SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 15, No. 9, pp. 1577–1583, 1996

Printed in the USA
0730-7268/96 $6.00 + .00

THE INFLUENCE OF pH AND MEDIA COMPOSITION ON THE UPTAKE OF INORGANIC SELENIUM BY *CHLAMYDOMONAS REINHARDTII*

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(*Received* 2 *January* 1996; *Accepted* 20 *March* 1996)

Abstract—The uptake of inorganic selenium species, selenate and selenite, by the green alga *Chlamydomonas reinhardtii* Dang was examined as a function of pH over the range 5 to 9 and in media with varying concentrations of major ions and nutrients using ^{75}Se as a radiotracer. Little difference was noted in the uptake of selenate as a function of pH, with the maximum uptake occurring at pH 8; however, selenite uptake increased substantially at the lower pH values. Selenate uptake was significantly decreased by higher sulfate concentrations and increased significantly by calcium, magnesium, and ammonium. Selenite uptake was significantly increased when the phosphate concentrations in the media were reduced. The results of these experiments demonstrate that varying water chemistry may significantly affect the uptake of inorganic selenium by phytoplankton and the subsequent transfer of the selenium to higher trophic levels.

Keywords—Selenium Phytoplankton uptake pH Sulfate Phosphate

INTRODUCTION

As a result of man's activities, selenium has been found to be an environmental problem in a number of terrestrial and freshwater systems in recent years. Power generation [1,2] and agricultural subsurface drainage water [3,4] have been implicated in the release of harmful levels of selenium to the environment. Sewage sludge and petrochemical refining are also significant sources of selenium [5,6]. Selenium has even been added to lakes to ameliorate high concentrations of mercury [7].

Selenium is found in aquatic systems in a variety of forms. Two inorganic species are common, selenite, Se(IV), and selenate, Se(VI) [5]. Selenite is the predominant form released by the power generation industry [5]; in agricultural drainage selenate is the most common form [8]. In addition, there are a plethora of organic selenium species, including selenium-containing amino acids and peptides, and methylated species, such as dimethyl selenide, dimethylselanone, and dimethylselenoxide [9,10].

Regardless of the original source, adverse environmental effects appear to result largely from transfer of selenium from lower to higher trophic levels. In the case of power generation, game fish populations have suffered reproductive failure after bioaccumulation of selenium from concentrations of about 10 μg/L dissolved selenium [1]. Dietary intake is the major source for uptake by fish [2,11]. Reproduction of several wetland bird species has been affected by mortality, gross malformations, and internal abnormalities of the young [3,12] in areas where high selenate concentrations (up to 350 μg/L) were found in ponds receiving agricultural drainage. As in lakes affected by selenite, dietary intake is believed to be the source of selenium [12].

Because of the importance of diet in the environmental effects of selenium, understanding the role of lower trophic levels in the uptake of selenium from inorganic forms is critical. One of the main routes from inorganic selenium in water to higher organisms is via uptake by phytoplankton, followed by further passage up the food chain [13,14] (other pathways include uptake of selenium from sediment by benthic organisms [11] and accumulation from the water by bacteria and microzooplankton [15]). Phytoplankton and periphyton accumulate both Se(IV) and Se(VI) [13,14,16], and zooplankton and benthic infauna are known to assimilate selenium from phytoplankton with unusually great efficiency [13,14,17,18], facilitating the passage of selenium to higher trophic levels. Efforts to model selenium biogeochemistry, bioaccumulation, and effects in contaminated lacustrine systems are currently being carried out [19].

It is quite common for the uptake of nutrients or trace elements by phytoplankton to be coupled to the transport of major ions. The active uptake of many ions is achieved through symport or antiport of H^+, Na^+, or K^+ [20]. In other cases, ions may be in competition for uptake at a transport site which will accept either ion. For example, the uptake and toxicity of chromate ion to phytoplankton is controlled by the concentration of sulfate in the growth medium; higher sulfate concentrations reduce the uptake and toxicity of chromate to phytoplankton [21]. Similarly, arsenate uptake and effects are dependent on phosphate concentration [22]. Therefore, it is possible that the composition of the medium will lead to significant variations in the uptake of selenium to phytoplankton and bioaccumulation in higher trophic levels. Thus, the wide variation in the composition of freshwaters worldwide could produce significant differences in selenium behavior and effects between different systems. The purpose of this study was to examine the possible influences of pH and medium composition on the uptake of selenium by a representative phytoplankter, the green alga *Chlamydomonas reinhardtii.*

METHODS

Algae and culturing

Chlamydomonas reinhardtii Dang (UTEX 89) was obtained from the University of Texas culture collection [23]. The culture

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Table 1. Composition of the growth medium used to culture and as the basis for experimental variations (modified from WC media [22])

	mg/L	μM
Major salts		
$CaCl_2 \cdot H_2O$	32.24	250
$MgSO_4 \cdot 7H_2O$	36.96	150
$NaHCO_3$	25.20	300
K_2HPO_4	8.71	50
$NaNO_3$	85.01	1,000
$Na_2SiO_3 \cdot 9H_2O$	28.42	100
KBr	0.12	1
NaF	0.42	10
Trace elements		
FeEDTA[a]		11.7
$CuSO_4 \cdot 5H_2O$	0.01	0.04
$ZnSO_4 \cdot 7H_2O$	0.022	0.08
$CoCl_2 \cdot 6H_2O$	0.01	0.05
$MnCl_2 \cdot 4H_2O$	0.18	0.90
$Na_2MoO_4 \cdot 2H_2O$	0.006	0.03
H_3BO_3	0.62	0.16
Vitamins and buffer		
Thiamin·HCl	0.1	
Biotin	0.0005	
B_{12}	0.0005	
HEPES[b]		2,500

[a] EDTA = ethylenediaminetetraacetic acid.
[b] HEPES = *N*-[2-hydroxyethyl]piparazine-*N*′-(2-ethanesulfonic acid).

was maintained in a variation of the freshwater medium WC [24], here denoted as HYCO (Table 1). With the exception of the pH experiments noted below, all cultures were buffered at pH 7.0 with 2.5 mM *N*-[2-hydroxyethyl]piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) [25]. The culture was axenic when obtained and cultured with sterile techniques; however, tests for subsequent bacterial contamination were not conducted.

Selenium uptake measurements

Selenium uptake measurements were carried out using ^{75}Se as a radiotracer. The details of isotope preparation and determination have been previously described [16]. For all the experiments reported here, concentrations of ^{75}Se added were 3 to 10 μg/L. For both selenate and selenite, these concentrations are within a range where uptake is approximately proportional to concentration. Selenate uptake versus time is virtually linear over 24 h; however, selenite uptake is initially very rapid and reaches a plateau near 4 to 6 h [16]. Therefore, for the study of pH on selenium uptake and the initial screening study of the effect of ions on selenium uptake, experiments were carried out using short term (4 to 6 h) incubations. In short-term experiments, uptake was examined in three 25-ml cultures, two live and one heat-killed at 70°C to check for adsorptive uptake [16]. The cultures were incubated in a 25°C incubator and then filtered onto 0.4-μm polycarbonate filters using three 5-ml rinses with buffered medium to remove unincorporated ^{75}Se. For the second study of the effects of medium composition on uptake, cells were inoculated at a low level into the media and allowed to grow over several days. Once cell densities had passed 1×10^5 cells/ml, samples from the cultures were filtered and rinsed as above to determine selenium uptake. Because of the low initial cell densities, heat-killed controls were not used.

Effects of pH on selenium uptake

The base HYCO medium was prepared at pH 5.0, 6.0, 7.0, 8.0, and 9.0 using 2.5 mM HEPES or 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer [25] (MES for pH 5.0 and 6.0; HEPES for pH 7.0, 8.0, and 9.0). *Chlamydomonas reinhardtii* was grown in medium buffered at pH 7.0 with 2.5 mM HEPES to a cell density of 1.9×10^5 cells/ml. For each pH tested, 80 ml of the *C. reinhardtii* culture was added to 320 ml of buffered medium (for a final cell density of 3.8×10^4 cells/ml), and the pH adjusted with dilute HCl and/or NaOH. From each different pH, six 50-ml test tubes received 25 ml of the buffered medium. Of the six, two tubes were heat-killed. One was used as a control for absorptive selenate uptake, and one for selenite uptake at each pH. The remaining tubes were used to determine selenate and selenite uptake by live cells (2 each). Selenium additions were 10 μg Se/L as selenate and selenite. Incubations lasted 6 h.

Effects of medium composition on selenium uptake—screening

Variations of the base medium were made up as follows: (1) control, (2) +1,000 μM Na_2SO_4, (3) +1,000 μM $CaCl_2$, (4) +1,000 μM $MgCl_2$, (5) +1,000 μM KCl, (6) +1,000 μM NaCl, (7) +500 μM NH_4Cl, (8) +1,000 μM $NaHCO_3$, (9) +500 μM Na_2SiO_3, (10) −KNO_3 (no added KNO_3), and (11) −K_2HPO_4 (no added K_2HPO_4). *Chlamydomas reinhardtii* was grown in 2 L of pH 7.0 HEPES-buffered HYCO medium to a cell density of 2.5×10^5 cells/ml. For each treatment above, 12 50-ml Pyrex® test tubes were prepared containing 20 ml of medium. Six tubes were used for selenate uptake and six for selenite. For each form two selenium concentrations, 3 and 10 μg/L, were tested. Treatment tubes were prepared by adding 5 ml of the culture to the 20 ml of prepared medium with isotope (final cell density = 5.0×10^4 cells/ml). Incubations lasted 4 h.

Both selenate and selenite results were analyzed by two-factor (treatment × Se concentration) analysis of variance (ANOVA) [26], and all treatments contrasted to the controls using Dunnet's test [27] to control the experimentwise error rate at 0.10. Based on the results of this experiment, a more detailed examination of the effects of a few constituents was carried out.

Effects of medium composition on selenium uptake—detailed examination of major effects

Batches of HYCO medium were made up with the following deletions: none (control), −$CaCl_2$, −$MgCl_2$, −Na_2SO_4, −K_2HPO_4, and −Na_2SiO_3. Twenty Pyrex 50-ml culture tubes were prepared with 30 ml of each medium. Treatment series were made in quadruplicate by adding substances back to the media from which deletions had been made, as follows: (1) control: +100, 200, 500, 1,000, or 2,000 μM NaCl; (2) −$CaCl_2$: +50, 100, 200, 500, or 1,000 μM $CaCl_2$; (3) −Na_2SO_4: +50, 100, 200, 500, or 1,000 μM Na_2SO_4; (4) −K_2HPO_4: +5, 10, 20, 50, or 100 μM K_2HPO_4; and (5) −Na_2SiO_3: +10, 20, 50, 100, or 200 μM Na_2SiO_3.

In addition, eight tubes containing the base medium were prepared to be used as controls. The addition series for $CaCl_2$, NaCl, and Na_2SO_4 received duplicate additions of [^{75}Se]selenate at 3 and 10 μg/L at each treatment concentration, while the addition series for K_2HPO_4 and Na_2SiO_3 received duplicate additions of [^{75}Se]selenite at 3 and 10 μg/L at each treatment concentration. Because of suspicions that a precipitate in the silicate addition treatment in the previous experiment may have led to an artifact in selenite uptake, we made the following changes: all treatment media were made up a week in advance to allow the dissolution of any precipitates formed during the sterilization of the media, and the maximum concentration of

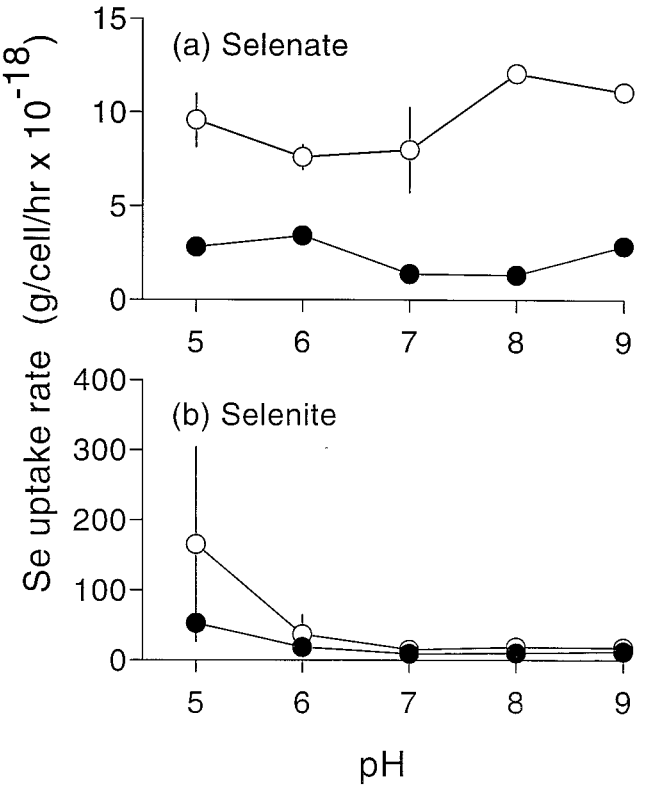

Fig. 1. The effect of pH on the uptake of inorganic selenium by *Chlamydomonas reinhardtii*. Incubation time, 6 h. (**a**) 10 μg Se/L selenate. (**b**) 10 μg Se/L selenite. Results for live cells show mean ± SD (n = 2) ○ = live cells, ● = heat-killed cells.

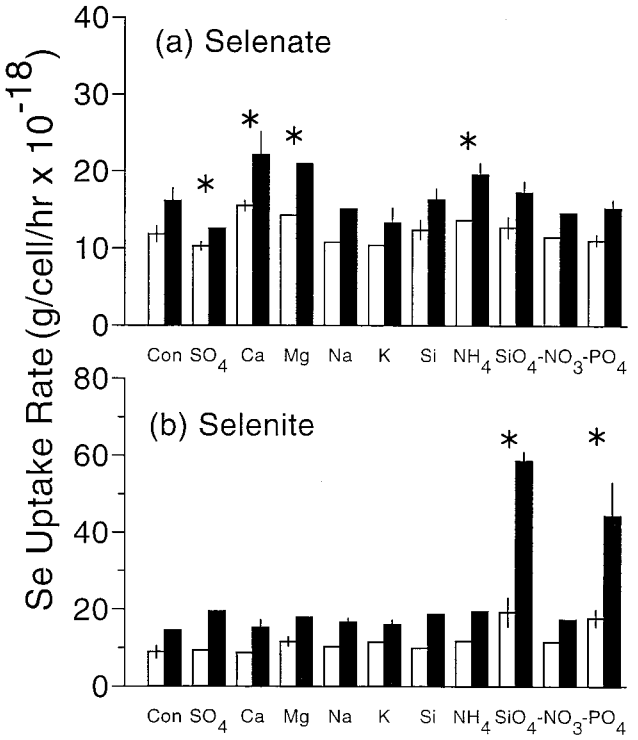

Fig. 2. Results of the initial screening for effects of media composition on inorganic selenium uptake. Incubation time, 4 h. (**a**) Selenate. (**b**) Selenite. Results for live cells show mean ± SD (n = 2). * = treatments statistically different from control by Dunnet's test ($p <$ 0.10, experimentwise error), open bars = 3 μg/L ^{75}Se additions, filled bars = 10 μg/L ^{75}Se additions.

silicate used was reduced from 1,000 μM to 200 μM. The eight control cultures were divided into four pairs, which received 3 and 10 μg/L of [^{75}Se]selenate or selenite.

Chlamydomonas reinhardtii was grown in pH 7.0 HEPES-buffered medium to a cell density of 5×10^5 cells/ml. Each treatment tube was spiked with 0.06 ml of culture, for an initial cell density of 1×10^3 cells/ml. Treatment and control tubes were incubated in a 25°C incubator with a 12 h light : 12 h dark cycle. Cell growth was monitored daily using in vivo fluorescence, resuspending each tube with a vortex mixer, and inserting the tube directly into a Turner Designs fluorometer. Cell densities in the treatment tubes were calculated from in vivo fluorescence from the ratio of cell density determined by cell counts to in vivo fluorescence of cells in extra control tubes without added ^{75}Se. Once cell densities reached approx. 1×10^5 cells/ml, 20- to 30-ml samples were filtered for determination of selenium uptake.

RESULTS

Effects of pH on selenium uptake

The uptake of selenate by *Chlamydomonas* was not strongly affected by pH (Fig. 1a). The average uptake rate of living cells varied from about 7.5×10^{-18} g Se/cell per h at pH 6.0 and 7.0 to 12×10^{-18} g Se/cell per h at pH 8.0. Sorptive uptake by heat-killed cells varied from 1.4×10^{-18} g Se/cell per h at pH 7 to 3.5×10^{-18} g Se/cell per h at pH 6.0. The variations across pH appeared not to be systematic.

The uptake of selenite was strongly pH-dependent at the lower pH values (Fig. 1b). At pH 7.0 or above, the average uptake rate was 17×10^{-18} g Se/cell per h. At pH 6.0 uptake doubled to approx. 37×10^{-18} g Se/cell per h, and at pH 5.0 average uptake had risen to 167×10^{-18} g Se/cell per h. Except for pH 5.0, selenite uptake by heat-killed cells was approximately half that of uptake by live cells.

Effects of medium composition on selenium uptake—screening

Mean control uptake rate for selenate in the live control treatment was 12×10^{-18} g Se/cell per h in 3 μg/L selenate and 16×10^{-18} g Se/cell per h at 10 μg/L selenate. Uptake rates of selenate by heat-killed algae were less than 10% of the rate by living algae. With selenite, the control uptake rate of living cells was 8.8×10^{-18} g Se/cell per h at 3 μg/L and 14.5×10^{-18} g Se/cell per h at 10 μg/L. Uptake of selenite by heat-killed cells varied from about 10 to 50% of live uptake, except for the added silicate treatment, where the rate for heat-killed algae was 88% of the rate of living algae for both 3 and 10 μg/L.

Only slight effects on selenate uptake by the compounds tested were noted (Fig 2a). $CaCl_2$, $MgCl_2$, and NH_4Cl caused significant increases in uptake (30, 23, and 13%, respectively), while added Na_2SO_4 caused a significant reduction (16%) (two-factor ANOVA, Dunnett's test, $\alpha = 0.10$). Added KCl, NaCl, reduced $NaNO_3$, and reduced K_2HPO_4 all reduced uptake 13, 7, 5, and 5%, respectively, but not significantly. Added $NaHCO_3$ caused little change (+3%).

Two treatments caused significant (two-factor ANOVA, Dunnett's test, $\alpha = 0.10$) increases in selenite uptake (Fig 2b). Reduced K_2HPO_4 caused an average 253% greater uptake, with uptake by heat-killed cells increasing proportionally. Added Na_2SiO_3 caused an average increase of 311%, with virtually all the added uptake also being present in the heat-killed treatment.

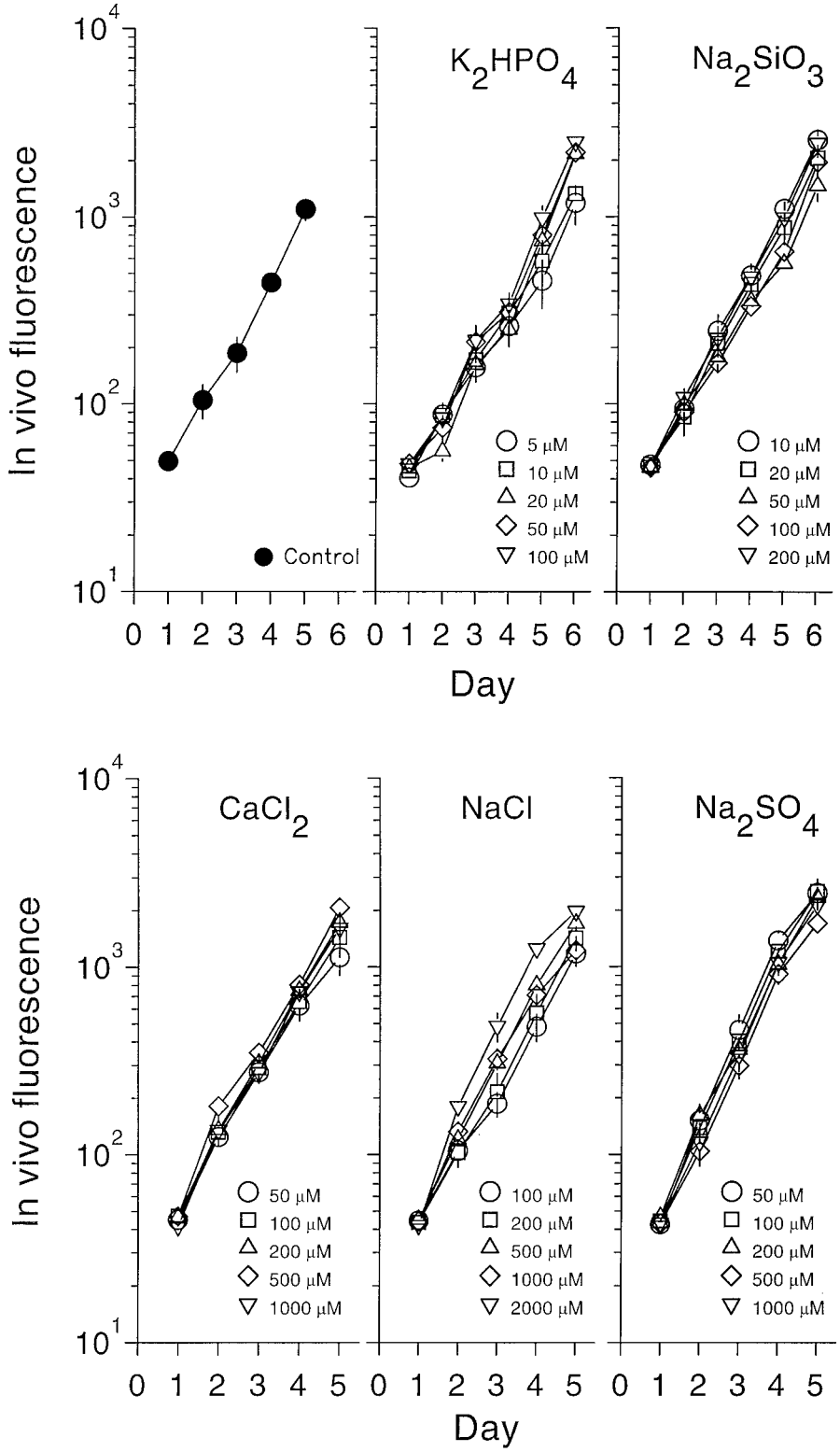

Fig. 3. Growth of cells during the long-term effect of ions on inorganic selenium uptake experiment. (**Top**) Control cultures and K_2HPO_4 and Na_2SiO_3 treatment cultures. (**Bottom**) $CaCl_2$, NaCl, and Na_2SO_4 cultures. Mean ± SD (n = 4).

The remainder of the treatments ranged from 102 to 134% of the control treatments.

Effects of medium composition on selenium uptake—detailed examination of major effects

In general, the variations in media composition did not substantially affect the growth of *C. reinhardtii*. However, there was evidence for somewhat enhanced growth in the three highest concentrations of the KH_2PO_4 series, and that added NaCl enhanced the growth rate slightly (Fig. 3).

In the Na_2SO_4 addition series, selenate uptake was lowest at the highest concentration (1,000 μM) and increased slowly as sulfate decreased (Fig. 4). At the lowest concentration (50 μM), selenate uptake increased substantially in both the 3- and

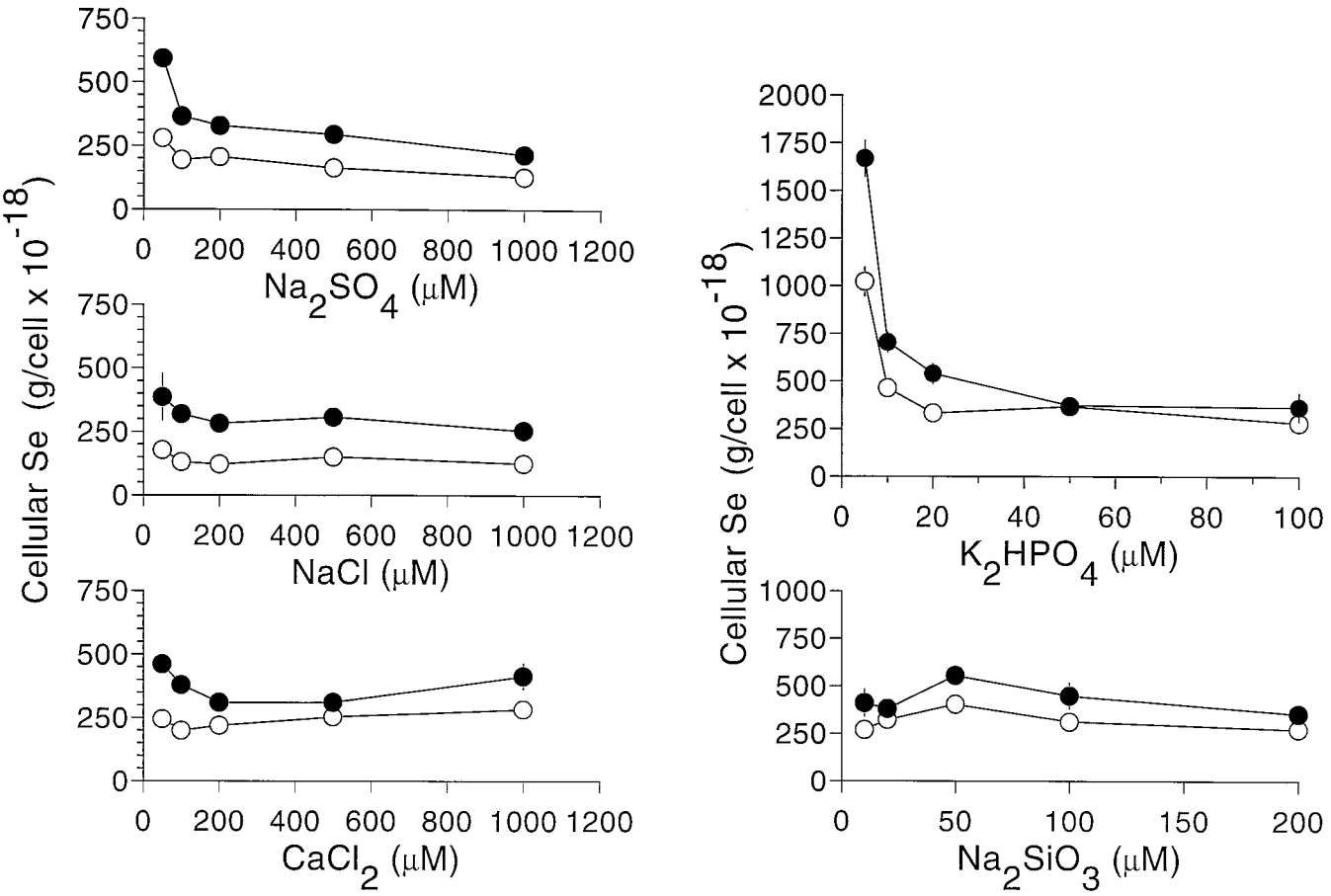

Fig. 4. The effect of media composition on inorganic selenium uptake in the long-term experiment. (**Left**) Effect of Na_2SO_4 (top), NaCl (middle), and $CaCl_2$ (bottom) on selenate uptake. (**Right**) Effect of K_2HPO_4 (top) and Na_2SiO_3 (bottom) on selenite uptake. ○ = 3 μg/L ^{75}Se additions, ● = 10 μg/L ^{75}Se additions.

10-μg/L treatments. As a test of whether the Na^+ or ionic strength increase with the added sulfate might be responsible for the observed effects of Na_2SO_4, a treatment with the same molarity of NaCl was made. In this treatment, there was also a slight decrease in selenate uptake with NaCl added (Fig 4), noticeably at the lowest concentrations. $CaCl_2$ demonstrated little effect on selenate uptake (Fig. 4).

Low K_2HPO_4 concentrations (below 20 μM) had strong stimulating effects on selenite uptake. At 5 μM K_2HPO_4, selenite uptake was two to four times greater than observed at 20 μM and above (Fig 4). Na_2SiO_3, however, showed little effect on selenite uptake (Fig 4), possibly because silicate was added long before the cell additions, allowing any precipitates formed during addition to redissolve and because of the lower, more realistic, concentration range.

DISCUSSION

Effect of pH on selenium uptake

The pH of the medium can influence the uptake of ions in several ways. First, the extent of protonation of ions in solution varies as a function of pH. Formation of a more biologically active form can increase uptake, while formation of a less active species can decrease uptake. Selenic acid is a strong acid (pK_2 = 2.05) and is essentially completely dissociated across the range of pHs examined here (and found in virtually all natural waters); thus, we would not expect the uptake of selenate to vary for this reason. The pH of the medium, over a range of 5 to 9, had little effect on selenate uptake. This suggests that over the pH range of many freshwater environments, selenate uptake can be effectively considered constant. Certainly, pH values outside this range on either side occur, and the generality of this relationship (or lack thereof) should be determined with other acid- and base-tolerant algae.

Selenous acid is a weak acid (pK_1 = 2.57, pK_2 = 7.31) [28], and thus important changes in its chemical form occur across the normal environmental range of pH. At pH values above 7.3, the selenite ion SeO_3^{2-} is predominant; at pH values between 2.5 and 8, $HSeO_3^-$ is predominant; while below pH 2.5 uncharged selenous acid (H_2SeO_3) predominates. The sharp rise in selenite uptake by cells at low pH values well below 7 suggests that the selenous acid molecule may readily enter the cell, while the charged species are largely excluded (or transported by a separate ion specific transport system). This increase in selenite uptake at low pH raises the question of whether the selenium concentrations in phytoplankton from relatively acid environments are enhanced relative to more neutral environments with similar selenite concentrations. No systematic studies of dissolved selenium occurrence, speciation, and bioaccumulation in lakes, across a gradient of pH, are available to determine whether there is environmental evidence for this hypothesis.

The other major effect of pH on the uptake of ions would be the effect of pH on uptake transport proteins. This would normally result in an optimum pH for uptake. For other enzymes

the shape of the pH activity profile is known to be quite variable, with optima ranging from pH 2 to 10 and from very narrow to quite broad [29]. For selenate, there is weak evidence for an optimum uptake near pH 8. For selenite there is no sign of an optimum, unless it lies somewhere below pH 5, a result better explained by changes in the inorganic speciation of selenite.

Effects of medium composition on selenium uptake

Of the ions tested in both ion experiments, only sulfate consistently demonstrated significant effects on selenate uptake. Mean sulfate concentrations of freshwater in the United States are about 100 μM [30], although concentrations can range from less than 10 to greater than 1,000 μM. For concentrations of sulfate near average or above, the effect on selenate uptake was rather small, with a decrease of about a factor of two for a 10-fold increase in sulfate concentration to 1,000 μM. Below 100 μM, the effect appears to be stronger, with a nearly twofold increase in cellular selenate concentration with a decrease to 50 μM. Sulfate ion is, therefore, an ion that should be considered in efforts to model selenate uptake of natural systems, particularly in soft-water systems with low sulfate ion concentrations.

We had, however, anticipated a proportional reduction of selenate uptake with elevated sulfate concentrations. Previous work has suggested that for several species of algae, selenate toxicity was approximately inversely proportional to sulfate concentration of the medium [31]. Analogous situations are found with sulfate and chromate [21] and arsenate and phosphate [22]. Given the similarity of selenate and sulfate, it was somewhat surprising that sulfate and selenate did not show a more strongly competitive pattern for uptake by *C. reinhardtii*. Given the environmental variability of sulfate concentrations, and the nonlinear response of selenate uptake by *C. reinhardtii* to sulfate concentration, this relationship should be examined in a wider variety of algae.

The two most notable effects observed in this study were the effects of silicate and phosphate on selenite uptake. The concurrent increase of selenite uptake in the heat-killed silicate treatments of the screening experiment suggested that the increased uptake was due to a silica precipitate in the medium at the rather high concentrations of silicate added. The second experiment, with a more reasonable range of silicate concentrations added in a manner to minimize precipitate formation, found no effect of silicate on selenite uptake by *Chlamydomonas* and lends credence to this conclusion.

Phosphate appears to exert strong control over the uptake of selenite and should be included in a biogeochemical model of selenium transport in lacustrine systems. Phosphate ion concentrations in lacustrine systems vary from undetectable levels in strongly phosphate-limited systems to very high concentrations in lakes with phosphatic geology or extreme nutrient additions [30], with normal concentrations of approx. 0.3 to 1.5 μM. However, because the effect of phosphate on selenite uptake occurs at low phosphate levels, systems in which phosphate is potentially limited are more likely to be influenced by changes in phosphate concentration. Many freshwater systems are at least potentially phosphate-limited [32]; hence, understanding and correctly modeling the relationship between phosphate and selenite uptake by algae is crucial to understanding the biogeochemistry of selenium. If phosphate-limited algae take up substantially more selenite than nitrogen- or light-limited algae, seasonal changes in selenite uptake, food web transfer, and transformations could be considerably different than predicted by selenium concentrations and biomass changes alone. From a practical point of view, this interaction may be extremely important to modeling the fate of selenite in lacustrine or riverine systems.

Previous experiments with three different species of phytoplankton, *C. reinhardtii*, the diatom *Cyclotella meneghiania*, and the cyanophyte *Anabaena flos-aquae*, suggest that selenate, selenite, and selenomethionine uptake among different taxa of algae have similar rates of uptake, adsorption, and substrate saturation [16] and thus likely share similar mechanisms of uptake. Further research should be carried out to determine whether the effects of sulfate and phosphate on selenium uptake are common features of algal selenium uptake. If so, models of selenium bioaccumulation in natural systems will need to incorporate this finding.

Acknowledgement—The laboratory assistance of Laura Cole, Douglas Talaber, and Dorothea Ferrier are gratefully acknowledged. This work was supported by the Electric Power Research Institute under RP2020/11.

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