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Raffinose Synthesis in *Chlorella vulgaris* Cultures after a Cold Shock[1]

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ABSTRACT

***Chlorella vulgaris* cultures have been submitted to a chilling shock, bringing down the growing temperature from to 24°C to 4°C. Growth was stopped immediately, and concomitantly there was an accumulation of sucrose and a decrease in the starch content. The enzymes involved in sucrose metabolism were differentially affected by the chilling shock. Sucrose phosphate synthase activity increased while sucrose synthase was not affected. Simultaneously with the chilling shock, raffinose began to accumulate. When algal cultures were returned at 24°C, raffinose disappeared. The presence of raffinose in algal cells has not been reported before.**

A sudden decrease in the temperature at which higher plants are growing is known as a chilling stress. Its effect in plant metabolism may be different according to plant species (11). However, a chilling stress is generally accompanied with an immediate stop in the plant growth while photosynthesis continues at a decreased rate (11). The result is a net accumulation of photosynthetic products due to the growth stoppage. In many plants this is seen as an accumulation of sucrose and of oligosaccharides derived from it (7). In lower photosynthetic organisms, like algae, the effect of a sudden decrease in temperature has been less well studied. However, it is known that cells of *Chlorella ellipsoidea*, hardened at 3°C for 48 h in the presence of glucose in the dark, also accumulate sucrose (5).

This paper reports the effect of a chilling shock in cultures of *C. vulgaris* showing that the accumulation of sucrose is concomitant with a decrease in the starch level while the activities of the enzymes involved in sucrose metabolism are differently affected. SPS[2] (UDP-glucose: D-fructose-6-phosphate-2-glucosyltransferase, EC 2.4.2.14) activity increases while SS (UDP-glucose: D-fructose-2-glucosyltransferase, EC 2.4.2.13) is not affected. Simultaneously after the chilling shock, raffinose, an oligosaccharide usually considered not present in algae, begins to accumulate.

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[2] Abbreviations: SPS, sucrose phosphate synthase; SS, sucrose synthase.

MATERIALS AND METHODS

Fine chemicals were purchased from Sigma Chemical Co, St. Louis, MO. UDP-[U-^{14}C]Glucose was obtained from the Instituto de Investigaciones Bioquimicas, Fundación Campomar, Buenos Aires, Argentina.

Algal Material

Chlorella vulgaris Beijerinck strain 11468 was obtained from the American Type Culture Collection, Rockville, MD. *C. vulgaris* cells were grown photoorganotrophically as described previously (16). Cells were collected by centrifugation at 5000*g* for 5 min and washed three times with 25 mM Tris-HCl (pH 7.0) containing 1 mM EDTA and 5 mM mercaptoethanol.

Enzyme Activities

These were determined either in toluene permeabilized cells according to Salerno (16) or in extracts prepared by sonication at 0°C in the presence of glass powder. SS and SPS were assayed by determining the labeled sucrose formed after retaining the unreacted UDP-[U^{14}C]glucose in an anion exchange resin (17). Invertase was assayed spectrophotometrically by coupling hexokinase, phosphoglucoisomerase, and glucose-6-phosphate dehydrogenase and following the appearance of NADPH (15). UDPase was assayed by measuring the inorganic phosphate liberated from UDP following the method of Fiske and SubbaRow as modified by Leloir and Cardini (10).

Sucrose, Raffinose, Starch and Chl

Cells (approximately 100 mg) were lyophilized and extracted with 80% (v/v) ethanol at 80°C thrice during 5 min. Ethanol extracts were evaporated and the residue was suspended in water. The solution was desalted by passage through Dowex 50 (H^+) and IRA-4B (OH^-) columns and a descending chromatography was performed on Whatman No. 1 paper using phenol/water (4:1 v/v) and ethyl acetate/pyridine/water (12:5:4 v/v) as developing solvents. The position of sugars on paper was ascertained with the silver nitrate reagent (20) and with the naphtoresorcinol reagent (3). Sucrose was estimated by the thiobarbituric acid method (13) and by measuring fructose and glucose after hydrolysis with invertase following the previously described procedure (15). Raffinose was first estimated by measuring its fructose content as for sucrose,

and was identified by its position on paper chromatograms, and by hydrolysis with alfagalactosidase from *Aspergillus niger*. The galactose and sucrose produced after enzyme action were identified again by paper chromatography against known standards. The oligosaccharide extracted from the *C. vulgaris* cells submitted to the cold shock was also submitted to the action of invertase. Fructose and melibiose produced were identified by their position on paper chromatograms. Starch was determined in the residue remaining after lyophilized cells were extracted with 80% ethanol followed by 80% (v/v) acetone extraction to remove Chl. The remaining dry residue was extracted with 70% perchloric acid according to the procedure of Hassid (1) and starch determined according to Krisman (9). Packed cells (30 mg) were extracted with 80% (v/v) cold acetone, and Chl was measured as previously reported (16).

RESULTS AND DISCUSSION

Chlorella vulgaris cells growing exponentially at 24°C were immediately transferred to 4°C. Figure 1 shows the change produced in the growth curve, while the inset presents the variation produced in the Chl content after the transfer to the lower temperature with reference to that of cells kept at 24°C. Algal cells transferred to 4°C rapidly attained the stationary phase. The Chl content initially diminished and reached a plateau a few hours later. These results are in agreement with those reported by Hatano for *Chlorella eliposidea* (5) and show a parallelism with what occurs in higher plants submitted to a cold shock. Pollock *et al.* (14, 19) have shown that growth in *Lolium temulentum* stops within a few minutes after being transferred from 24°C to 4°C. Similarly, previous experiences with *Zea mays* leaves indicated that Chl content decreased when plants grown at 24°C were exposed to 4°C (12).

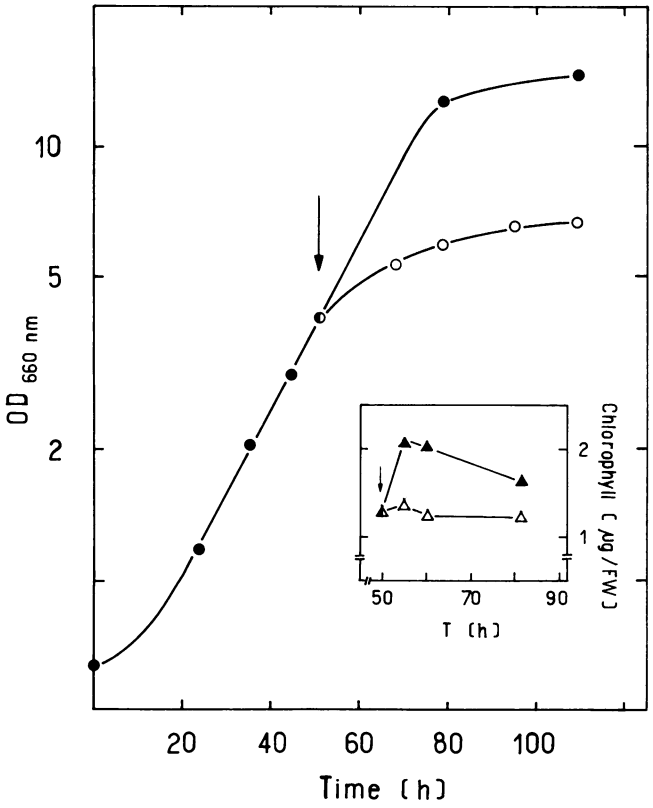

Figure 1. Effect of transferring *C. vulgaris* cells growing at 24°C (●) to 4°C (○). Inset, Chl content at 24°C (▲) and at 4°C (△).

The parallelism found for *C. vulgaris* and higher plants for growth and Chl content was also encountered on analysis of the effect of the chilling shock on the carbohydrate metabolism of algae.

Steponkus (18) and Garber and Steponkus (4) have contended that cold acclimation in higher plants involves both alterations in membranes and an accumulation of sucrose. In this work membranes were not studied, but Table I presents results obtained when the content of monosaccharides, sucrose, and starch were measured. The values indicate that algae reacted similarly to higher plants and thus supported the contention of Steponkus. Sucrose increased in the cold about 400%, reaching a maximum level 24 h after the cells were transferred to the lower temperature. Starch level on the other hand decreased, and it may indicate that its hydrolysis (or lack of synthesis) is providing the necessary sugar phosphates for enhanced synthesis of sucrose. The activity of the enzymes involved in sucrose metabolism supports this idea. Figure 2 presents the levels found for SPS, SS, invertase and UDPase in *C. vulgaris* cells after the algae were transferred to 4°C. No modification is observed in the activity values for the three last enzymes while there is a clear enhancement for those of SPS. Again, in algal cells it seems that there is also an inversely related activity of SPS and starch content which agrees with the observations of Kerr *et al.* in soybean leaves (8).

The increased activity of SPS, and the slight effect upon activity of SS after the cold shock, differs markedly with the results reported by Calderón and Pontis for wheat (2). This difference may be explained taking into account that sucrose accumulation leads to fructan synthesis in wheat while this does not occur in *Chlorella*.

Paper chromatography of the sugars present in algae before and after the cold shock revealed the appearance of a sugar with a mobility similar to that of raffinose. Initial visual observations of the chromatograms indicated that this sugar was apparently increasing its level with the time the culture remained at 4°C. The data presented in Table II show that indeed this is the case. Raffinose is not present in the cultures

Table I. *Content of Soluble Sugars and Starch in C. vulgaris Cells Grown at 24 °C and in Cells Transferred at 4 °C during Different Times*

T = 0 corresponds to the moment of transfer as indicated by arrow on Figure 1.

Time	Sugars Containing Fructose		Reducing Sugars		Starch	
	24°C	4°C	24°C	4°C	24°C	4°C
h	*nmol/mg fr wt*				*μg/mg fr wt*	
0	1.4	1.4	2.35	2.35	33	33
11	3.2	7.3	2.10	2.53	22	12
22	2.2	9.9	1.64	2.11	37	23
46	4.3	7.0	1.57	2.15	31	20

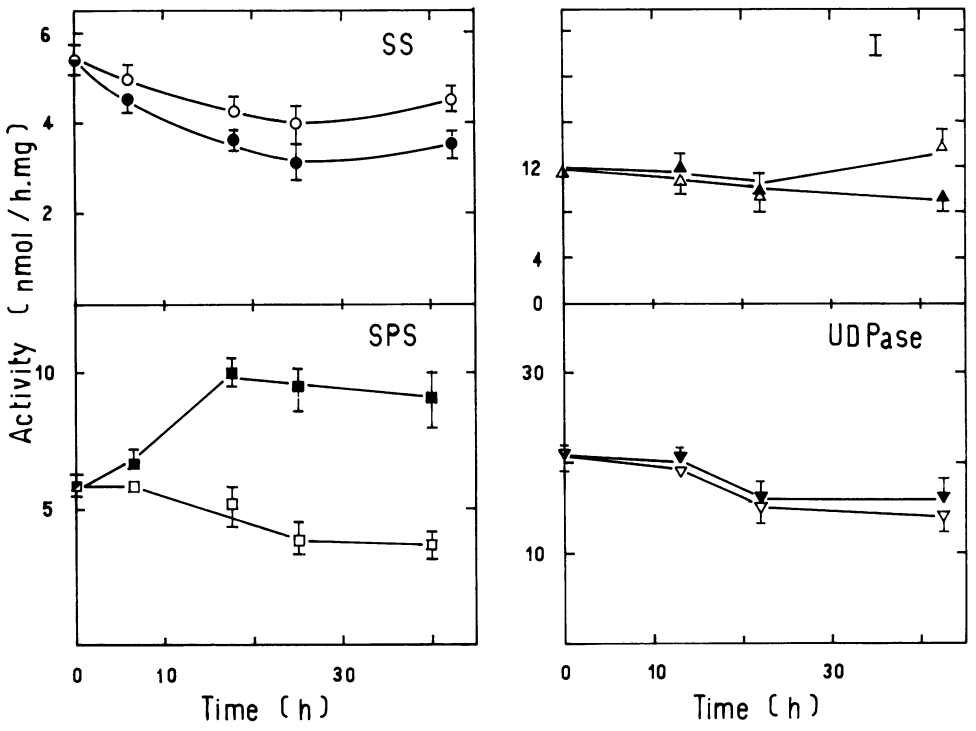

Figure 2. Activities of enzymes involved in sucrose metabolism. Cells grown at 24°C were transferred to 4°C. T = 0 corresponds to the moment of transfer as indicated by arrow on Figure 1. SPS, sucrose phosphate synthase; SS, sucrose synthase; I, invertase; UDPase, uridine diphosphatase. Closed symbols, 4°C; open symbols, 24°C.

Table II. *Sucrose and Raffinose Content in Cells Kept at 24 °C and in Cells Transferred (at T = 0 h, Indicated by Arrow on Fig. 1) to 4 °C*

Time	Raffinose		Sucrose	
	24°C	4°C	24°C	4°C
h	*nmol/mg fr wt*			
0	0.05		0.54	
24	0.08	0.51	0.70	4.10
30	0.10	0.70	0.53	5.20

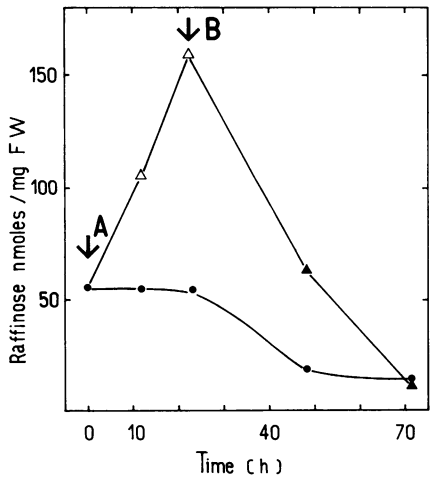

Figure 3. Raffinose content in *C. vulgaris* cells grown at 24°C. At the time indicated by arrow A (time 0 h), cells grown 50 h at 24°C were transferred to 4°C (△) or kept at 24°C (●). Arrow B (time 24 h) cells were transferred back to 24°C (▲).

grown at 24°C, but it starts to appear once the culture is moved to a lower temperature. Moreover, the data show that the amount of raffinose increases with the time the algae remains at 4°C.

Is the appearance of raffinose connected with the cold shock or is just an indication that growth is stopping? This question may be answered by examining to the level of raffinose when algae growing at 24°C enters into the stationary phase. The results presented in Table II show that there is no raffinose present up to 30 h after the algae have stopped growing. It is tempting to speculate that the observed accumulation of raffinose is associated with the cold shock. It should be remembered that in gymnosperms it has been shown that during the cold season sucrose is transformed into raffinose, and that the next member of the raffinose oligosaccharide family, stachyose, also appears following a very cold period (7). If this interpretation is correct, raffinose should disappear on transfer of the algal cultures at 4°C back to 24°C. Experiments following this idea indicate that raffinose level does indeed decrease and tends to disappear when *C. vulgaris* was moved to 24°C (Fig. 3).

It should be pointed out that raffinose is not considered to be present in algae (6). Therefore, to the authors' knowledge this is the first report of its occurrence. It remains to be determined experimentally whether the enzymes involved in raffinose synthesis are induced by the cold shock, or if there is a very low basal level of raffinose synthesizing enzymes, that are activated at low temperature. Work is in progress to examine these questions.

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