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ISOLATION OF SILICATE IONOPHORE(S) FROM THE APOCHLOROTIC DIATOM NITZSCHIA ALBA

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SUMMARY An organic extract of Nitzschia alba cells possesses ionophoretic activity towards silicate, as it induces silicate transport across an organic phase or across synthetic lipid membranes. The activity is dependent upon Na^+ and prefers silicon to germanium, a congener. The activity can be resolved into two apparently pure fractions by a combination of high performance liquid chromatography and thin-layer chromatography. Preliminary characterization indicates that the compound(s) contains vicinal hydroxyl groups but is devoid of amino, sugar or phosphate groups.

Since the report of the isolation of a Na^+/K^+ ionophore from beef heart mitochondria by Blondin et al. (1), interest in natural ionophores has increased and several ionophoretic compounds have been isolated from organelles as well as from purified active transport proteins (2,3). It has been postulated that these compounds are part of the active transport systems (e.g., the binding sites) or that they act in conjunction with the carrier protein (3,4). It has previously been shown that diatoms possess an inducible active transport system for silicate (5-7), and in the apochlorotic marine diatom, Nitzschia alba, silicate transport occurs by symport of $Si(OH)_4$ with Na^+(7). The mechanism by which Na^+ interacts with the silicate transport system is still unclear. The present paper reports the isolation, purification and characterization of two ionophoretic coumpounds in N. alba; such information will be useful in understanding the "silicate pump" and the mechanism of silicate transport.

MATERIALS AND METHODS

Growth and harvest of cells. N. alba cells were grown to late log phase ($7\text{-}8\times10^5$ cells/ml; growth stops at 10^6 cells/ml) in a synthetic sea water medium (8), and harvested at 23°C by centrifugation at 3,000 x g for 3 min. Because the yield of the ionophore(s) was very low, cells were grown on a

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large scale in 80 liter medium in 10 carbuoys (10 liter capacity) at 30°C, aerated by vigorous stirring on magnetic stirrers. Cells were then washed in medium-free of silicate, resuspended to $2x10^6$ cells/ml in the same medium without any additional silicate and incubated for 3 h at 30°C, resulting in silicate starved cells that were then harvested and stored at -80°C until used.

Preparation of lipid vesicles. Lipid vesicles were prepared by the reverse-phase evaporation method (10). 25 mg egg lecithin (Calbiochem-Behring) in 0.25 ml chloroform solution was dried under nitrogen and dissolved in 12 ml diethyl ether to which was added 4 ml saline buffer (12.7 mM NaCl, 0.24 mM KCl, 0.64 mM K-phosphate, pH 7.0). The solution was sonicated in a bath sonifier (Ultrasonic Industries, Inc.) at 4°C until the two layers were thoroughly mixed, after which it was evaporated under suction with vigorous stirring in a rotavapor (Buchler Instruments) for 30 min. The resulting suspension was dialyzed overnight at 5°C against 1 liter of the same saline buffer and redialyzed in a fresh dialysis bag against fresh saline buffer for 4 h and stored in ice until use the same day. For preparation of $Ge(OH)_4$-loaded vesicles, 1 mM $Ge(OH)_4$ with 0.5 μCi [^{68}Ge] was added before sonication and evaporation. For $Si(OH)_4$ uptake assay vesicles were prepared by replacing saline buffer with KCl buffer (12.94 mM KCl and 0.64 mM K-phosphate, pH 7.0).

Assay for the ionophoretic activity. (a) Bulk-transport of $Ge(OH)_4$. Ionophoretic activity was assayed by a slight modification of the Bulk-transport method (9) using [^{68}Ge] (a congener of silicon) as a tracer. One ml each of three solutions was layered in a 13 mm x 100 mm siliconized glass test tube as follows: the bottom layer consisted of 60% sucrose, 100 mM NaCl, 10 mM Tris-HCl (pH 7.0) and 1 mM $Ge(OH)_4$ in double distilled water with 5 μCi [^{68}Ge] (New England Nuclear); the middle layer contained 49% CCl_4 and 51% heptane with the ionophoretic activity in solution; the top layer contained 10 mM NaCl, 10 mM Tris-HCl (pH 7.0). To retard oxidation, 1 mM dithiothreitol was added to each layer and the tube was placed in a water bath at 23°C and stirred with a small magnetic stir bar. Samples were periodically withdrawn from the top layer for counting in a scintillation counter in a cocktail made of toluene and Triton X-100 (2:1) and 4 g/liter omnifluor (New England Nuclear). For assay of Na^+ transport, the NaCl component in the layers was replaced by KCl, and in the bottom layer 1 mM $Ge(OH)_4$ was replaced by 1 mM NaCl while 5 μCi [^{68}Ge] was replaced with 1 μCi [^{22}Na] (New England Nuclear).

(b) Silicate uptake by lipid vesicles. Because [^{31}Si] decays rapidly (half-life 156 min), it is not suitable for use in the Bulk-transport method. For assaying the ionophoretic activity with [^{31}Si], therefore, synthetic lipid vesicles were used. The uptake reaction was started by adding 0.2 ml saline buffer (12.7 mM NaCl, 0.24 mM KCl, 0.64 mM K-phosphate, pH 7.0) containing 6 mM Na_2SiO_3 solution with ca. 15 μCi [^{31}Si] prepared as described before (5) and up to 20 μl dimethylsulphoxide (DMSO) solution of the ionophoretic extract to 0.2 ml of lipid vesicles. After 3 min incubation at room temperature (23°C), the mixture was cooled in ice, immediately passed through a 7 mm x 100 mm Sephadex G 75 column, and washed with 8 ml cold saline buffer. Four drops per fraction were collected and counted in a scintillation counter. The column excludes vesicles while retarding free silicate. Radioactivity eluting with the vesicles is considered uptake by the vesicles.

(c) $Ge(OH)_4$ release from lipid vesicles. The ionophore-induced release of $Ge(OH)_4$ (using [^{68}Ge] as tracer) from preloaded lipid vesicles proved both to require the least amount of ionophore and to be most sensitive assay. Up to 20 µl of DMSO solution of the ionophore was added to 0.2 ml preloaded vesicles, diluted with 1.8 ml KCl buffer (12.94 mM KCl and 0.64 mM K-phosphate, pH 7.0) and incubated for 5 min at 23°C. The mixture was then cooled in ice and immediately centrifuged at 70,000 x g for 20 min in an ultracentrifuge and the supernatant quickly removed for counting.

Extraction and purification of the ionophoretic activity. Frozen log phase cells were resuspended in 2 vol (200 ml/100 g wet wt cells) of 50 mM K-phosphate buffer (pH 7.0), and extracted overnight by 3.75 vol chloroform: methanol (1:2) with stirring. Cells were then filtered through Whatman filter paper No. 1 on a Buchner funnel by suction and resuspended in 2 vol distilled water; residual $CHCl_3$ was removed from the suspension by vacuum distillation followed by bubbling with N_2 for 1 h. If necessary, the suspension was restored to original volume with water and after the pH was brought to 11 by NaOH, it was incubated at 23°C for 5 min. HCl was added to readjust the pH to 8.5, trypsin (Type III, Sigma Chemical Co.) was added at mg/ml, and the cell suspension was incubated at 23°C for 4 h. The incubation was stopped by adding conc. HCl to pH 3 and the suspension was extracted with 3.75 vol chloroform:methanol (1:2) overnight at 23°C with continuous stirring and bubbling with N_2. The cells were filtered, 1.25 vol of water and of chloroform were added to the filtrate and vigorously stirred in a magnetic stirrer for 2 h. The suspension was then allowed to separate in a separatory funnel. The chloroform layer containing the activity was evaporated to dryness in a rotary evaporator under reduced pressure at 25-30°C, and the activity in the dried residue was extracted with absolute ethanol. Distilled water was added to bring the solution to 87% alcohol and the mixture, in small portions, was shaken with petroleum ether (b.p. 40-60°C) to remove neutral lipids. The alcoholic layers containing the activity were pooled, evaporated to dryness, and the residue dissolved in methanol. This methanolic extract was used for the ionophore assay in Fig. 1 and was further purified according to the following scheme.

(i) The methanolic solution of the extract was injected into a 25 cm x 10 mm Sepralyte C_{18} reverse-phase column (5 µ, Analytichem International) and eluted 2 ml/min with a gradient of methanol (MeOH) in water (starting with 50% MeOH, rising to 100% in 45 min). The high performance liquid chromatography (HPLC) system consisted of a solvent delivery system (Spectra-Physics SP8700), computing integrator (System I, Spectra-Physics), a variable wavelength monitor (Spectra-Physics SP8400). Two regions of activity are eluted at 20-28 min and 38-50 min, respectively.

(ii) The active fractions were concentrated and run on the same column isocratically. Active fraction I was eluted with 55% MeOH at 15-18 min while active fraction II was eluted with 85% MeOH at 11-13 min.

(iii) Each of the two fractions were resolved into 6-10 components by thin-layer chromatography on cellulose plates (Eastman Chemicals) with hexane:ethyl acetate:methanol:acetic acid (60:20:20:1). Spots were detected by UV and iodine vapors. Fraction I stayed on the origin and fraction II gave an R_f of 0.7.

(iv) Active spots were eluted from the chromatogram by chloroform:methanol (1:1) and concentrated by evaporation. Each of the two fractions could then be resolved into only two components on further TLC using different combinations of solvents. Fraction I gave an R_f of 0.3 in ethyl acetate:methanol: acetic acid (80:20:1) and fraction II gave an R_f of 0.25 in dioxane:acetonitrile:acetic acid (50:40:1).

(v) When run on a 25 cm x 4.6 mm analytical column (Whatman Partisil 5 ODS-3 C_{18}, reverse-phase), each fraction gave a single major peak.

RESULTS AND DISCUSSION

Silicate ionophoretic activity in N. alba cells. The ionophoretic activity of the methanolic extract is demonstrated by its ability to induce $Ge(OH)_4$ transport across an organic phase as well as by its ability to induce $Si(OH)_4$ uptake in synthetic lipid vesicles (Fig. 1). In the Bulk-phase transport assay, virtually no $Ge(OH)_4$ is transported in the absence of the extract, whereas addition leads to a concentration-dependent linear transport

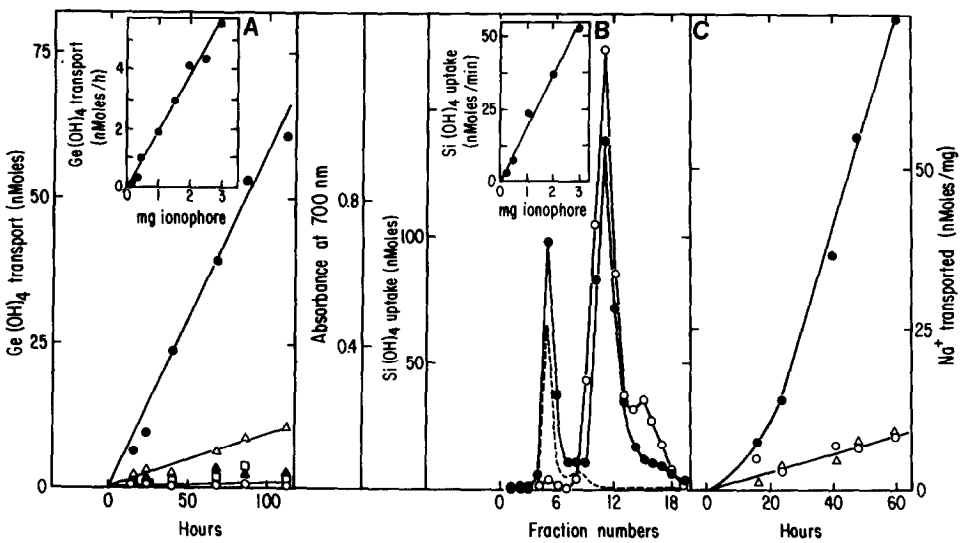

Fig. 1. Germanate, silicate and Na^+ ionophoretic activity in an organic extract of Nitzschia alba cells. A. Bulk-transport of $Ge(OH)_4$:○ , no addition; △ , 1 mg extract; ● , 3 mg extract; ▲ , 3 mg extract but no Na^+, NaCl component in the assay system (MATERIALS AND METHODS) was replaced by KCl at the same concentrations; □ , 3 mg extract + 200 μM silicate. Inset shows the rate of $Ge(OH)_4$ transport as affected by different concentrations of the extract. $Ge(OH)_4$ transport was measured for 20 h in the presence of indicated amounts of the extract. B. $Si(OH)_4$ uptake by lipid vesicles: --- turbidity; ○ , no addition; ● , +3 mg extract. Inset shows the rate of $Si(OH)_4$ uptake by lipid vesicles as affected by different concentrations of the extract. Uptake was measured for 3 min in the presence of the indicated amouunts of the extract. C. Bulk-transport of Na^+:○ , 500 μM silicate; ● , 500 μM silicate + 1 mg extract; △ , 1 mg extract.

(Fig. 1A). Increasing the amount of the extract increases the rate of the transport (Fig. 1A, inset). Ge (a congener of silicon) can mimic silicon and is transported by the same transport system though silicate is the preferred substrate (5,7). This is true for the ionophore-induced $Ge(OH)_4$ transport system, as Bulk-transport of 1 mm $Ge(OH)_4$ is inhibited by 200 μM silicate. Apparently the ionophoretic activity of the extract is 5 times more specific for silicate than for germanate. As transport of $Ge(OH)_4$ is insignificant in the absence of Na^+, Na^+ is required for ionophoretic activity towards silicate. The converse is also true, as the extract induces Na^+ transport across an organic phase in the presence of silicate (Fig. 1C). The ionophore may have binding sites for both Na^+ and silicate, and binding of either Na^+ or silicate to the ionophore may facilitate the binding of the other. Or it might be that while the ionophore may have excellent binding capacity for either Na^+ or silicate, for successful ionophoretic activity, both Na^+ and silicate must be bound to the molecule simultaneously. The silicate transport system in N. alba is Na^+-dependent and occurs by symport of $Si(OH)_4$ with Na^+ (7). The Na^+-dependence of the silicate ionophoretic activity might indicate that the Na^+-dependence of the silicate transport system resides in the binding site. This strongly suggests a relationship between the silicate transport system and the ionophoretic activity. Further work is necessary to firmly establish this point.

Optimization of the ionophoretic activity. Log phase cells, when starved for silicate, show 6 times more ionophoretic activity than do untreated cells (Table 1). As silicate starvation is necessary for full manifestation of the silicate transport activity in the cells, it indicates that the ionophoretic compound(s) may be a component of the transport system. For maximum release of the ionophoretic activity, cells must be treated with trypsin before extraction. This may mean that the ionophoretic compound(s) is linked covalently to some component in the cells, possibly the membrane. However, it may be that the active component is free in the membrane or the cells but perturbation in the membrane by trypsin is necessary for extraction. Activity increases about twofold when a few drops of con. HCl are added to the extracting solvents (e.g., chloroform:methanol 1:2). This treatment increases the

Table 1. Effect of treatments on the ionophoretic activity in N. alba

Treatment	$Ge(OH)_4$ Bulk-Transport pMoles/mg/h
Control[a]	196
Non-starved[b]	34
Non-trypsinized[b]	55
HCl omitted before extraction[b]	76

[a]Ionophoretic extract was prepared as described in MATERIALS AND METHODS which included chloroform:methanol (1:2) extraction after HCl was added to trypinsized delipidated silicate-starved cells.

[b]Conditions of ionophore preparation was same as in "a" except the particular step as mentioned was omitted.

extractability of lipids from cells, possibly by inducing more perturbation in the membrane (11).

Purification of the ionophoretic activity. When delipidated cells are treated with trypsin and extracted with chloroform and methanol (1:2), the activity appears in the chloroform phase (MATERIALS AND METHODS). This excludes simple lipids as the ionophoretic principles. The chloroform and methanol extract of the delipidated cells contains more than 20 different compounds as revealed by HPLC (Fig. 2). The active compounds comprise a small fraction of the extract (less than 5%) and reside in two well-separated regions that can be further resolved in 6-10 compounds; the active principle in each fraction is a chromatographically (HPLC and TLC) pure compound. Typically, 100 g wet wt cells yields ca. 45 µg compound I and 70 µg compound II. In the Bulk-transport assay, purified compound I shows a specific activity of 9.1 nMoles $Ge(OH)_4$ transported/mg/h, and compound II has a specific activity of 7.1 nMoles $Ge(OH)_4$ transported/mg/h, whereas the crude extract has a specific activity of only 196 pMoles/mg/h.

These experiments demonstrate a Na^+-dependent silicate ionophoretic activity in N. alba for which two distinct and chromatographically pure compounds may be responsible. Preliminary characterization revealed that both compounds may contain vicinal hydroxyl groups since they react positively with Schiff reagent (12), only after periodate treatment. Tests for sugar with α-napthol, phosphate by Hack and Ferrans procedure and free amino groups with ninhydrin (12) were all negative with either compounds. Both HPLC and TLC

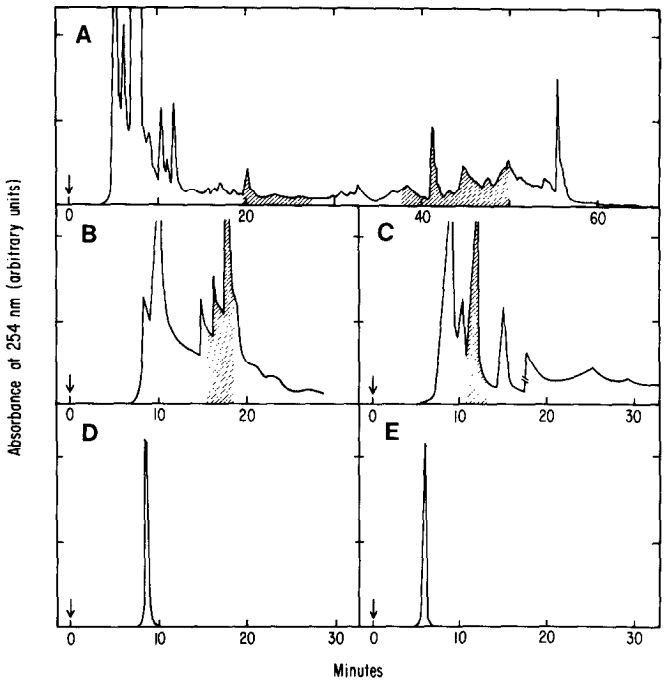

Fig. 2. Purification of the ionophoretic activity in an organic extract of N. alba cells by high performance liquid chromatography. A. HPLC of the crude extract. 15 mg of the crude extract in 50 µl methanol was applied to the 25 cm x 10 mm Sepralyte C_{18} column and eluted with a gradient of methanol and water starting at 50% methanol and changing to 100% methanol at the rate of 1.11% per min. The shaded areas contain the ionophoretic activity. B. HPLC of the first active fraction eluting from the column at 20-28 min in A. The shaded area represents the active fraction. C. HPLC of the second active fraction eluting from the column at 38-50 min in A. The shaded area represents the active fraction. D and E. HPLC of the purified compounds I and II. 20 µl of each of the purified compounds in methanol was injected to a 25 cm x 4.6 mm Whatman ODS-3 Partisil 5 analytical column and eluted with 55% (D) and 85% (E) methanol, respectively.

chromatographic behavior indicate that the two compounds are different and that one is more polar than the other. For example, in HPLC, when a 25 cm x 10 mm Sepralyte C_{18} column is eluted with a gradient of methanol and water, compound I is eluted at ca. 69% methanol while compound II is eluted at 92% methanol. In TLC in the solvent system ethyl acetate:methanol:acetic acid (80:20:1), compound I gives an R_f of 0.3 and compound II gives an R_f of 1.0; in the solvent system dioxane:acetonitrile:acetic acid, compound I gives an R_f of 0 and compound II an R_f of 0.25. However, one or both compounds may be degradation product(s) or precursor(s) of a natural component in the cells, or one may be a break-down product of the other. The biological significance of

the presence of the ionophores in N. alba is not fully understood yet. One or both may be involved in the active transport of silicate as a part of the carrier protein (e.g., the binding site) or may act in conjunction with the carrier, much the way periplasmic proteins are involved in some transport systems (13). On the other hand, either or both may be involved in cytoplasmic silicate transport as silicate carriers from the site of entry, the cytoplasmic membrane, to the site of utilization, i.e., silica deposition vesicles involved in the diatom wall formation (14) or may act as sequestering agents to retain and regulate the silicon-pool in the cell required for wall formation and the other silicon-dependent cellular processes.

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