Plant & Cell Physiol. 23(2): 333–341 (1982)

Effect of Temperature on Starch Degradation in *Chlorella vulgaris* 11h Cells

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Analysis of products formed in *Chlorella vulgaris* 11h cells during photosynthesis in air containing 3,000 ppm $^{14}CO_2$ at various temperatures revealed that the level of ^{14}C-starch was maximum around 20–24°C and decreased with further rise in temperature until 40°C, while ^{14}C-sucrose greatly increased at temperatures above about 28°C. Elevating the temperature from 20 to 38°C during photosynthetic $^{14}CO_2$ fixation resulted in a remarkable decrease in ^{14}C in starch and a concomitant increase in ^{14}C in sucrose. This conversion of starch to sucrose when shifting the temperature from 20 to 38°C proceeded even in the dark. Hydrolysis of sucrose by β-fructosidase showed that, irrespective of the experimental conditions, the radioactivities in sucrose were equally distributed between glucose and fructose. The enhancement of starch degradation with temperature rise was more remarkable than that of the activity of ribulose bisphosphate carboxylase from the same cells. When *Chlorella* cells which had been preloaded with ^{14}C-starch after photosynthesis for 30 min at 20°C were incubated in the dark for an additional 30 min at 20°C, ^{14}C-starch was degraded by only about 4%. However, the values after 30-min dark incubation at 28, 32, 36 and 40°C were increased by about 10, 19, 36 and 50%, respectively. During the temperature-dependent conversion of starch to sucrose, no significant amount of radioactivity accumulated in free glucose and maltose.

Key words: *Chlorella vulgaris* — CO_2-fixation — Starch — Sucrose — Temperature (starch degradation).

Sachs (1862) established that starch is an end product of photosynthesis in green plants. However, the regulatory mechanisms of starch metabolism are not fully understood. Recently, Steup et al. (1976) and Heldt et al. (1977) demonstrated that starch synthesis and degradation in isolated spinach chloroplasts are strictly regulated by the concentrations of inorganic phosphate in the medium. When ^{14}C-starch which had been preloaded photosynthetically in isolated spinach chloroplasts was degraded in the presence of orthophosphate in the dark, the major ^{14}C-labeled intermediates were 3-phosphoglycerate, triose-phosphates and phosphate esters in the Embden-Meyerhof pathway (Levi and Gibbs 1976, Peavey et al. 1977, Heldt et al. 1977). Therefore, α-glucan phosphorylase is thought to play a key role in the primary reaction of starch degradation in chloroplasts (Stitt and Heldt 1981). Steup and Latzko (1979) and Steup et al. (1980) separated a chloroplastic phosphorylase from a non-chloroplastic one in spinach and pea leaves. Amylolytic cleavage also seems to be involved in the starch breakdown in spinach chloroplasts since the radioactivity from ^{14}C-starch accumulated in maltose and glucose (Levi and Gibbs 1976, Peavey et al. 1977, Heldt et al. 1977, Stitt and Heldt 1981).

In photoautotrophically grown green algae, a large amount of starch accumulates during

Abbreviation: RuBP, ribulose bisphosphate.

the light period and is rapidly degraded to serve as the main energy source during the subsequent dark period (Wanka et al. 1970, Takeda and Hirokawa 1978). However, only a little information on the regulation of starch metabolism in algae is available (Sanwal and Preiss 1967, Preiss and Levi 1980). Nakamura and Miyachi (1977, 1980a) have explored the influence of temperature on the level of glucose-polymer (starch) during photosynthetic $^{14}CO_2$ fixation in *Chlorella vulgaris* 11h cells. Preliminary experiments (Nakamura and Miyachi 1977) indicated that conversion of glucose-polymer to sucrose was greatly stimulated by temperatures above 30°C. This was the first report on the specific role of temperature in starch degradation in algae as well as in higher plant leaves.

The present paper represents a detailed analysis of the influence of temperature on the rate of starch degradation and the labeling of intermediates and products of photosynthesis during the degradation process in *Chlorella vulgaris*. Photosynthetic $^{14}CO_2$ fixation was carried out under continuous bubbling of air containing 3,000 ppm $^{14}CO_2$ since under higher CO_2 concentrations, radioactivity was incorporated more into starch, and not into glycolate, which was the major product at lower (300 ppm) CO_2 concentrations (Nakamura and Miyachi 1980a, b).

Materials and Methods

Algal culture—Chlorella vulgaris 11h cells were grown photoautotrophically in an inorganic culture medium (Ogasawara and Miyachi 1970) at 20–23°C under bubbling of air enriched with 3% CO_2 (v/v) as described previously (Nakamura and Miyachi 1980a). Cells were harvested, washed once with one-tenth volume of the culture medium, and suspended in the same medium at the cell density of 0.1 ml packed cell volume per ml. The suspension was kept on ice until use.

Photosynthetic $^{14}CO_2$ fixation—Photosynthetic $^{14}CO_2$ fixation was carried out in a flat sided vessel 'lollipop' (20 ml) which was submerged in a flat plastic tank filled with circulating water at the given temperatures. Three minutes after 10 to 15 ml of the above-mentioned suspending medium had been bubbled with air containing 3,000 ppm $^{14}CO_2$, the reaction was started by injecting *Chlorella* cell suspension at a final cell density of 3 ml packed cell volume per liter. Bubbling with $^{14}CO_2$ was continued during the experiments. The lollipop was illuminated from one side at 24,000 lux with a metal halide lamp (Yoko Lamp, Toshiba Electric Co., Ltd., Tokyo). At specified intervals, 1.0 ml of the algal suspension was quickly transferred to 4 ml of methanol.

Effects of temperature on the further metabolism of $^{14}CO_2$ fixation products—Thirty minutes after the photosynthetic $^{14}CO_2$ fixation (20°C) described above, the algal suspension in the lollipop (15 ml) was transferred into a glass tube which had been placed in ice in the dark. Suspension samples of 2 ml each were placed in seven test tubes and kept at 0°C in the dark until use. Next, 0.2 ml of each suspension was taken into 2.5 ml of 87% (v/v) methanol, and the remaining portion was immediately transferred to a water bath kept at various temperatures from 20 to 44°C in the dark. The test tubes were occasionally shaken by hand. At various time intervals, 0.2 ml of each suspension was transferred to 2.5 ml of 87% (v/v) methanol.

Analysis of the labeled compounds—Radioactivities incorporated in the resulting 80% methanol-soluble and -insoluble fractions were determined with a gas-flow counter. The soluble fraction was chromatographed in two dimensions on Whatman No. 1 filter paper, first with phenol : acetic acid : 1 M EDTA : water (740 : 10 : 1 : 260, v/v) and then with *n*-butanol : propionic acid : water (140 : 71 : 100, v/v). The radioactivity of each radioactive spot was measured with a GM counter. The spots containing glutamate, serine and glycine which overlapped were eluted with water and further separated by high-voltage electrophoresis (for these details, see Nakamura and Miyachi 1980a).

The content of ^{14}C-starch in the insoluble fraction was determined by digestion with *α*-

amylase and amyloglucosidase. Aliquots of the 80% methanol-insoluble fractions were washed twice with distilled water and resuspended in 1.0 ml distilled water. Each suspension was kept in a boiling water bath for 60 min and then homogenized with a sonicator (Ohtake Works Co., Ltd., Tokyo) at 150 W for 6 min. Next, 0.2 ml each of the insoluble fraction was incubated with 0.2 ml of 100 mM sodium acetate buffer (pH 5.0) containing α-amylase (34U) and amyloglucosidase (1.5U) for 3 hr at 30°C. The reaction was terminated by adding 1.6 ml methanol. The methanol suspension was centrifuged, and then the radioactivities in the resulting 80% methanol-soluble and -insoluble fractions were determined. The soluble fraction was further analyzed by one-dimensional paper chromatography with *n*-butanol : acetic acid : water (12 : 3 : 5, v/v). Each autoradiogram gave only one spot corresponding to glucose.

In some experiments, ^{14}C-sucrose was hydrolyzed by β-fructosidase (invertase) as follows. The radioactive sucrose on the two-dimensional chromatogram was eluted with distilled water and then purified by one-dimensional paper chromatography with *n*-butanol : acetic acid : water (12 : 3 : 5, v/v). The purified ^{14}C-sucrose was eluted again with distilled water. A portion of the eluate was dissolved in 1 mM potassium phosphate buffer (pH 5.5) containing β-fructosidase (180U) at 20°C. After 1 hr the sample was cochromatographed with authentic sucrose, glucose and fructose using *n*-butanol : acetic acid : water. Radioactivities in the respective spots on the paper were measured with a GM counter.

Assay of RuBP carboxylase—RuBP carboxylase was isolated from *Chlorella vulgaris* 11h cells and its activity was assayed with 20 mM $NaH^{14}CO_3$ (pH 7.8) according to Hogetsu and Miyachi (1979).

Results and Discussion

Effects of temperature on radioactivities incorporated into starch and sucrose during photosynthetic $^{14}CO_2$ fixation—Fig. 1 shows that under continuous bubbling of air containing 3,000 ppm $^{14}CO_2$, the total radioactivity fixed by *Chlorella* cells for 30 min increased with the rise in temperature from 4 to 24°C, but it became nearly constant when the temperature was further increased until 40°C. The radioactivities in the insoluble fraction and starch also increased up to 24°C, then decreased with the further rise in the temperature. At temperatures lower than 20°C, the radioactivity incorporated into starch accounted for more than 95% of that incorporated into the insoluble fraction. The percent incorporation of ^{14}C into starch decreased as the temperature was raised above 20°C (at 40°C the radioactivity in starch was 83% of the total radioactivity in the insoluble fraction). In sharp contrast, the radioactivity incorporated into sucrose greatly increased at temperatures above 28°C. The results indicate that temperatures above 20°C inhibited starch biosynthesis and inversely stimulated sucrose formation or that starch degradation to form sucrose was greatly accelerated by higher temperatures.

Changes in ^{14}C-incorporation into major products with temperature rise from 20 to 38°C during the course of $^{14}CO_2$ fixation—To examine the basis for the temperature-dependent change in starch and sucrose labeling, the temperature of a *Chlorella* suspension was quickly raised from 20 to 38°C during the course of photosynthetic $^{14}CO_2$ fixation (Fig. 2). The radioactivity in the insoluble fraction immediately started to decrease, although there was practically no change in the rate of total ^{14}C-incorporation. On the other hand, the level of ^{14}C-sucrose rose greatly on elevation of the temperature and that of phosphate esters decreased for several minutes and then recovered almost to the original level.

To test whether or not photosynthetic electron transport and photophosphorylation are responsible for this change, the light was turned off and the temperature was quickly raised from 20 to 38°C. Fig. 3 shows that the temperature-induced breakdown of the radioactive insoluble fraction and the concomitant increase in sucrose occurred even in the dark. Surprisingly, the

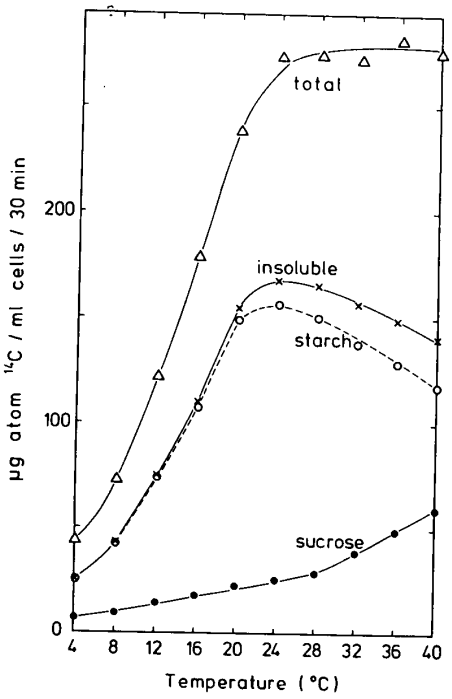

Fig. 1

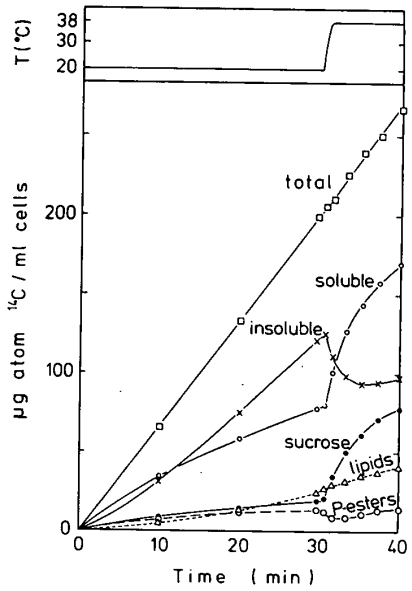

Fig. 2

Fig. 1 Effect of temperature on the levels of starch and sucrose during photosynthesis for 30 min in air containing 3,000 ppm $^{14}CO_2$ in *Chlorella vulgaris* 11h cells.

Fig. 2 Change in the major products with temperature elevation to 38°C in light after photosynthetic $^{14}CO_2$ fixation for 30 min at 20°C by *Chlorella vulgaris* cells. Bubbling with 3,000 ppm $^{14}CO_2$ was continued throughout the experiment. P-esters, phosphate esters.

greater part (nearly 83%) of the radioactivity, which decreased in the insoluble fraction (starch), was recovered in sucrose about 10 min after the temperature transition. The level of phosphate esters declined and did not recover to the original level in the dark. These results indicate that conversion of radioactivity from starch to sucrose in *Chlorella* cells is greatly accelerated at higher temperatures (38°C) and the photosynthetic electron transport system is not involved in this process.

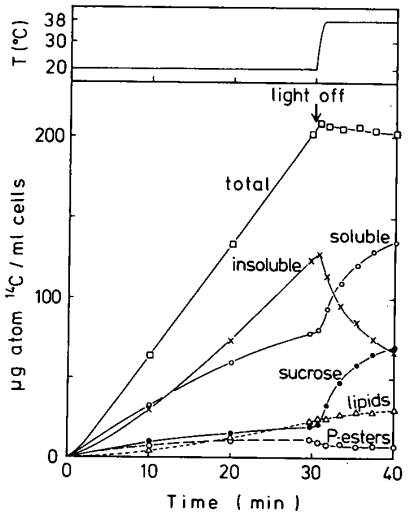

Fig. 3 Change in the major products on temperature elevation to 38°C in the dark after photosynthetic $^{14}CO_2$ fixation for 30 min at 20°C by *Chlorella vulgaris* cells. Air containing 3,000 ppm $^{14}CO_2$ was constantly bubbled throughout the experiment. P-esters, phosphate esters.

Table 1 Hydrolysis of radioactive sucrose by β-fructosidase

Exp.	^{14}C-Sucrose obtained after	Addition of β-fructosidase	cpm in Sucrose	Glucose (G)	Fructose (F)	G/F
1	$^{14}CO_2$ fixation for 30 min, 20°C, L	−	1,865	38	36	—
		+	49	949	936	1.01
	$^{14}CO_2$ fixation for 30 min, 40°C, L	−	1,664	33	22	—
		+	53	677	677	1.00
2	$^{14}CO_2$ fixation for 30 min, 20°C, L and 10 min, 38°C, L	+	47	1,118	1,159	0.96
3	$^{14}CO_2$ fixation for 30 min, 20°C, L	+	31	815	820	0.99
	$^{14}CO_2$ fixation for 30 min, 20°C, L and 5 min, 38°C, D	+	12	513	511	1.00
	$^{14}CO_2$ fixation for 30 min, 20°C, L and 10 min, 38°C, D	+	54	662	642	1.03

The ^{14}C-sucrose samples in Exp. 1, 2 and 3 were obtained from the experiments shown in Fig. 1, 2 and 3, respectively. L, light; D, dark. Experimental conditions are described in **Materials and Methods**.

Hydrolysis of radioactive sucrose by β-fructosidase—The radioactivity in sucrose formed during photosynthesis for 30 min in air containing 3,000 ppm $^{14}CO_2$ at either 20 or 38°C was equally distributed between glucose and fructose (Table 1). In addition, the same amount of radioactivity was detected in both moieties when a great amount of sucrose was produced from starch at higher temperatures either in light or dark. This indicates that during the temperature-dependent conversion of starch to sucrose, both glucose and fructose portions in sucrose were synthesized from radioactive starch in *Chlorella* cells.

Effects of temperature on ^{14}C-starch degradation and the associated changes—To examine the temperature dependence of the conversion of starch to sucrose, *Chlorella* cells which had been allowed to fix $^{14}CO_2$ photosynthetically for 30 min at 20°C were exposed to various temperatures in the dark (Fig. 4a–c). Fig. 4a shows that the decrease in the radioactivity in the insoluble fraction was insignificant from 20 to 28°C, but was marked at 32 through 44°C. The percent decrease in the radioactivity in the insoluble fraction (starch) after incubation for 30 min at 20, 24 (data not shown in the figure), 28, 32, 36, 40 and 44°C was about 4, 7, 10, 19, 36, 50 and 50%, respectively. The loss of the total radioactivity, which would be due to dark respiration, was relatively small, and the effect of temperature on the loss was insignificant.

Fig. 4b shows that ^{14}C in sucrose greatly increased with the rise in temperature up to 40°C. The decrease in radioactivity in the insoluble fraction at 32, 36, 40 and 44°C for 30 min was recovered by accumulation in the sucrose fraction by 85, 82, 78 and 62%, respectively. The level of glutamate also increased with the rise in temperature until 44°C. In contrast, the level of ^{14}C label in the lipid fraction during the dark period slightly increased similarly for all temperatures tested. No significant differences in the time courses of the labeling patterns of phosphate esters (Fig. 4b), alanine, aspartate, serine and glycine (Fig. 4c) were observed in the dark period at all temperatures from 20 to 44°C.

No significant amounts of glucose and maltose were detected during starch degradation in *Chlorella vulgaris* cells in the dark as well as light (data not shown). Thus, polyglucan phosphorylase seems to be responsible for the starch breakdown induced by high temperatures in *Chlorella vulgaris* 11h cells. However, the possibility that α-amylase and/or debranching enzyme is involved in this temperature-dependent process can not be excluded.

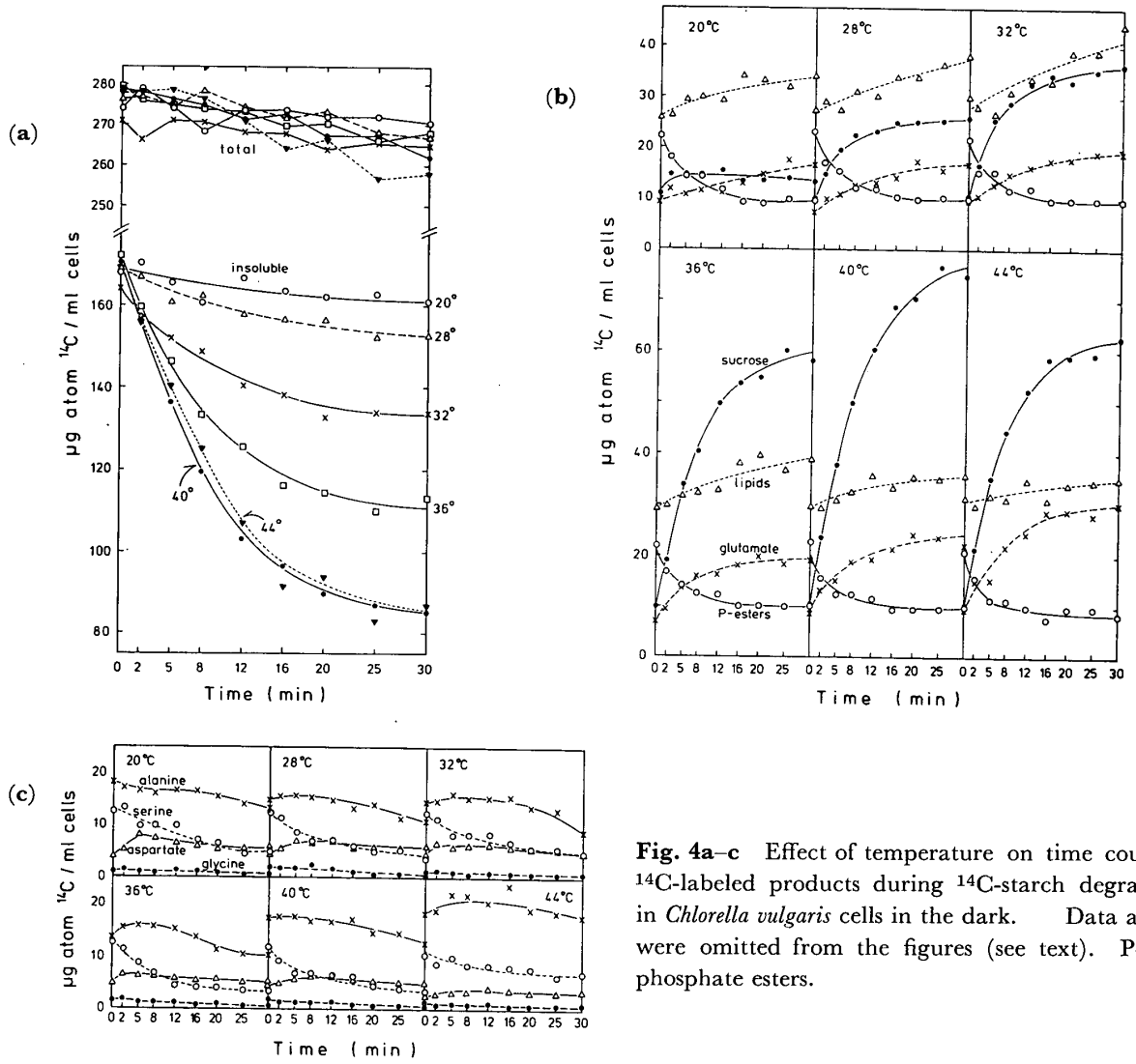

Fig. 4a–c Effect of temperature on time courses of ^{14}C-labeled products during ^{14}C-starch degradation in *Chlorella vulgaris* cells in the dark. Data at 24°C were omitted from the figures (see text). P-esters, phosphate esters.

When ^{14}C-starch was degraded in spinach chloroplasts in the dark, ^{14}C principally accumulated in 3-phosphoglycerate, triose-phosphates, maltose and glucose (Levi and Gibbs 1976, Peavey et al. 1977, Heldt et al. 1977, Stitt and Heldt 1981). In spinach chloroplasts, starch degradation was strictly controlled by the concentrations of inorganic phosphate (Steup et al. 1976, Heldt et al. 1977), suggesting that starch phosphorylase plays a key role in the breakdown of starch in chloroplasts. This idea was supported by recent evidence with spinach and pea leaves from Steup and Latzko (1979) and Steup et al. (1980) that α-glucan phosphorylase is located in the chloroplasts. However, observations that ^{14}C-incorporation into free glucose and maltose increased during starch degradation in spinach chloroplasts (Levi and Gibbs 1976, Peavey et al. 1977, Heldt et al. 1977, Stitt and Heldt 1981) suggest that amylolytic cleavage also plays a role in the starch degradation in the dark.

If starch degradation in *Chlorella* cells is catalyzed by phosphorylase, the possible essential reactions involved in the temperature-dependent conversion of starch to sucrose would be as follows:

$$(\text{glucose})_n + P_i \longrightarrow (\text{glucose})_{n-1} + \text{glucose 1-P}$$
$$\text{glucose 1-P} + \text{UTP} \longrightarrow \text{UDP-glucose} + PP_i$$

glucose 1-P ⟶ glucose 6-P ⟶ fructose 6-P

fructose 6-P + UDP-glucose ⟶ sucrose 6-P + UDP

sucrose 6-P + H_2O ⟶ sucrose + P_i

Therefore, when 1 mol of sucrose is formed, 1 mol of UTP is required. On the other hand, if starch is degraded by the catalysis of amylases, another 2 mol of ATP are required to form 2 mol of phosphorylated glucose. Involvement of phosphorylase in starch degradation in *Chlorella* would result in a more energetically efficient transfer of carbon from starch to sucrose at high temperatures.

Whichever enzyme is responsible for the degradation of starch in *Chlorella*, the starch degradation would be a temperature-sensitive step in the conversion of starch to sucrose since this conversion proceeded at high temperatures even in the dark and seemed not to be limited by the supply of ATP and/or UTP.

To determine in detail the temperature dependence of starch degradation in *Chlorella*, the decrease in the radioactivity in starch during 30-min incubation (ΔC) and the initial rate of that decrease (v_0) from Fig. 4a were replotted as a function of temperature in Fig. 5. Fig. 5 also illustrates the temperature dependence of RuBP carboxylase extracted from the same *Chlorella vulgaris* cells. The enhancement of both ΔC and v_0 by the rise in temperature was similar to that of RuBP carboxylase activity from 20 to 28°C. However, the acceleration of ^{14}C-starch degradation became remarkable from 28 to 40°C, and was much more responsive to increased

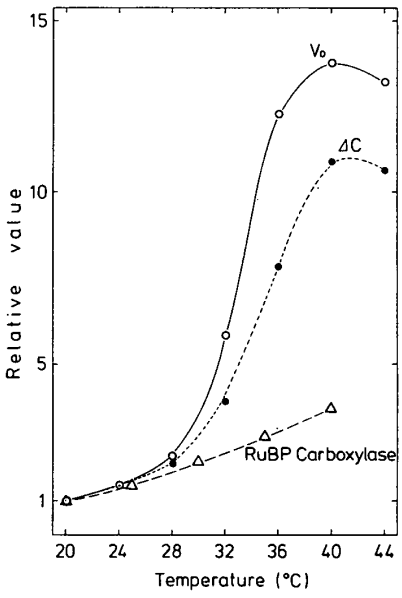

Fig. 5

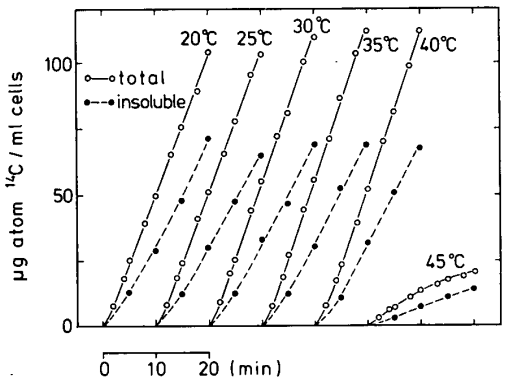

Fig. 6

Fig. 5 Temperature dependence of starch degradation and RuBP (ribulose bisphosphate) carboxylase in *Chlorella vulgaris* cells. v_0 is the initial rate of the decrease in the ^{14}C-insoluble fraction at various temperatures. ΔC is the amount of ^{14}C lost in the insoluble fraction during the dark incubation for 30 min at various temperatures. The values of v_0 and ΔC were obtained from Fig. 4a. The activity of RuBP carboxylase from *Chlorella vulgaris* cells was measured as described in **Materials and Methods**. The respective values at 20°C were normalized to 1.0. The activity of RuBP carboxylase at 20°C was 37 nmol $^{14}CO_2$/mg soluble protein/min.

Fig. 6 Effect of 30-min temperature treatment of *Chlorella* cells on the subsequent photosynthetic $^{14}CO_2$ fixation and starch formation. Immediately after the *Chlorella* cell suspension had been preincubated for 30 min at various temperatures from 20 to 45°C in the dark, it was subjected to the photosynthetic reaction in air containing 3,000 ppm $^{14}CO_2$ at 20°C.

temperature than RuBP carboxylase activity. Further rise in temperature to 44°C caused a slight decrease in ΔC and v_0. This would be due to the irreversible damage of *Chlorella* cells at 44°C (disccussed below for Fig. 6).

Effects of preincubation of Chlorella *at various temperatures on the subsequent* $^{14}CO_2$ *fixation*—To test whether high temperatures caused irreversible damage of *Chlorella* cells, the cell suspension was exposed to various temperatures from 20 to 45°C in the dark for 30 min prior to subsequent photosynthetic $^{14}CO_2$ fixation at 20°C. Fig. 6 shows that preincubation of temperatures up to 40°C had no effect on $^{14}CO_2$-fixation capacity or on the rate of ^{14}C-incorporation into the insoluble fraction. The activities were severely inhibited when the cells had been preincubated at 45°C. The results suggest that the conversion of starch to sucrose induced by high temperatures up to 40°C is a reversible phenomenon in *Chlorella* cells.

Starch metabolism in Chlorella—One of the general questions about starch metabolism in green plants is whether any degradation occurs during photosynthesis in light. Therefore, when plants accumulate low levels of starch, it is often not clear whether this is due to a limited rate of biosynthesis or to the rate of starch breakdown occurring at an appreciable rate. The results of the present study with *Chlorella* suggest that at least under some conditions, the capacity for the net synthesis of starch and sucrose during photosynthesis may be in part regulated by the rate of starch degradation.

This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Agriculture, Forestry and Fishery (GEP-57-II-1-2).

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(Received October 27, 1981; Accepted January 9, 1982)