Plant Physiol. (1967) 42, 1601-1606

Studies on the Biochemistry and Fine Structure of Silica Shell Formation in Diatoms. Chemical Composition of Navicula pelliculosa during Silicon-Starvation Synchrony[1]

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Received July 7, 1967.

Summary. Changes are reported in total cellular organic carbon, nucleic acids, proteins, carbohydrates, lipids and chlorophylls during the course of silicon-starvation synchrony of *Navicula pelliculosa.* All constituents increased at the same rate, relative to cell number, for 30 hours of exponential growth during which silicon was depleted from the medium. Increase in cell number then stopped, but net synthesis of most components continued for a further 5 to 7 hours before ceasing. Deoxyribonucleic acids and lipids accumulated throughout the 14 hour silicon-starvation period. When silicon was resupplied, lipid synthesis ceased and organic carbon and carbohydrates decreased slightly. Net synthesis remained low during the 4 hour silicon uptake period but was resumed at higher rates as cell number began to rise. In cultures transferred to the dark 1 hour prior to readdition of silicon, total carbon, carbohydrates, and lipids decreased markedly during silicon uptake and cell separation. This was due in part to conversion of protein which maintained the protein level of the dark cells close to that of cells kept in the light. Mechanisms by which silicon starvation and reintroduction of silicon might affect rates of cellular synthesis are discussed.

Silicon is a major component of the cell wall in almost all diatom species and is essential for their growth, but whether it is required only as a wall constituent or affects other metabolic processes as well is not known. Studies conducted in this laboratory (4, 17), primarily to investigate the metabolism of silicon in wall formation, have shown that in the fresh water diatom, *Navicula pelliculosa,* cell separation ceases once the medium is depleted of silicon. However, cellular development is not arrested until after cytokinesis has taken place and deposition of new walls has begun. Thus after 14 hours of silicon starvation more than 70 % of the cell population consists of biprotoplastic cells, i.e., 2 daughter protoplasts, each surrounded by a new plasmalemma and separated by an intercellular space, contained within the parent frustule. When silicon is resupplied there is a 4 hour period of rapid silicon uptake after which new walls are completed and synchronous cell separation takes place.

Further studies have shown that with the onset of silicon deficiency ^{32}P-(6) and ^{35}S-incorporation (3), and photosynthesis (6) decrease. After several hours of silicon starvation pigment synthesis also decreases (11). Following the reintroduction of silicon a marked decrease in nucleoside triophosphates (4) coupled with increased oxygen consumption and ^{32}P-uptake suggests increased utilization of cellular energy. Presumably this is used in the biochemical processes associated with silicon uptake, deposition, or completion of the wall. It is not known, however, to what extent the synthesis of other cellular components is affected by silicon-starvation and by the reintroduction of silicon. This paper reports changes in the content of RNA, DNA, proteins, carbohydrates, lipids, total cellular carbon, and chlorophylls during exponential growth, silicon-starvation, silicon uptake, and synchronous cell separation of cultures kept in the light, or transferred to the dark after silicon starvation.

Materials and Methods

Silicon-Starvation Synchrony. Axenic cultures of the fresh water diatom *Navicula pelliculosa* (Breb) Hilse were grown in 50 ml of fresh water Tryptone

[1] This study was supported by grant, GM-08229-6, from the National Institutes of Health, United States Public Health Service, and in part by contract AT (11-1)-34, project 108 of the United States Atomic Energy Commission.

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(Difco) medium in 125 ml Erlenmeyer flasks on a reciprocal shaker at a light intensity of 5000 lux as previously described (4). The synchrony experiments were carried out in a 4-liter polycarbonate bottle containing 3 liters of the Tryptone medium, which includes a limited amount of silicon (8 μg per ml of medium) and an excess of all other essential nutrient elements. Sufficient volume of culture was inoculated into the bottle to give an initial cell concentration of 6 to 8 $\times$ 10^5 cells per ml. The culture was maintained at 20°, illuminated at 17,000 lux with cool white and warm white (Sylvania) fluorescent lights, and aerated at a rate of 10 liters per minute. Silicon was reintroduced to a concentration of 17 μg per ml culture 14 hours after the termination of exponential growth. In some experiments the culture was placed in the dark 1 hour prior to reintroduction of silicon. Due to the limited volume of culture, it was not possible to investigate all parameters on a single synchrony; the results reported here are the combined data obtained from several synchronies. All parameters were investigated in at least 2 synchronies, and the same trends were found in all cases. Duplicate samples were taken and the average reported. To maintain the starvation period at 14 hours in each of the synchronies, silicon was introduced after 47 to 51 hours of growth to accommodate slight variations in the initial innoculum. For convenience, all results are presented so that reintroduction of silicon is shown at hour 50 on the graphs.

Total Organic Carbon. One ml samples were filtered on 24 mm Whatman GF/C glass paper filters, washed twice with 4 ml inorganic medium, and the cellular organic carbon combusted to carbon dioxide by wet oxidation by the modification of the method of Menzel and Vaccaro (20) as described previously (4). The carbon dioxide was measured on a Beckman infrared analyzer and converted to μg carbon equivalent, using glucose as a standard.

Nucleic Acids. Cell samples containing about 10^9 cells for the DNA determinations, and about 5 $\times$ 10^8 cells for RNA, were centrifuged at 1600 $\times$ g for 5 minutes and the cell pellet extracted by modification of the method of Smillie and Krotkov (26). In each of the extractions 10 ml of solvent was used for the DNA samples, and 5 ml for RNA. The extractions were carried out on the centrifuged pellet in the following sequence: A) twice with absolute methanol at 0°; B) twice with 5 % trichloroacetic acid at 0°; C) with 95 ethanol saturated with sodium acetate at 0°; D) with 95 % ethanol boiled for 20 seconds; E) with ethanol-diethyl ether (2:1, v/v) boiled for 20 seconds; and F) with diethyl ether boiled for 20 seconds. The residue was air-dried and stored at −27° until it was extracted for nucleic acids.

DNA was extracted from the residue with 1.5 ml 5 % perchloric acid at 70° for 15 minutes, and the amount of deoxyribose determined by the Burton modification (2) of the diphenylamine reaction. Highly polymerized calf thymus DNA sodium salt (Sigma) was used as a standard.

RNA was extracted from the residue with 1.5 ml of 0.3 M KOH at 37° for 16 hours. The sample was then cooled to 0° and acidified with 5 % perchloric acid to precipitate most of the residual protein and DNA. After centrifugation, the pH of the supernatant was adjusted to approximately 8.0 with KOH. tris-HCl buffer (pH 7.8), was added to a final concentration of 0.025 M, and about 3 mg Dowex 2 (Cl^-) resin (50–100 mesh) was added per μg nucleotides. After the suspension was filtered through a sintered glass filter, the resin was washed with 0.025 M tris-buffer pH 7.8 and the ribonucleotides then eluted with 10 ml of 1 N HCl (8). RNA content was determined by optical density at 260 mμ. A reference curve was obtained with RNA prepared from yeast nucleic acid which had been purified by methods of Vischer and Charggaff (31) and Sevag et al. (25). Results obtained by this method were in good agreement with determinations of phosphate and ribose by standard colorimetric analysis.

Proteins. A) Samples containing 4 to 6 $\times$ 10^6 cells were harvested by centrifugation at 1600 $\times$ g, washed twice with Tryptone-free medium, and the protein content determined by the method of Lowry et al. (18). Crystalline bovine serum albumin (Pentex, Inc.) was used as a standard.

B) Samples containing about 10^7 cells were filtered through glass paper filters, washed twice with Tryptone-free medium, and the residue digested with sulfuric acid as described by Strickland and Parsons (28). The resulting ammonia was determined by a modification of the hydrindantin-ninhydrin colorimetric method of Moore and Stein (19). The amount of protein was calculated as N $\times$ 6.25.

Carbohydrates. Samples containing about 5 $\times$ 10^7 cells were filtered through Whatman glass paper filters, washed twice with Tryptone-free medium, and hydrolized with 3 ml 0.5 N HCl for 15 minutes at 105°. The hydrolysate was filtered through a second glass paper filter and the filtrate analyzed for sugar by the phenol-sulphuric acid method (9). Optical density was measured at 490 mμ; glucose served as the standard.

Lipids. Samples containing 2 to 3 $\times$ 10^7 cells were harvested by centrifugation at 1600 $\times$ g, washed with Tryptone-free medium, and extracted with 1 ml chloroform-methanol (2:1, v/v) overnight. After centrifugation, the pellet was further extracted twice with 1 ml chloroform-methanol, and the extracts combined and transferred to a 10 ml glass ampule. The organic solvent was removed by heating at 40° overnight. The organic carbon of the dried lipid fraction was oxidized to carbon dioxide and the latter was measured by infrared absorption. Lipid carbon was converted to dry weight of lipid using the factor 1.7 (12).

Chlorophylls. One ml samples were pipetted directly into 9 ml of absolute acetone containing a small drop of 1 % $MgCO_3$ suspension. The cell suspension was kept in the dark overnight to allow complete extraction, after which the amount of chlorophyll was

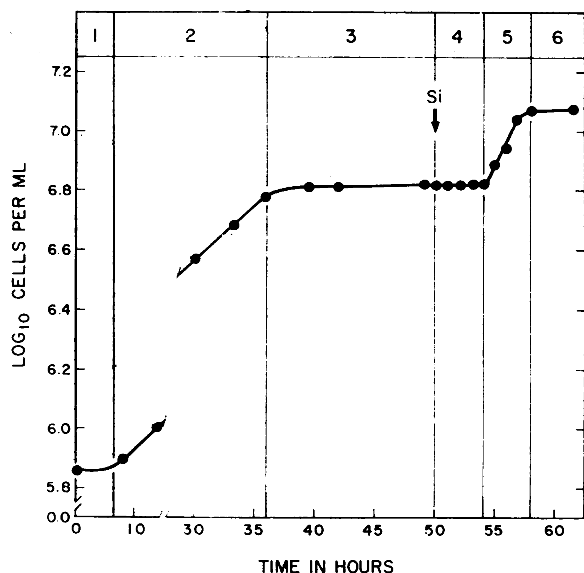

FIG. 1. Changes in cell number during the course of a silicon starvation synchronous culture of *Navicula pelliculosa*. 1) lag period; 2) exponential growth; 3) silicon starvation; 4) silicon uptake; 5) cell separation; 6) start of new growth cycle.

determined fluorometrically (13).

Silicon. The silicon content of the medium was determined by a modification (4) of the method of Tuma (30).

Results

Changes in cell number during the course of the silicon-starvation synchrony are shown in figure 1.

Exponential Growth. The concentrations of various cellular components, determined periodically during exponential growth, is shown in table I. Within the limits of experimental error, all components increased at the same rate as cell number. Protein values are those determined from Kjeldahl nitrogen values. Determination by the Lowry method gave values 30 % lower, as would be expected since the Kjeldahl method includes non-protein amino nitrogen, small peptides, free amino acids, and amines.

Silicon Starvation. Effects of silicon starvation on cellular components are shown in figure 2. After about 35 hours of exponential growth, silicon-content of the medium falls from its initial value of about 8 μg per ml to less than 0.2 μg per ml.

This residual amount, which could be detected at all times, may represent a small fraction of the added silicon which is present in a polymerized or complexed form and is thus not available.

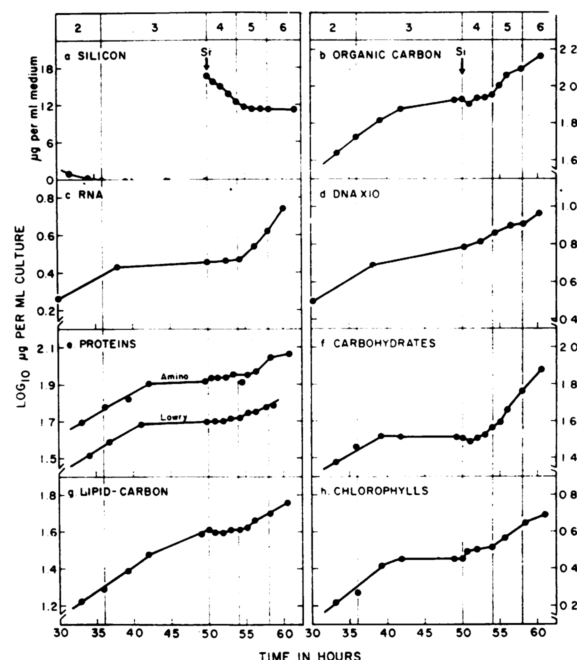

FIG. 2. Changes in concentrations of silicon, organic carbon, RNA, DNA, proteins, carbohydrates, lipids and chlorophylls during silicon-starvation synchrony of *N. pelliculosa*. Growth periods as in figure 1.

Table I. *Changes in Gross Chemical Composition of* N. pelliculosa *during Silicon-Starvation Synchrony of a Culture Kept in Continuous Light*

Constituents	Exponential Growth $\mu g/10^6$ cells	% of total	End of Si starvation (50 hrs) $\mu g/10^6$ cells	% of total	End of Si uptake (54 hrs) $\mu g/10^6$ cells	% of total	End of cell separation (58 hrs) $\mu g/10^6$ cells	% of total
DNA	0.09	0.37	0.09	0.31	0.11	0.33	0.08	0.31
RNA	0.46	1.90	0.42	1.58	0.42	1.28	0.38	1.46
Proteins	10.0	41.5	11.3	39.3	13.0	39.7	10.2	39.0
Carbohydrates	4.8	21.0	4.4	15.4	5.3	16.2	5.1	19.5
Lipids	6.1	25.1	9.7	33.8	9.7	29.5	7.5	28.5
Chlorophylls	0.43	1.78	0.42	1.57	0.44	1.34	0.4	1.54
SiO_2	2.2	8.2	2.3	8.0	3.8	11.6	2.6	9.8
Organic carbon	10.1	...	11.8	...	13.0	...	10.2	...
Total*	24.08	100	28.63	100	32.77	100	26.26	100

* Organic carbon not included.

Although silicon becomes limiting at about the thirty-sixth hour of growth, when increase in cell number ceases (fig 1), exponential increase in all cell components investigated continues. Increase in the concentration of RNA, proteins, carbohydrates and chlorophylls stops after 5 to 7 hours of silicon starvation, whereas the concentration of DNA continues to increase throughout the latter part of the starvation period. Net lipid synthesis continues almost exponentially until silicon is reintroduced. During the latter part of the starvation period the increase in carbon content of the cells can be accounted for largely by the increase in lipids.

Reintroduction of Silicon in the Light. Immediately following the reintroduction of silicon to the medium, net lipid synthesis ceases. At the same time there is a slight but consistent decrease in total organic carbon and carbohydrates, and an increase in the amount of DNA and chlorophylls. However, the concentration of proteins and RNA remains more or less constant until the start of cell separation.

Once silicon uptake ceases, at the start of the cell separation period, net synthesis of all cellular constituents is resumed at rates which approach or exceed those of the original exponential culture.

The variation in rates of net synthesis of constituents during the course of the experiment led to marked changes in the percentage composition of the cells (table I). The relative rates of synthesis of the various components during the silicon uptake and cell separation periods is such that by the end of the experiment the percentage composition of the cells was close to that of the exponential culture.

Reintroduction of Silicon in the Dark. Previous observations (4) have shown that cells transferred to the dark prior to addition of silicon can complete silicon uptake and cell division, presumably at the expense of cell reserve material. In order to determine to what extent such reserves are consumed or converted to other cellular components during wall formation in the dark, measurements of organic carbon, proteins (by the amino-nitrogen method), carbohydrates and lipids were repeated on cultures which had been transferred to the dark 1 hour prior to the reintroduction of silicon. The results of these experiments are shown in table II.

During the 1 hour dark period, prior to silicon addition, the composition of the cells did not change significantly (cf. table I, column 2; table II, column 1). During both silicon uptake and cell separation periods, the cellular content of carbohydrates and lipids decreased. This decrease could be accounted for in part by respiratory loss, indicated by the decrease in total organic carbon, and in part by conversion to proteins. The increase in protein was such that protein content was only slightly less in dark as compared to light daughter cells.

The loss of carbohydrates and lipids per ml of culture was greater during the silicon uptake period, when little protein was formed, than in the cell separation period. These results are consistent with our previous observations that addition of silicon stimulates aerobic respiration which is presumably coupled to active silicon transport.

Discussion

During exponential growth in batch cultures the chemical composition of microorganisms may change (1). The composition of *N. pelliculosa*, however, was constant throughout exponential growth and did not differ significantly from that reported for exponential cultures of diatoms (see 16) and other algae (14, 22).

Although increase in cell number ceased once silicon had been depleted from the medium, concentration of all components measured continued to rise during the next 5 to 7 hours. Net synthesis of RNA, proteins, carbohydrates and chlorophylls then ceased. Similar changes in cellular metabolism have been reported for partially synchronized cultures of *Cyclotella cryptica* transferred to a silicon free medium (32). Increase in DNA persisted throughout the silicon starvation period, as did that of lipid. The latter appears to be typical of algae in which growth is limited by mineral deficiencies, since it has been shown that lipids accumulate in nitrogen-starved cultures of *N. pelliculosa* (10) and in nitrate (27) or sulfate-starved algae (21).

Other metabolic activities have been shown to decrease as *N. pelliculosa* becomes silicon starved (3, 6, 11). However, evidence that silicon is needed for metabolic processes, apart from wall formation, is lacking. Recent studies on the incorporation of radioactive silicon (^{31}Si) indicate that diatoms contain a cytoplasmic pool of silicon (Coombs and Volcani, unpublished observations). In addition, the existence of such a silicon pool can be inferred from the fact

Table II. *Changes in Cell Constituents in* N. pelliculosa *during Silicon-Starvation Synchrony of a Culture Transferred to the Dark 1 Hour Prior to Re-addition of Silicon*

Constituents	End of Si starvation (50 hrs) µg/ml	End of Si starvation (50 hrs) µg/10^6 cells	End of Si uptake (54 hrs) µg/ml	End of Si uptake (54 hrs) µg/10^6 cells	End of cell separation (60 hrs) µg/ml	End of cell separation (60 hrs) µg/10^6 cells
Organic carbon	83	11.8	70	10.0	68	6.2
Proteins	81	11.6	86	12.2	100	9.1
Carbohydrates	33	4.6	25	3.6	22	2.0
Lipids	68	9.7	56	8.0	51	4.6

that all silicon starved biprotoplastic cells examined in the electron microscope show that silicon deposition has been initiated at the site of the new walls (Halicki, Kiethe, and Volcani, unpublished observations). Thus the increase in cellular components during the first half of silicon-starvation might take place at the expense of the silicon pool, and cease as the initiation of new walls exhausts the pool. A similar explanation was suggested to account for continued cellular synthesis in *C. cryptica* transferred to a silicon free medium (32). Attempts to study changes in the size of the pool in *N. pelliculosa* have been hampered by difficulty in determining how much silicon is leached from the walls during extraction. This difficulty has been overcome in part by the use of ^{31}Si, and further studies on its incorporation may provide firmer evidence as to the role of silicon in synthetic processes other than wall formation.

It is possible that the decreased metabolism of the culture during silicon starvation reflects the rising percentage of morphologically arrested cells. That is, in the course of normal cell development net synthesis of nucleic acids, proteins, and pigments may cease once the various cell organelles have been duplicated, recommencing only when the daughter cells are liberated. This is suggested by a number of studies on the life cycle of algae synchronized by light/dark regimes which show that syntheses of these cellular components is halted prior to cell division, when cells have reached a certain stage of maturity. For example, in cultures of *Chlorella* left in the light throughout a complete cycle of growth and division, synthesis of protein and RNA stops about 2 hours prior to increase in cell number and recommences as the daughter cells are released (24). In cultures of *Euglena,* protein content doubles in the early part of the life cycle and then remains constant for several hours before cell number increases (7). Other studies of various algae (see 23, 29) report major changes in carbohydrates and lipids, which accumulate in the light and are subsequently used during the dark period for protein or pigment synthesis. However, since the cells are placed in the dark prior to cell division, it is not possible to determine whether the changes in cell composition result from the light/dark transition or from changes in the maturity of the cells.

Little information is available on the rates of synthesis at various stages of morphological development in diatoms. In cultures of *Cylindrotheca fusiformis,* synchronized by a single dark period, carbon mass increases during the first half of the growth cycle but levels off during nuclear division, wall formation, and cell separation (5). Further studies on the metabolism of *N. pelliculosa,* now under way with cultures synchronized by a light/dark regime, should provide more information on the correlation between rate of synthesis and stage of morphological development.

In cultures transferred to the dark prior to the reintroduction of silicon the contribution of photosynthesis is excluded, and hence the changes in cellular components during silicon uptake are predominantly associated with wall formation. In the light cultures, immediate metabolic changes are indicated by the slight decrease in cellular carbon carbohydrates and lipids. In the dark cultures, the decrease in these components is marked. This is consistent with the observation (6, 15) that the addition of silicon to starved cells markedly stimulates dark respiration, which is associated with decreased nucleoside triphosphates and increased ^{32}P incorporation. Thus an increased production and utilization of adenosine triphosphate, associated with increased synthetic metabolism, is indicated.

The present study shows that little net synthesis of organic constituents occurs during silicon uptake, and thus supports previous suggestions that the decrease in nucleoside triphosphates indicates that energy is utilized in active silicon uptake, deposition, or wall formation.

Acknowledgments

We thank Dr. J. D. H. Strickland for his interest in this work. We are grateful for the technical assistance of Miss Hester Kobayashi and Miss Lynn Peterson.

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