, as in the case of glucose, whereas it was strongly inhibited by cycloheximide, as mentioned earlier.

Discussion

Fraction I protein, which displays the activity of RuDP carboxylase, has been shown to be present in plant cells in concentrations as high as about 50% of the total chloroplast soluble protein (*14*). Our results suggest that the level of this enzyme in algal cells is sharply regulated by environmental conditions. The marked rise in activities of PEP-carboxylase and -carboxykinase observed on addition of glucose to green cells seems to be related to a regulatory mechanism operating in the glucose metabolism.

Chloramphenicol has been reported to repress protein synthesis on chloroplast ribosomes (70S-type), and cycloheximide that on cytoplasmic ribosomes (80S-type) in plant cells (*15*). The fact that both antibiotics inhibited development of RuDP carboxylase in glucose-bleached algal cells suggests that subunits of the enzyme protein might be synthesized separately at different cell sites, in the chloroplast and cytoplasm.

[2] The concentration of cycloheximide was 15 μg/ml in the experiments shown in Fig. 4 and 5. Further experiments (M. Arai: unpublished) showed that similarly strong inhibition was brought about by this antibiotic at a concentration of 3 μg/ml.

Fuller and Gibbs (*1*) reported that extracts from bleached cells of *Chlorella variegata* obtained by growing the alga on an organic carbon source in the light, showed no RuDP carboxylation to give PGA. They also demonstrated that dark-grown *Euglena* extracts showed a slight activity of RuDP carboxylation. Smillie, Scott and Graham (*15*) showed that when dark-grown cells of *Euglena gracilis* were illuminated, a marked increase of RuDP carboxylase activity was induced concomitant with an increase in the level of Fraction I protein. The increase was inhibited by chloramphenicol, but not by cycloheximide; this observation differs in part from ours with *Chlorella protothecoides*. Graham, Grieve and Smillie (*16*) found that when etiolated pea seedlings were exposed to red light for short time periods, the activities of not only RuDP carboxylase and other Calvin cycle enzymes but also enzymes associated with respiratory metabolism, considerably increased. They also found that the increases induced by red light were reduced by subsequent irradiation with far red light. They suggested that phytochrome participates as the primary photoregulator in the syntheses of these enzymes. The nature of the light-dependency of RuDP carboxylase development in *Chlorella* cells must be elucidated in the future; we can only note here that in algal cells, light does not seem to affect so widely different types of enzymes as in pea seedlings, and that light is continuously required for the development of RuDP carboxylase in *Chlorella*.

App and Jagendorf (*17*) reported that synthesis of alkaline fructose 1,6-diphosphatase, as well as chlorophyll, in etiolated *Euglena gracilis* cells in the light was repressed by metabolizable organic compounds. Recently McMahon and Bogorad (*18*) reported that when etiolated leaves of *Zea mays* were illuminated, a rapid increase in the activity of RuDP carboxylase occurred, and in the presence of a high concentration of sucrose (0.5 M) the increase in enzyme activity was suppressed.

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