SILICON METABOLISM IN DIATOMS

I. Evidence for the Role of Reduced Sulfur Compounds in Silicon Utilization*

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INTRODUCTION

Each diatom cell is characteristically enclosed in a perforated siliceous skeleton or frustule composed, in the simplest forms, of two valves. According to Rogall (1939), the frustules consist of pure silicic acid in a "subcolloidal" state, as shown by chemical analysis and x-ray crystallography. Cooper (1952) states that the chemical constitution of diatom skeletons is similar to that of hydrated silica dispersed in water.

Although ecologists have long been concerned with the quantitative relationship between silicon utilization and diatom growth (Gardiner, 1941; Lund, 1950; Jørgensen, 1952, 1953), there has apparently been no study of the biochemical aspects of silicon metabolism in diatoms, as has been made for phosphorus metabolism in many organisms.

Most workers have found that, in order to culture diatoms successfully, soluble silicon, in the form of silicate, must be added to the medium. All species cultivated by the author have shown silicon to be essential for growth. Such a requirement can only be demonstrated in liquid culture, since commercial agar itself serves as a source of silicon (Bachrach, 1927; Lewin, unpublished).

In an investigation of silicon metabolism in diatoms the following questions arise. (*a*) Does silicon play any role in cellular activity other than that of frustule formation? (*b*) By what processes are soluble silicates converted into the insoluble silica of the frustules? (*c*) Do these processes involve organosilicate compounds or enzymes, or is silica deposited externally by some inorganic reaction at the cell surface?

In the course of preliminary investigations and the evolution of techniques for such studies, some evidence was obtained for the role of sulfhydryl groups in silicon utilization. The present paper deals with this aspect of the problem.

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Materials and Methods

A bacteria-free clone of *Navicula pelliculosa* (Bréb.) Hilse was chosen as the test organism in this study since it grows rapidly in synthetic inorganic media, and since some aspects of its physiology have already been investigated (Lewin, 1953). Whereas in most diatoms which have been investigated experimentally the valve dimensions decrease as a consequence of repeated asexual division (see Fritsch, 1935), the cells of *N. pelliculosa* remain of constant size in culture, possibly as a consequence of the absence of a continuous siliceous girdle. This feature makes *N. pelliculosa* a particularly suitable species for cultural studies.

In order to obtain the diatoms in a condition in which they would readily remove silicon from the surrounding medium, they were first grown autotrophically in a nutrient solution containing a low concentration of silicon. After the available silicon had been utilized, further growth was prevented by limitation of this element, and the cells in the culture became "silicon-deficient." When such cells were transferred to a solution containing a further supply of silicon and were incubated in the dark under non-growing conditions, they removed silicon from the medium. A similar technique was used by Harvey (1953) in his study of phosphorus and nitrogen absorption by *Nitzschia closterium*.

The medium used for cultivation of silicon-deficient cells contained the following concentrations of salts (grams/liter): K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $Ca(NO_3)_2 \cdot 4H_2O$, 1.0, dissolved in glass-distilled water. Traces of B, Cu, Fe, Mn, Mo, and Zn were added. Silicon was supplied as potassium silicate ("solution silicate of potash," Eimer and Amend, New York) to establish a concentration of 1.0 mg. soluble silicon per liter. Large pyrex glass Erlenmeyer flasks containing 1.5 liters of medium were sterilized by autoclaving and inoculated under sterile conditions. The cultures were constantly aerated by filtered air and illuminated at 300 ft.-c., at 23°C., for 10 days.

Cells were concentrated from the culture medium, and after each washing (see below), by centrifugation. Aliquots of cells were then suspended in a solution of potassium silicate to give a final cell concentration of from 6 to 10 million cells/ml. and a final Si concentration of 5 to 10 mg. Si/liter. Cells heated to 100° for 2 minutes served as controls. The cells were then incubated at 23° under non-growing conditions (*i.e.* in N-free medium in darkness). At intervals, 3 ml. samples were withdrawn. The cells were removed by brief centrifugation and a silicon determination was carried out on 2 ml. of the supernatant, employing a modification of the method proposed by Harrison and Storr (1944). The silicomolybdate complex was "developed" by addition of 2 ml. H_2O and 0.4 ml. of 5 per cent ammonium molybdate (made up in 1 N H_2SO_4), and 2 ml. of 6 N H_2SO_4 were added to remove the effect of traces of phosphomolybdate. Reduction to molybdenum blue was effected by adding 0.4 ml. of 5 per cent aqueous hydroquinone. Each sample was made up to 10 ml. with distilled water, and after standing for 20 minutes the color intensity was measured in a Klett-Summerson photoelectric colorimeter with the red filter (No. 66). Sodium silicofluoride (Na_2SiF_6), which hydrolyzes in water to give silicic acid (King, 1939), was used to provide a standard solution containing 2.34 mg. Si/liter.

For respirometry studies a Braun circular Warburg apparatus was employed. Each vessel held 2 ml. of a cell suspension containing 1 to 2 $\times 10^8$ cells in potassium silicate (in order to duplicate conditions during silicon uptake experiments), 0.2 ml. of 10 per cent KOH in center well, and 0.5 ml. of inhibitor or other reagent in the side arm. Experiments were carried out in darkness at 25°.

RESULTS

1. General.—The utilization of silicon as measured in this way was shown in preliminary experiments to be: (*a*) a property of living cells; (*b*) an aerobic phenomenon, inhibited in the absence of oxygen, and stimulated by turbulent aeration; (*c*) more rapid at 20° than at 10° or 30°; (*d*) not appreciably affected by the addition of potassium phosphate buffer (0.003 M, pH 5.4 or 8.6) to the silicate solution. (It was impracticable to employ higher concentrations of buffer since phosphate interferes with the colorimetric determination of silicate.)

2. Silicon Uptake by Washed Cells.—It was observed that deficient cells washed in distilled water (see Fig. 1) or saline (0.02 per cent NaCl plus 0.02 per cent $CaCl_2$) took up silicon more slowly than did unwashed cells. The ability to take up silicon could be partially restored to washed cells by resuspending them either in fresh nutrient medium or in the medium in which the cells had originally grown. When each of the original constituents was tested individually, it was found that $MgSO_4$ alone was as effective as the complete medium (see Fig. 2); a further experiment revealed that it was the sulfate ion which was necessary, since K_2SO_4 functioned as efficiently as $MgSO_4$. Cells washed in medium in which all sulfate was replaced by equimolar chloride behaved in the same manner as cells washed with distilled water.

Whereas sulfate (2×10^{-3} M) only partially restored the ability of washed cells to utilize silicon, Na_2S (4×10^{-5} M) was found to give complete restoration even after five washings. Glutathione (2×10^{-4} M), *l*-cysteine (2×10^{-4} M), *dl*-methionine (2×10^{-3} M), and $Na_2S_2O_3$ (4×10^{-5} M) were equally effective (see Fig. 3).

The fact that reduced sulfur compounds were more effective than sulfate suggested the role of a reducing agent in addition to a source of sulfur. Ascorbic acid alone (2×10^{-4} M) was found to induce only partial recovery in washed cells, as did sulfate, but ascorbic acid plus sulfate gave almost complete recovery (see Fig. 4).

It could be shown that washing and centrifuging did not *per se* damage the cells, since cells washed in dilute Na_2S (4×10^{-5} M) fully retained their ability to absorb silicon. Furthermore, in cells so treated there was no reduction of activity upon subsequent washing with distilled water, indicating a protective action of the sulfide.

3. Comparison of the Respiration of Washed and Unwashed Cells.—It was

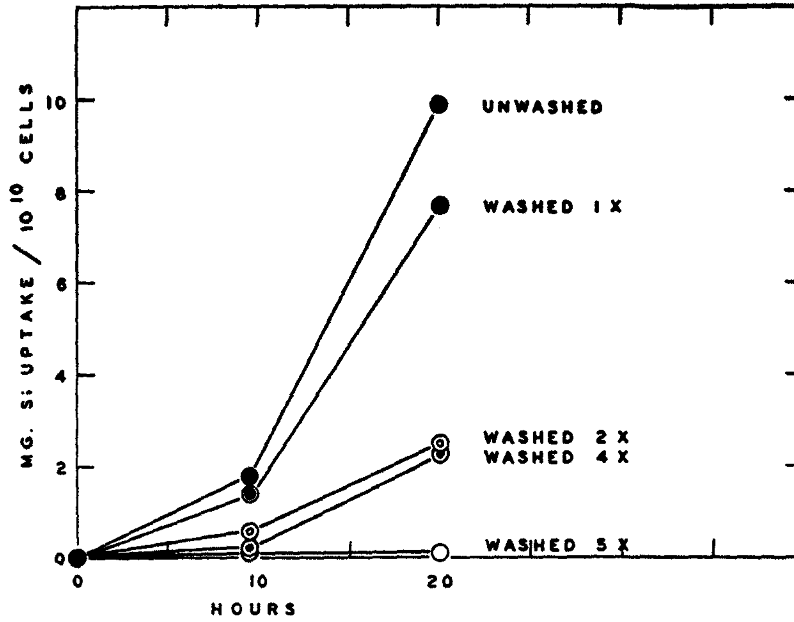

FIG. 1. Effect of washing cells with water.

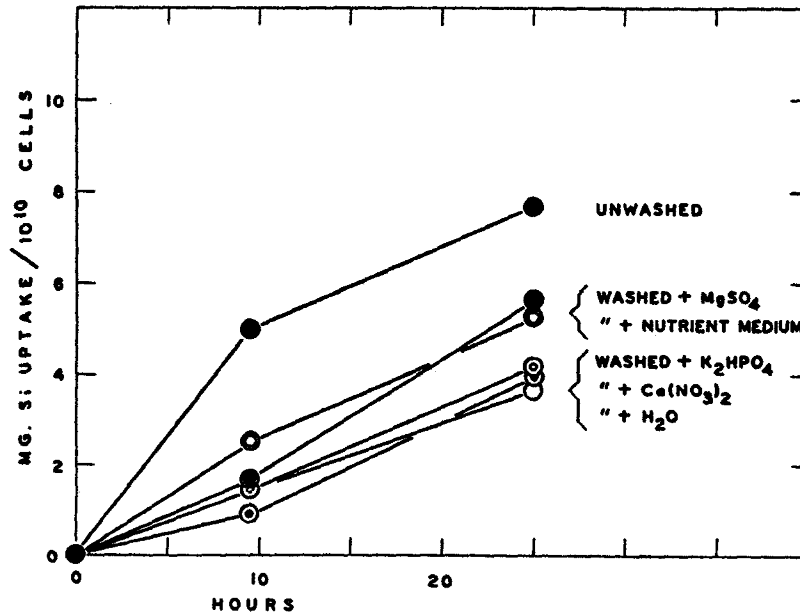

FIG. 2. Effect of mineral nutrient constituents on cells washed twice with water, showing partial restoration by $MgSO_4$ (2×10^{-3} M).

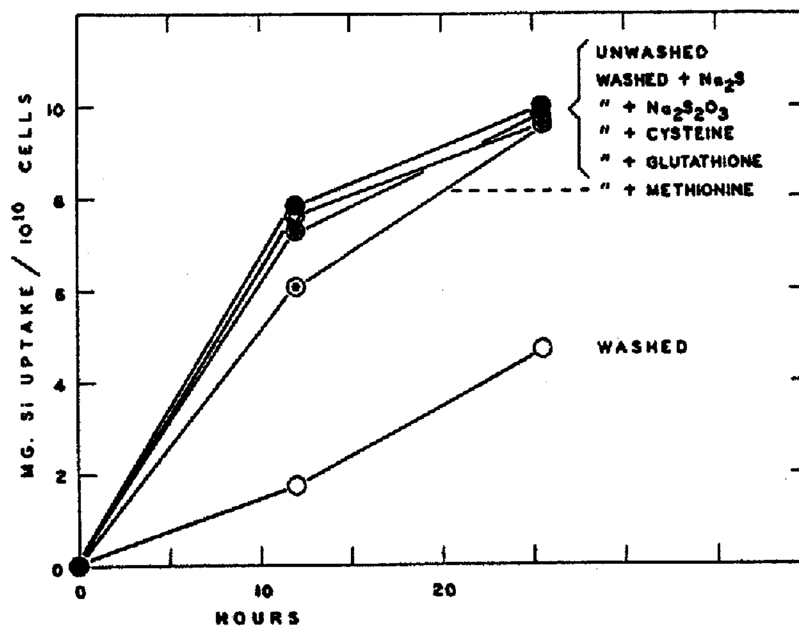

FIG. 3. Effect of sulfur compounds on cells washed twice with water, showing complete restoration by Na_2S (4×10^{-5} M), $Na_2S_2O_3$ (4×10^{-3} M), *l*-cysteine (2×10^{-4} M), glutathione (2×10^{-4} M), or *dl*-methionine (2×10^{-3} M).

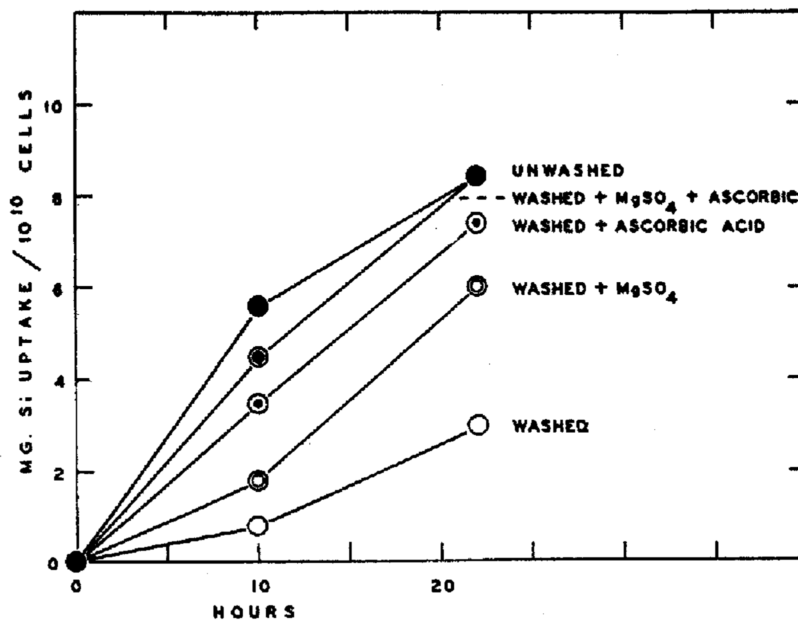

FIG. 4. Effect of $MgSO_4$ and ascorbic acid on cells washed twice with water, showing complete restoration by $MgSO_4$ (2×10^{-3} M) plus ascorbic acid (2×10^{-4} M).

necessary to exclude the possibility that the effect of washing was to decrease the rate of cell respiration, and thus indirectly to affect silicon uptake, already shown to be an aerobic process. The respiration of washed and unwashed cells was compared and it was observed that the respiration rate of twice washed cells was slightly higher than that of unwashed cells during the first 2 hours, after which the rates were identical. Furthermore, the addition of fresh nutrient medium, or of Na_2S, $MgSO_4$, glutathione, or *l*-cysteine (at the above concentrations) had no effect on the respiration of washed cells. These experiments indicated that the impairment of silicon absorption by washing was not an indirect result of decreased respiratory activity.

4. Effect of Washing Cells with EDTA.—It was suggested that washing might have upset the ionic balance and resulted in the inhibition of certain surface enzymes by heavy metal ions, and that reduced sulfur compounds, by removing these metal ions, might have reversed this inhibition, thus restoring silicon assimilation. If this had been so, then chelation of the toxic cation with an agent such as ethylenediamine tetraacetic acid (EDTA) might have been as effective as sulfide. However, samples of cells washed in 1×10^{-2} or 1×10^{-3} M EDTA (pH adjusted to 6.5 with NaOH) or in distilled water were all equally inhibited in their ability to take up silicon.

5. Examination of Wash Water.—Attempts to detect the presence of a sulfhydryl compound or other reducing agent in the wash water were unsuccessful. Measurements of the absorption spectrum of the supernatant between 3,000 and 7,000 A showed no indication of the presence of any pigmented compound. Nitroprusside and lead acetate tests for sulfide, a biuret test for protein, and iodine titration gave negative results even with concentrated (10 times) supernatant.

6. Effect of Sulfhydryl Inhibitors on Unwashed Cells.—Since the utilization of silicon might involve a —SH protein at the cell surface, attempts were made to find an inhibitor of silicon uptake which did not penetrate to impair the action of respiratory enzymes within the cell. Among sulfhydryl inhibitors, iodoacetate acts as an alkylating reagent of —SH groups, while cadmium and trivalent arsenic inhibit by mercaptide formation, and are considered highly specific reagents for —SH groups (Barron, Nelson, and Ardao, 1948). The results of experiments using these three inhibitors are summarized in Table I.

Cadmium chloride appears to be a specific inhibitor of silicon utilization at concentrations (1×10^{-2} M, 1×10^{-3} M) which are not inhibitory to respiration. Furthermore, treatment of cells in 1×10^{-3} M $CdCl_2$ for 20 hours did not affect their viability. The inhibitory effect of $CdCl_2$ (1×10^{-3} M) was found to be reversed by either glutathione (2×10^{-4} M) or *l*-cysteine (2×10^{-4} M) (see Fig. 5). Unless the respiratory enzymes of *N. pelliculosa* are all resistant to Cd^{++}, which seems unlikely, the cell membrane appears to be impermeable to Cd ions or $CdCl_2$, indicating that —SH groups at or near the cell surface may be involved in the mechanism of silicon utilization.

7. The Destination of Silicon after Uptake.—Before attempting an explanation for the mechanism of inhibition of uptake by washing or by $CdCl_2$ and

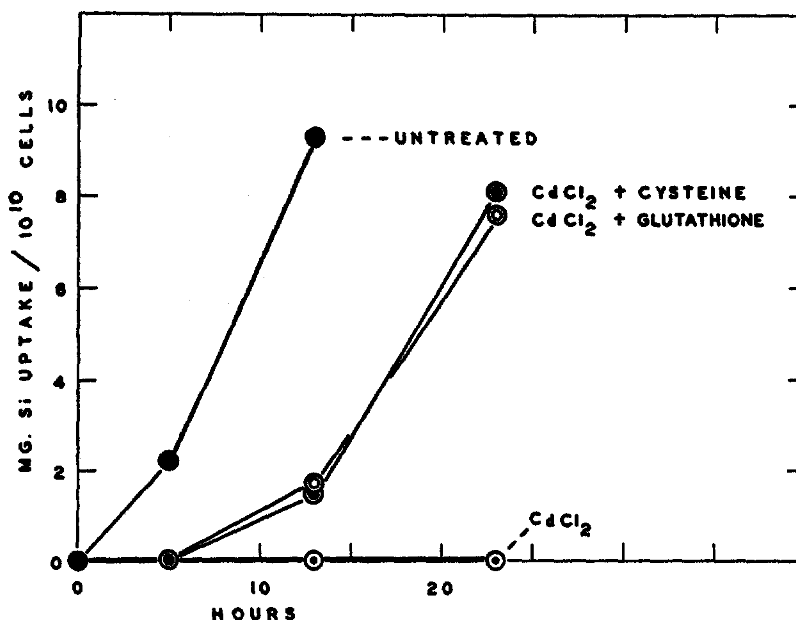

Fig. 5. Inhibitory effect of $CdCl_2$ (1×10^{-3} M) on unwashed cells and reversal of inhibition by *l*-cysteine (2×10^{-4} M) or glutathione (2×10^{-4} M) added 10 minutes after $CdCl_2$.

TABLE I

Effect of Sulfhydryl Inhibitors on Respiration and Si Utilization

Each reagent was tested at concentrations of 10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M.

Reagent	Lowest concentration inhibitory to respiration in 4 hrs.	Lowest concentration inhibitory to silicon utilization in 5 hrs.
Sodium iodoacetate	1×10^{-4} M	1×10^{-4} M
Cadmium chloride	No inhibition	1×10^{-3} M
Sodium arsenite	1×10^{-3} M	1×10^{-3} M (1×10^{-4} M, slight inhibition; 1×10^{-6}, 1×10^{-7}, stimulation)

for its restoration by reduced sulfur compounds, it was necessary to determine where the silicon was actually going in the cell. The silicon taken up by depleted cells might either be retained intracellularly in some intermediate form or might be deposited at once as SiO_2.

Preliminary studies (Lewin, unpublished) indicated that virtually all the silicon taken up by deficient cells could be accounted for in the insoluble SiO_2 fraction. This being so, it was necessary to determine whether the new material was laid down as a thickening of the existing silica frustules or whether it became organized in the form of new valves within the parent frustule preparatory to further cell division. For this reason cell and valve counts were made before and after silicon uptake (24 hours). Whole cells were counted directly using a hemacytometer in the usual fashion. Valve counts were made on an aliquot after digestion of the organic material with concentrated HNO_3, using high power phase contrast microscopy, since the "cleaned" valves of *N. pelliculosa* are otherwise very difficult to distinguish.

In view of the fact that both cell number and valve number remained unaltered in the course of the uptake experiment (see Table II), it seems safe

TABLE II

Destination of Silicon after Uptake

	Control	+K silicate
Cells/liter, initial	2.18×10^{10}	2.10×10^{10}
Silicon (mg./liter) taken up (24 hrs.)	0.0	7.00
Cells/liter, final	2.00×10^{10}	1.88×10^{10}
Valves/liter, final	3.63×10^{10}	3.65×10^{10}

to assume that the silicon taken up was deposited on the already existing silica frustules, presumably by activity at the protoplast membrane.

DISCUSSION

Since silicon utilization in *N. pelliculosa* can be inhibited either by washing the cells in distilled water or by treatment with $CdCl_2$, whereas respiration is not affected by either of these treatments, a site of inhibition other than that of the respiratory mechanism is indicated.

This site may well be the superficial membrane closely associated with the production of the silica frustule. The activity of this membrane has been demonstrated by Küster-Winkelmann (1938), who observed that when cells of *Achnanthes* spp. are placed in hypertonic media, the cell lining becomes detached from the inside of the valve, and a new silica valve may be formed on the freshly exposed protoplast. However, the nature of this membrane is in some doubt, since the silica wall of diatoms is closely associated with a mucilaginous material usually referred to as pectin. This can be readily stained with toluidine blue, etc., and generally appears as a layer of variable thickness exterior to the frustule. However, Liebisch (1929), among others, has presented evidence that there is also a pectin layer beneath the frustule

on which an impression of the valve markings is left when the silica is dissolved by hydrofluoric acid. The exact relationship between protoplast surface, pectin layers, and silica must be determined before the site of silica deposition can be resolved.

With regard to the sulfhydryl nature of the silicon uptake mechanism in diatoms, it is worth mentioning that reduced sulfur compounds have been found to stimulate growth in certain diatoms. Thus Harvey (1939) found that

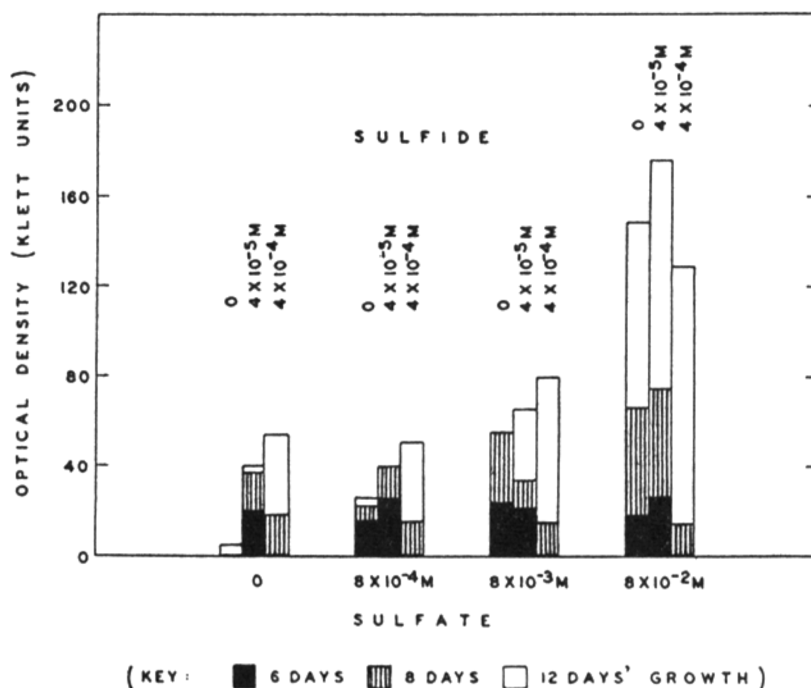

FIG. 6. Effect of sulfide on growth of *N. pelliculosa* in the presence of various concentrations of sulfate. (10 mg. Si/liter).

growth of *Ditylum brightwelli* was stimulated by compounds such as cystine, glutathione, or methionine (1 to 20 mg./liter). Matsudaira (1942) showed that the addition of small quantities of inorganic sulfides (0.1 to 1 mg. $Na_2S \cdot 9H_2O$/liter) to natural or artificial sea water greatly stimulated growth of *Skeletonema costatum*. Likewise, in the present investigation it was observed that sulfide (4×10^{-5} M) stimulated growth of *N. pelliculosa* (see Fig. 6) even in a medium in which abundant sulfur was present as sulfate. It is possible that the stimulatory effect of reduced sulfur compounds may prove to be a general phenomenon among diatoms associated with their requirement for silicon.

We may conclude that washing of cells impairs their ability to take up silicon, by removing sulfate, and in addition, by impairing their ability to form a reduced sulfur compound of some sort, which is presumably active at the surface where silica is deposited.

SUMMARY

1. Cells of the fresh water diatom *Navicula pelliculosa* may be grown in a mineral medium containing a low concentration of silicon. When transferred to a fresh silicate solution and incubated under non-growing conditions such deficient cells rapidly take up silicon from the medium.

2. The utilization of silicon is an aerobic process.

3. When deficient cells are washed with distilled water or saline, their ability to utilize silicon is impaired whereas respiration is unaffected.

4. The ability of washed cells to take up silicon can be partially restored with sulfate or ascorbic acid, and is completely restored by Na_2S, $Na_2S_2O_3$, glutathione, *l*-cysteine, *dl*-methionine, or ascorbic acid plus sulfate.

5. The sulfhydryl reagent, $CdCl_2$, inhibits silicon utilization of unwashed cells at concentrations which do not affect respiration. This inhibition similarly is reversed by glutathione or cysteine.

6. However, sodium iodoacetate or sodium arsenite inhibits respiration and silicon utilization at the same concentrations.

7. The silicon taken up by deficient cells is deposited at the cell surface as a thickening of the existing silica frustules.

8. Sulfhydryl groups in the cell membrane may be involved in silicon uptake by diatoms.

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