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SILICON METABOLISM IN DIATOMS. II. SOURCES OF SILICON FOR GROWTH OF NAVICULA PELLICULOSA[1,2,3]

JOYCE C. LEWIN

OSBORN BOTANICAL LABORATORY, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT AND MARITIME REGIONAL LABORATORY, NATIONAL RESEARCH COUNCIL, HALIFAX, NOVA SCOTIA, CANADA[4]

Richter (22), Bachrach (2), and Pringsheim (21) have reported that small amounts of agar tend to stimulate growth of diatoms in liquid cultures, and both Richter and Bachrach attributed this effect at least in part to the presence of silicon compounds. Likewise Brieger (4) observed in commercial agar the presence of insoluble siliceous matter, including diatom frustules, which might slowly dissolve to furnish sufficient silicon for growth of diatom cells. Patrick and Wallace (20) found that agar, pyridine extract of agar, and ashed extract contained a factor stimulatory to the growth of *Nitzschia linearis*. They concluded that some trace element in the agar was responsible for stimulation, but they did not suggest the possibility that this factor might be silicon.

Apparently there has been no published account of quantitative studies on the availability to diatoms of the soluble silicon in agar and agar ash. This paper deals with evidence indicating that silicon is the factor in agar ash stimulatory to the growth of *Navicula pelliculosa*, and with experimental investigations on the ability of this diatom to utilize various silicon compounds for growth.

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[2] Submitted as National Research Council No. 511-527.
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[4] Present address: Guest research worker.

MATERIALS AND METHODS

The diatom used throughout these studies was originally isolated from fresh water in 1949 and has been maintained as a bacteria-free clone on agar slants. It has proved to be a suitable organism for studies of diatom physiology (17, 18).

The basic medium, to which agar, agar ash, or various silicon compounds were added, contained the following concentrations (gm/l) of salts dissolved in distilled water: 0.2 K_2HPO_4, 0.2 $MgSO_4 \cdot 7\,H_2O$, 1.0 $Ca(NO_3)_2 \cdot 4\,H_2O$. Trace elements were added as follows: 0.1 ppm each B, Co, Cu, Mn, Mo; 0.3 ppm Zn; 0.5 ppm Fe. Unless otherwise stated, silicon was added to culture media in the form of orthosilicate, (Fisher, table II). All media were adjusted to pH 7.0 to 7.5 with HCl or KOH. Sterilization was carried out by autoclaving at 15 lbs pressure for 15 minutes.

Special precautions to exclude contamination by silicon leached from glass surfaces were unnecessary. Throughout the experiments described below, controls were run routinely, and at no time was there any evidence that appreciable quantities of silicon from the Pyrex glassware were contaminating the media sufficiently to affect the growth of diatoms. In the absence of added silicon compounds, growth was negligible.

For each concentration of agar, agar ash, or silicon compound tested, 3 or 4 replicates were set up in

125-ml Pyrex Erlenmeyer flasks containing 25 ml of liquid medium. Flasks were inoculated with one loopful from a homogeneous suspension of diatom cells, and shaken constantly at 60 oscillations per minute on a glass-bottomed tray. An illumination of 300 fc was provided by four parallel "White" fluorescent lamps below the tray, and the position of the flasks was changed daily to insure a uniform average illumination. The cultures were maintained at a constant temperature of 23° C.

Throughout these experiments growth of diatoms was virtually uniform in replicate flasks. When growth appeared, one flask of each series was removed from the shaker, the volume of liquid made up to 25 ml to replace that lost by evaporation, 4 drops of 10 % HCl added to dissolve any precipitate of $Ca_3(PO_4)_2$, and the light absorption measured with a Klett-Summerson photoelectric colorimeter, fitted with a ruby glass filter No. 66. Before densities were determined, clumpy suspensions were homogenized by a double passage in a Potter-Elvehjem homogenizer with a Teflon pestle. The sister flasks of the series were treated similarly after intervals of a few days. In those flasks which contained agar or agar ash, the final Klett readings were corrected by subtracting the Klett values given by the medium prior to inoculation. Growth curves were obtained by plotting Klett units against time. A Klett reading of 100 is equivalent to 1.2×10^{10} cells/l.

Silicon (as orthosilicic acid) was determined colorimetrically by a modification of the method proposed by Harrison and Storr (12). Four tenths ml of 5 % ammonium molybdate in 1 *N* H_2SO_4 was added to 4 ml of the silicate solution to form the yellow silicomolybdate complex, and 2 ml of 6 *N* H_2SO_4 were added to eliminate interference from phosphate. Reduction to molybdenum blue was effected by adding 0.4 ml of 5 % aqueous hydroquinone. Each sample was finally made up to 10 ml with distilled water, allowed to stand for 20 minutes, and the color intensity then measured photocolorimetrically. Sodium silicofluoride (Na_2SiF_6), which hydrolyzes in water to give silicic acid (15), served to provide a standard solution containing 2.34 mg Si/l.

Agar ash was prepared by incinerating samples of Bacto-Difco agar in platinum crucibles at 700° to 900° C for two hours. The ash was then added directly to H_2O or mineral medium for silicon determinations and to mineral medium for growth experiments. To remove silicon from the ash, HF was added to the sample and the mixture was heated in a platinum crucible, with additions of H_2SO_4 to convert fluorides to sulfates.

Results

Growth on Various Concentrations of Silicon: Silicon, as potassium orthosilicate, was added in a range of concentrations to both distilled water and mineral medium. Quantitative determinations of the soluble silicon were made both before and after autoclaving the solutions. When silicate was added to mineral medium approximately 17 % disappeared from solution and no longer responded to the molybdate test. On the other hand, the concentration of soluble silicon, both in water and in mineral medium, increased by about 40 % after autoclaving. *N. pelliculosa* was then grown in media containing silicon at various concentrations (fig 1). Growth was proportional to silicon content at low concentrations; 35 mg Si/l gave the best growth and 70 mg Si/l was appreciably inhibitory.

The Effect of Agar on Growth: If 0.1 % agar was added to a mineral medium containing no other source of silicon, some growth of the diatoms occurred (fig 2). Determinations of soluble silicon made on autoclaved solutions of agar in water and in mineral medium showed that 0.1 % agar added 1.5 mg Si/l to the solution. However, agar was stimulatory to growth even in the presence of adequate amounts of silicon (fig 2). It was observed that the cell suspension in the media supplemented with agar was evenly dispersed, whereas that in the media without agar tended to be clumpy. Possibly the increased viscosity of the agar medium facilitated the dispersal of the cells, and thereby promoted growth in some way.

The Effect of Agar Ash on Growth: Agar ash, added to a mineral medium containing no other source of silicon, was found to contain an inorganic factor essential for growth (fig 3). This concentration of ash (equivalent to 1.0 % agar) was found to supply 24 mg soluble Si/l to the mineral medium. The diatom growth in agar ash media was comparable to that in media containing orthosilicic acid of equivalent silicon concentrations.

After treatment with HF to remove all silicon as the volatile SiF_4, agar ash would no longer support growth. It could be shown that this was not a toxic effect caused by HF treatment, since excellent growth took place when the HF-treated-ash media were supplemented with 35 mg Si/l as orthosilicic acid (fig 3). Thus, after removal of silicon from agar ash, there was no evidence for any other inorganic factor stimulatory to growth of *N. pelliculosa*. The addition of vitamins to the medium supplemented with agar ash produced no additional stimulation of growth.

Orthosilicate as a Source of Silicon: In the colorimetric determination of silicon, ammonium molybdate reacts with orthosilicate (7) but not with colloidal silica (9, 11). By comparing the relative growth of *N. pelliculosa* in media containing various silicon preparations with the titres obtained by the molybdate test, it should be possible to determine whether the silicon taken up by diatoms for the formation of their frustules is in the form of orthosilicic acid.

Growth of N. pelliculosa on alkali-treated colloidal silica: Untreated colloidal silica ("Ludox," du Pont Inc.) does not react to give a color with ammonium molybdate, nor does it constitute a source of available silicon for growth of *N. pelliculosa*. As the alkali : silica ratio in a solution increases, the colloidal SiO_2 particles become smaller, until dispersion into SiO_4 tetrahedra is complete (5). It was therefore of in-

terest to test alkali-treated Ludox both in the molybdate reaction and for diatom growth.

The sample of Ludox used in these studies contained 38.64 % SiO_2 or 18.03 % Si. Aliquots of 1.0 ml Ludox, theoretically containing 180 mg total Si, were treated at 25° C for 3 days with KOH in various molar ratios as shown in table I. Each sample was then made up to 10 ml with distilled water, and the soluble silicon was determined with ammonium molybdate. This has been expressed as a percentage of total silicon in table I. It can be seen that only after Ludox has been treated with alkali did it give a color test with ammonium molybdate, indicating the presence of silicon in a soluble form.

Aliquots of each sample were added to mineral nutrient medium to give total silicon concentrations of

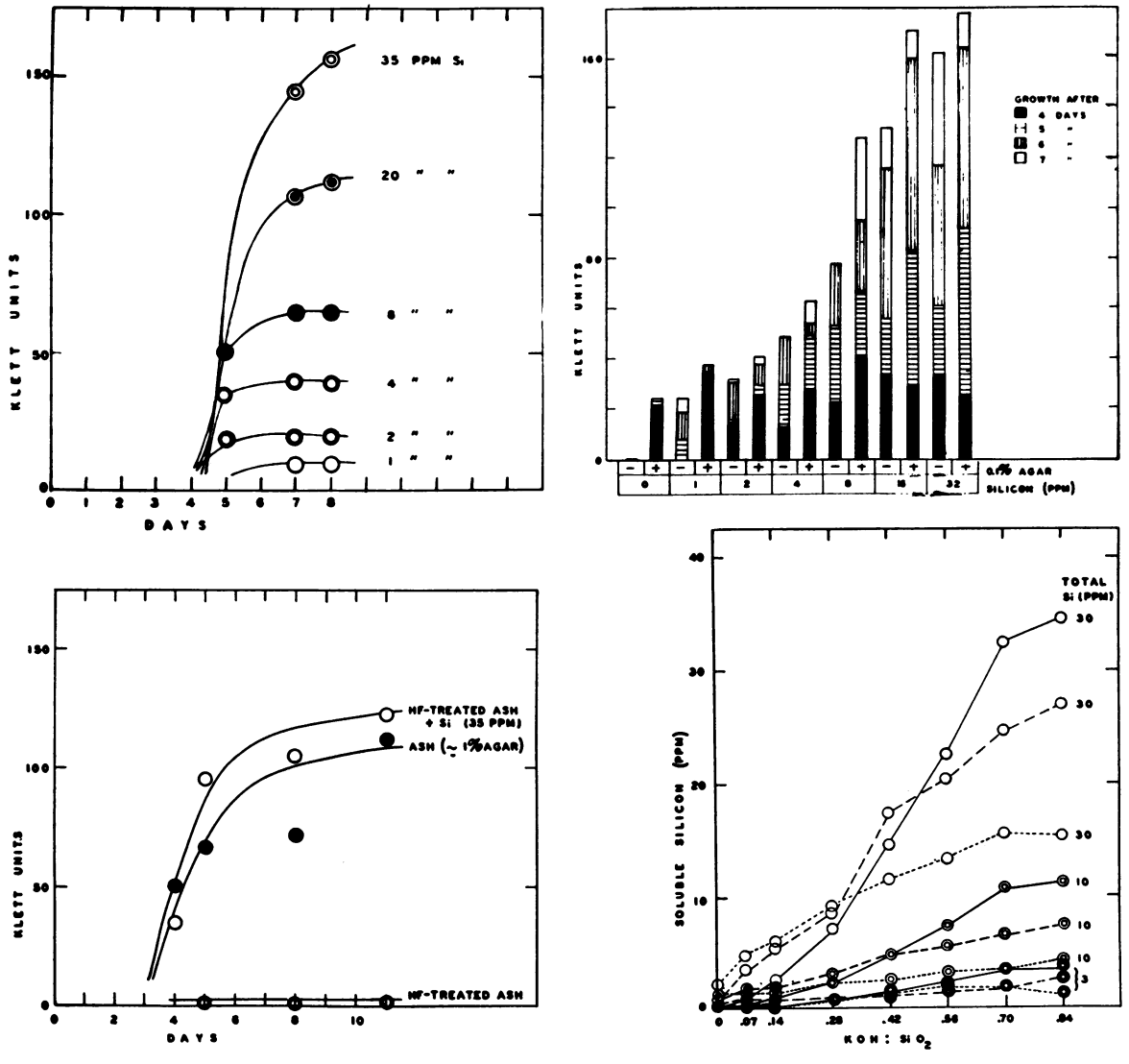

FIG. 1 (*upper, left*). Growth (cell density) of *N. pelliculosa* in media supplemented with orthosilicate. (The Si conc shown is that determined in mineral media after autoclaving.)

FIG. 2 (*upper, right*). Growth (cell density) of *N. pelliculosa* in media supplemented with orthosilicate and in the presence and absence of agar. (The Si conc shown is that determined in mineral media before autoclaving.)

FIG. 3 (*lower, left*). Growth (cell density) of *N. pelliculosa* in media supplemented with the ash of agar and HF-treated ash with and without added silicon.

FIG. 4 (*lower, right*). Soluble silicon liberated upon treatment of Ludox with various amounts of alkali. KOH : SiO_2 represents molar ratios.

Silicon determinations made on samples added to water ————

Silicon determinations made on samples added to mineral medium before autoclaving — — — —

Silicon determinations made on samples added to mineral medium after autoclaving - - - - - - - -

TABLE I

ORTHOSILICATE CONTENT OF MINERAL MEDIA AFTER ADDITION OF ALKALI-TREATED LUDOX SOLUTIONS

KOH : SiO_2 (MOLAR RATIO) EMPLOYED	% SOLUBLE SI IN AQUEOUS SOLN	TOTAL SI CONC (MG/L)					
		3.3		10.1		30.4	
		A*	B	A	B	A	B
0.00	0	0.5	0.5	1.2	1.2	0.6	2.4
0.07	3	0.8	0.5	1.9	1.2	3.6	4.8
0.14	8	..	0.6	2.1	1.5	5.4	6.0
0.28	23	0.9	0.8	3.2	2.3	8.6	9.1
0.42	48	1.3	0.9	4.7	2.5	16.9	11.5
0.56	74	1.6	2.0	5.5	3.2	20.4	13.3
0.70	110	1.8	1.9	6.5	3.6	24.7	15.5
0.84	112	2.5	1.3	7.3	4.2	27.0	15.3

* Amount (mg/l) detected after addition to mineral media. A—before autoclaving. B—after autoclaving.

3.3, 10.1, and 30.4 mg/l. The amount of silicon in true solution depended on the extent to which colloidal SiO_2 had been broken down to silicate and on the quantity of Ca silicate precipitated. The amounts of soluble silicon, based on molybdate determination, present in each solution before and after autoclaving, are shown in table I. The orthosilicate concentrations and growth of diatoms in media prepared using such treated Ludox samples are illustrated graphically in figure 4 and figure 5, respectively.

From these data it can be concluded that the more extensively the colloidal silica has been hydrolyzed by alkali, the greater is the resulting growth.

Growth of N. pelliculosa on other silicon compounds: Other compounds tested in various concentrations as sources of silicon for *N. pelliculosa* are listed in table II. Apparently only those compounds which in solution yield silicon in a form available to react with ammonium molybdate, presumably orthosilicic acid, can serve as sources of silicon for *N. pelliculosa*. The cells are not able to extract silicon from large colloidal particles such as those of Ludox, nor are they able to use a substituted orthosilicic acid in which one hydroxyl group has been replaced by a methyl group.

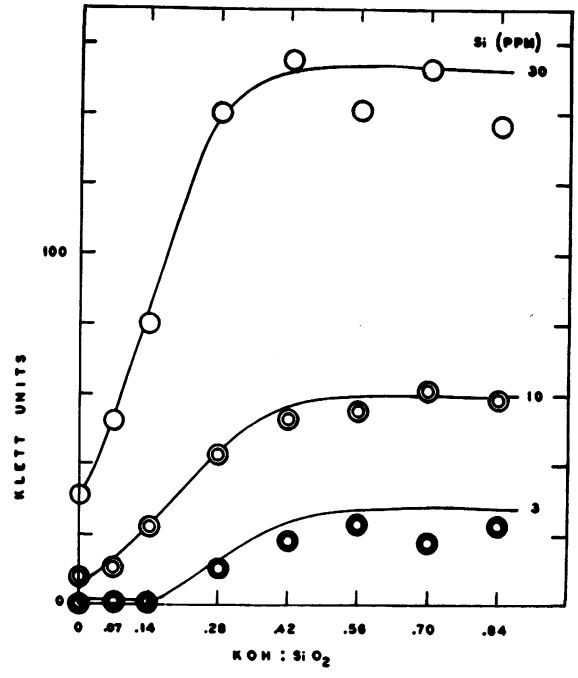

FIG. 5. Growth (cell density) of *N. pelliculosa* after 12 days in media supplemented with alkali-treated Ludox employed to give a total silicon concentration of 3, 10 and 30 mg/l. KOH : SiO_2 represents molar ratios.

DISCUSSION

Virtually all species of diatoms cultured in the laboratory on synthetic media have been reported to exhibit an absolute requirement of silicon for growth. Certainly this is true for all those investigated in this laboratory, in which 60 pure strains, representing at least 6 genera and 15 species, have been tested. Hy-

TABLE II

COMPOUNDS TESTED AS SOURCES OF SILICON FOR NAVICULA PELLICULOSA

COMPOUND	CONC	SOLUBLE SI	GROWTH AFTER 10 DAYS
	mg dry wt/l	*mg/l*	*Klett units*
Orthosilicate, $K_2O : SiO_2 = 2 : 1$ (Fisher, 423990/F33)	200	20	150
Mixture of $K_2Si_2O_5$ and $K_2Si_3O_7$ (Fisher "K_2SiO_3," C.P., P-298/510740)	200	1.4	10
"Metasilicate," $K_2O : SiO_2 = 1 : 1.5$ (Fisher)	200	18	99
Potassium silicate, $K_2O : SiO_2 = 1 : 3.3$ (Sylvania, PS-4, TT-Q-3) 32.7 % SiO_2 found	60	11	93
Ludox colloidal silica, $SiO_2 : Na_2O = 285 : 1$, 29.2 % SiO_2; found 32.7 % SiO_2 (du Pont) 38.6 % SiO_2 found	260, 26	0	0
Sodium methyl siliconate, $CH_3SiO_{1.5}$—20 %, Na_2O—9.5 % (Canadian Gen. Elec. Co., SC-50)	200, 70, 20	0	0
Sodium methyl siliconate powder, $CH_3SiO_{1.5}$—60 %, Na_2O—25.5 % (Canadian Gen. Elec. Co., 81362)	200, 70, 20	0	0

drated silica exists in a number of different forms, from large colloidal particles to the simpler dissolved silicic acid and orthosilicate ions. Although a few investigators have tested various sources of silicon for their availability to diatoms (2, 8), no attempt has been made to determine more precisely the form of silicon utilized for diatom growth. This is in part attributable to the fact that pure silicon compounds of known chemical composition are not readily available. Some of the published results are summarized in table III. It will be seen that there are many discrepancies, some of which can be explained by the various concepts of "good" growth in terms of the rate of cell division and final cell density.

At the pH of media employed in these investigations the dissolved silicon would be predominantly in the form of orthosilicic acid, or hydrated SiO_2, some of which might be slightly aggregated. The equilibrium would be shifted toward the monomer as the orthosilicate is removed from solution by diatoms. The large colloidal particles of Ludox are free neither to react with ammonium molybdate nor to be utilized for diatom growth. However, untreated Ludox depolymerized slightly in the alkaline medium (fig 4, 5) and this breakdown might be expected to continue slowly at room temperatures until finally diatom growth could occur even in media supplemented with untreated Ludox.

The amount of silicate in the medium is a vital factor controlling the density of a diatom culture when other nutrilites are present in sufficient quantities (14). Although silicon in natural waters is present at concentrations ranging from about 0.1 to 3.0 mg Si/l, it was found that very dense cultures of *N. pelliculosa*, containing approximately 10^{10} cells/l, could only be obtained when the concentration of silicon added to the medium was of the order of 15 to 30 mg/l. In most recent studies of diatom physiology, it would appear that adequate amounts of silicon have been incorporated in the nutrient media, although some of these may not have been sufficient for maximum growth. Examples of the concentrations, given as mg Si/l, employed by various workers are: 4.7 for *Navicula distans* (16), 23 for *Navicula minima atomoides* (25), 10, 14, 23 for *Nitzschia palea* (6, 13, 28), 4.5 for *Nitzschia linearis* (20), and 12 for *Asterionella formosa* (19).

Bacto-Difco agar is stated to contain 0.9 % SiO_2, equivalent to 4.2 mg silicon per gram. In the original material the silica may be present largely in the form of frustules of epiphytic and other associated diatoms. The process of incineration leaves a quantity of free alkali in the ash, which aids in the solution of these frustules. Virtually all of the silica in agar is thereby liberated in a soluble form.

Although it has been demonstrated that the stimulatory action of agar ash is to be attributed to its content of silicon in a soluble form, some residual effect in the case of agar itself (0.1 %) remains to be explained. Though agar has been shown to contain small quantities of vitamins (23, 24), it has not been possible to demonstrate any stimulating effect of vitamin mixtures on the growth of *N. pelliculosa*. Some authors (10, 28) have suggested that particles of such substances as agar or glass wool may promote diatom growth in liquid culture by providing a solid substratum, but it is difficult to understand how this would be beneficial. Agar may also act as a buffer (26) and this property may contribute to its favorable action when added to such weakly buffered media as those customarily employed for the cultivation of

TABLE III

GROWTH STUDIES OF DIATOMS

SPECIES	SOURCE OF SI	GROWTH	REF
Nitzschia palea	$CaSi_2O_5$	Best	(22)
	$K_2Si_2O_5$, $Na_2Si_2O_5$	Good	
	Glass walls of culture flasks only	Some	
Coscinodiscus excentricus	Glass walls of culture flasks only	Some	(1)
	K silicate fragments	Some	
	Precipitated Ca silicate	Some	
	Pure dialyzed silica	Some	
Nitzschia linearis	Washed kaolin, Powdered orthoclase feldspar, Pure pulverized potter's clay	Excellent	(8)
	Vitreous silica, Silica gel, Powdered glass	None	
	K or Na silicate	None (possibly conc. or pH inhibitory), (3))	
Fragilaria elliptica, *Navicula* sp., *Nitzschia palea*, *Rhopalodia gibba*, *Melosira varians*, *Nitzschia palea*, *Nitzschia sigmoidea*	$K_2Si_2O_5$	Good	(4)
Navicula sp.	Silica gel	Good (no pit formation)	
Fragilaria elliptica	Silica gel	Good (pit formation noted)	
Nitzschia palea	Glass walls of culture flasks only	Poor	(2)
	Agar	Excellent	
	Precipitated SiO_2	Good	
	Powdered quartz, Powdered opal	Some	
Nitzschia palea, *Navicula minuscula*	Nacrite crystals	Good, dissolution of crystals noted	(27)
Nitzschia palea	Na_2SiO_3 (impure)	None	(13)
	Na_2SiO_3 (pure)	Excellent	

algae. Finally, the favorable effect of small quantities of agar on the growth of diatoms may be attributable in part to its action as a dispersing agent. Wetter (29) has found that 0.1 % agar promotes growth of *Aspergillus niger* owing to better nutrient conditions for the conidia resulting from their homogeneous dispersion in a medium of higher viscosity.

Summary

Navicula pelliculosa (Bréb.) Hilse has an obligate requirement for silicon. Growth is proportional to soluble silicon at low concentrations, and is most dense in media containing about 35 mg Si/l. Since the addition of 1.0 % agar supplies 1.5 mg soluble Si/l to the nutrient medium, such media can support some diatom growth in the absence of any other source of silicon. The ash of agar, employed at a concentration equivalent to 0.1 % agar, supplies 24 mg soluble Si/l and thus supports good diatom growth. By treatment with HF this silicon can be removed from agar ash, which thereby loses its essential growth factor. When 0.1 % agar is added in the presence of adequate amounts of silicon, there is some acceleration of growth, possibly attributable to its action as a dispersing agent.

Orthosilicates, which react with ammonium molybdate, serve as suitable sources of silicon for growth of *N. pelliculosa*. Colloidal silica (Ludox) neither reacts with ammonium molybdate nor supports diatom growth unless it has first been depolymerized with alkali.

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