

# Mucus Secretion by the Freshwater Snail *Lymnaea stagnalis* Limits Aluminum Concentrations of the Aqueous Environment

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Extracellular mucopolysaccharide (EPS) is a significant component in many waters. Its role in the cycling and mobilization of metals is unclear. In vitro studies were conducted to examine the influence of EPS, secreted by the freshwater pond snail, *Lymnaea stagnalis*, on soluble water Al concentrations at near-neutral pH. Snails maintained in aerated water of known ion content (pH 7.3, 10 °C) and added aluminum (500 µg/L Al), significantly ( $p < 0.05$ ; at 24 and 48 h) reduced Al (measured by inductively coupled plasma optical emission spectroscopy) in solution as compared to controls (absence of snails). Although snails accumulated Al into soft tissue, this only accounted for a small percentage of the total reduction. The remaining Al was recovered following acidification of the water. This observation was attributed to pedal EPS secreted by *L. stagnalis* (quantified by periodic acid Schiff staining), which is chiefly insoluble and substrate bound. The Al that remained in solution was *more* labile, possibly due to the influence of soluble EPS. Further experiments with isolated EPS, confirmed that this poorly soluble film binds and reduces Al in solution. The influence of EPS on the solution chemistry and bioavailability of Al and possibly other metals may be important in natural waters.

## Introduction

The solubility and mobility of metals such as aluminum in natural water is controlled by pH and complexation (1). Aluminum (Al) for example is the most abundant metal in the Earth's crust, present largely as insoluble aluminosilicates (2), but its concentration in the hydrosphere is usually low due to high chemical stability of Al-containing minerals. Furthermore, any solubilized Al readily undergoes hydrolysis to form strong complexes with the hydroxide ion ( $\text{HO}^-$ ), and around neutral pH this is predominant as the insoluble  $\text{Al}(\text{OH})_3$  species. However, Al may also form complexes with a number of other inorganic ligands, so the presence of these ligands in natural waters may alter the aqueous chemistry of Al, usually enhancing its solubility.

Fluoride ( $\text{F}^-$ ) interacts strongly with Al, particularly under acidic conditions (1, 2), and the complex formed is soluble and persists at neutral pH. However the percentage of Al bound to fluoride is usually low in natural waters due to the low levels of  $\text{F}^-$  (1, 2). The sulfate ( $\text{SO}_4^{2-}$ ) concentration in natural waters is usually much higher than  $\text{F}^-$ , but its interaction with Al is weaker at acidic pH (1, 2) and insignificant in near-neutral waters. Complexes formed with phosphate ( $\text{PO}_4^-$ ) are notably insoluble and hence decrease soluble Al concentration, but since phosphate is also required by many aquatic organisms, the phosphate concentration is often limited in natural waters. Soluble silica ( $\text{Si}(\text{OH})_4$ ) is present in natural waters at high concentrations, and complexes with Al are suggested to be present in solution (1, 3). However Si uptake by diatoms can limit water Si concentration, and recent work has shown that the interaction between monomeric silica and Al is weak, suggesting that this complex is negligible in many waters (4, 5). Finally, chloride, nitrate, and bicarbonate have only weak affinity for Al, so apart from hydroxide ions, complexes formed with inorganic ligands are likely to be negligible in most waters (1) at around neutral pH and especially in the presence of dissolved organic matter.

Indeed complexation of Al occurs mainly with organic carbon moieties, of which humic and fulvic acids are considered most important (6, 7). Extracellular polysaccharide (EPS) (8) is another significant source of total organic carbon (TOC) in natural waters (9, 10) which, in the form of mucus glycoprotein, is a ubiquitous secretion by aquatic algae, bacteria, fungi, and molluscs (11, 12). The role of EPS in the cycling of environmental metals has not been considered, although it is widely known that EPS interacts with metals and sequesters metals from natural waters (8). In the mammalian gut, soluble mucins (EPS) regulate the luminal solubility of hydrolytic metal ions, while at the tissue–water interface the gelatinous mucus layer (an EPS matrix) regulates their uptake (13). Recent work suggests that there is such a microenvironment at the sediment–surface interface of natural waters (14). Here we study the effect of EPS in the water column on the aqueous chemistry of Al.

The concentration of Al in natural waters is increased upon acid weathering of Al-containing minerals, as seen with acid rain and acid mine drainage. Elevated concentrations of soluble Al are toxic in the aquatic environment, especially to fish (micromolar concentrations), causing gill damage and loss of osmoregulative capacity (15). Aluminum is also toxic to plants (16), algae (17), and invertebrates (18). Aquatic invertebrates, in particular molluscs, accumulate toxic metals from their environment (19). The pond snail *Lymnaea stagnalis*, which is a grazing omnivore, was chosen for the present study since it accumulates Al from its environment (20) and has a well-characterized behavior and physiology that are adversely affected by the metal (18). During investigations on the accumulation of Al in *L. stagnalis*, we noted that the snails markedly reduced the concentration of the metal in their aqueous environment, more so than could be explained by uptake into the animals. *L. stagnalis* secretes mucopolysaccharide into the aquatic environment, much of which remains as a biofilm on the substrate. This study examined the influence of *L. stagnalis* and its secreted pedal mucopolysaccharide on the aqueous chemistry of Al.

## Experimental Section

All reagents were of AnalaR grade (BDH Ltd., Poole, U.K.), unless indicated otherwise. Water was ultrapure (UHP) 18 MΩ cm<sup>-1</sup>, from an Elga (High Wycombe, U.K.) water purifier.

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Three separate experiments were carried out: first, to determine the distribution of Al in the water column in the presence and absence of snails. Both the concentration and lability of Al in solution were measured, as was EPS secretion into the water by *L. stagnalis* and Al uptake by the snails. In experiment 2, snails were removed prior to the addition of Al to investigate the effect of their EPS secretions alone; the initial water Al concentration was lower to avoid the significant Al(OH)<sub>3</sub> precipitation observed in controls of experiment 1. Finally, in experiment 3, the effect of isolated, substrate-bound, pedal mucopolysaccharide on the distribution of Al in the water column was confirmed.

**Experiment 1.** Acid-washed, 10-L virgin polypropylene containers were used throughout, each containing 5 L of standard snail water (SSW) (222 mg/L CaCl<sub>2</sub>, 9.6 mg/L MgSO<sub>4</sub>, 4 mg/L KHCO<sub>3</sub>, 5.1 mg/L KNO<sub>3</sub>, and 58 mg/L NaHCO<sub>3</sub> in ultrapure water, pH 7.3) (21). Solutions were aerated for 24 h at 10 °C prior to the introduction of 10 *L. stagnalis* (Sciento, Salford, U.K.) to each experimental tank (*n* = 3). Snails were not fed and were maintained at 10 °C, with 12/12 h light/dark regime for the duration of the experiment. Control tanks (*n* = 3) were identical but without the introduction of *L. stagnalis*. All tanks were aerated throughout the experiment. Two hours after the introduction of snails to experimental tanks, 500 µg/L Al was added to both experimental and control tanks. Aluminum nitrate nonahydrate (Aldrich Chemical Co., Gillingham, U.K.) was used, prepared in acidified SSW (pH < 3). Following introduction of Al, the pH of the solutions was unaffected and remained at pH 7.3 ± 0.05 for 48 h. The solutions were sampled repeatedly over 48 h, 5 cm below the surface of the solutions at the center of the containers. Where indicated, surface water was also removed and was taken within 3 mm of the surface (water–air interface). Aliquots (10 mL) were removed and acidified to 0.7% with nitric acid (Aristar, BDH Ltd) for the determination of Al in solution, while determination of labile Al used 5 mL of unacidified water. Aliquots (10 mL) were also removed, again not acidified, for the determination of EPS in solution. At the end of the experiment (i.e., 48 h after addition of Al), Al was recovered from the containers by acidification of the bulk water to 5% HNO<sub>3</sub> for 24 h and removal of an aliquot for analysis of Al. Aluminum taken up by the snails was determined, while a group of snails, maintained similarly in SSW but without added Al, served as controls. All analyses were performed in duplicate (see Analyses).

**Experiment 2.** Eight *L. stagnalis* were maintained overnight (16 h) in 0.2 L of SSW in a polypropylene beaker. Snails were then removed and 0.3 L of MOPS (4-morpholinepropanesulfonic acid, Aldrich Chemical Co.) buffered SSW was added to give a final solution of 0.5 L of SSW with 2.1 g/L MOPS buffer at pH 7.2. An identically treated beaker, but without exposure to snails, served as the control. Aluminum nitrate (250 µg/L) was added to both beakers. The beakers were sampled over a period of 48 h by the removal of 3 mL of bulk water that was then acidified to 0.7% with HNO<sub>3</sub> for analysis of Al in solution. At the end of the experiment, Al in the beakers was recovered by acidification of the solutions for 24 h and analysis of an aliquot for Al.

**Experiment 3.** Three *L. stagnalis* were maintained on the surface of a 5 cm<sup>2</sup> polystyrene plate for 90 min, leaving a thin film of pedal mucopolysaccharide on the plate. Snails were removed, and the plate was immersed in a beaker containing 0.5 L of MOPS (2.1 g/L) buffered SSW at pH 7.2. An identical plate in a separate beaker, not exposed to snails, served as the control. Aluminum nitrate (350 µg/L) was added to both beakers. At 48 h, the plates were removed and the adsorbed Al was determined after washing the plate in 5% HNO<sub>3</sub> for 24 h. Aluminum concentrations of the waters were also determined as in experiment 2. A similar plate, exposed

to snails but not to Al, served as a control for the determination of background Al in the snail pedal mucopolysaccharide. The experiment was performed in triplicate.

**Analyses. (a) Aluminium in Solution.** Total Al in the acidified samples was analyzed by inductively coupled plasma optical emission spectroscopy (ICPOES; Jobin-Yvon JY24, Instruments SA, Longjumeau, France) with a v-groove nebulizer and conventional Scott-type spray chamber for Al at 396.152 nm, as previously described (22).

**(b) Labile Aluminum.** Lability of Al in solution was measured with the UV-active M<sup>3+</sup> chelator, 1,2-dimethyl-3-hydroxy-4-pyridinone (DMHP) as described previously (23). The free DMHP absorbs strongly at 274 nm, distinct from the Al-bound chelator, which absorbs at 289 nm. Briefly, 150 µL of 83.5 mg/L DMHP (gift from Dr. G. Tilbrook, King's College, London), prepared in SSW, was added to 2.85 mL of the water sample in a 1-cm quartz cell (DMHP in slight excess to Al). The solution was immediately shaken, and the absorbance at 274 nm was measured (LKB Biochrom Ultraspec II spectrophotometer, Pharmacia Biotech, U.K.) with time to a constant value at 25 °C. The initial rates of reaction were obtained from the raw data to give a measure of Al lability, as these rates are more informative and reliable than single arbitrary points that are commonly taken to measure labile aluminum (24). Control experiments confirmed that soluble EPS (0–2 mg/L) did not interfere with the assay.

**(c) Extracellular Polysaccharide (EPS).** Water samples were freeze-dried, reconstituted with 1 mL of ultrapure water, and sonicated for 20 min to aid dissolution of the EPS. Samples were measured colorimetrically with a modification (25) of the periodic acid Schiff (PAS) method that is used commonly in histochemical staining of mucopolysaccharide (26). Briefly, 100 µL of 19.7 g/L sodium periodate (Sigma Chemical, Poole, U.K.) in 49.04 g/L sulfuric acid was added to the reconstituted preheated samples (37 °C water bath). After thorough mixing, the samples were incubated at 37 °C for 30 min with occasional shaking, and 200 µL of 27 g/L sodium arsenite (Sigma Chemical) in 24.8 g/L HCl (Aldrich Chemical Co) was added to remove excess unreacted periodic acid. Immediately after the disappearance of the yellow color, 500 µL of Schiff's reagent was added, and the samples were incubated at 37 °C for a further 30 min, with occasional shaking. The resulting purple coloration was quantified with a computer-interfaced UV/VIS spectrophotometer (LKB Biochrom Ultraspec II). Absorbances were scanned between 450 and 650 nm, at 25 °C, within 1 h of the addition of Schiff's reagent. A standard curve was similarly produced, with solutions containing partially purified porcine stomach mucin (EPS) (type III, Sigma Chemical). Direct proportionality was found between the absorbance at λ<sub>max</sub> (565 nm) and the concentration of porcine mucin. The absorbance at 565 nm was therefore used in calculating the glycoprotein (EPS) concentration in solution. Control experiments confirmed that Al (0–600 µg/L) had no effect on the assay for EPS (0–250 µg/L).

**(d) Recovered Aluminum.** Aluminum recovered (into solution) from the base and walls of the containers, following acidification, was analyzed by ICPOES. Aluminum removed from solution by uptake into the snails was also determined. Snails were removed from solution and rapidly killed with ultrapure boiling water, and the whole tissue was separated from the shell. The total snail tissue was oven dried at 60 °C to a constant dry weight (0.12–0.23 g of snail) in individual acid-washed polypropylene containers and then digested with 5 mL of 30% hydrogen peroxide (Aristar) plus 5 mL of 65% nitric acid (p.a. plus, Riedel-de-Haen, Flurochem, Derbyshire, U.K.). Identical digest blanks were also undertaken with ultrapure water (0.18 mL) in place of snail tissue. Digests were diluted (1 + 1) with ultrapure water, and the

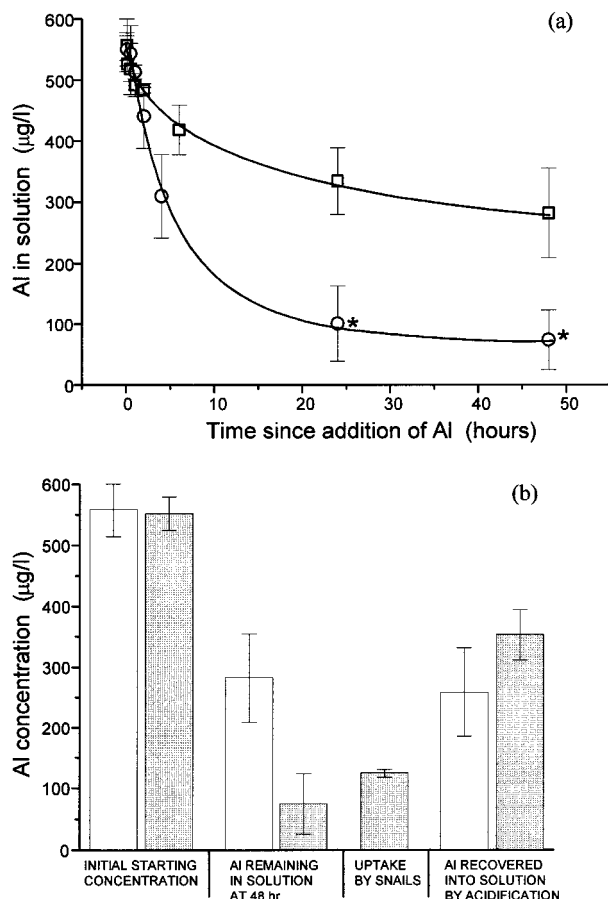


FIGURE 1. (a) Aluminum in solution as a function of time, following addition of aluminum nitrate (500 µg/L) to SSW alone (square, control) or to SSW containing *Lymnaea stagnalis* (circle, experimental). Data are mean  $\pm$  SD of three experiments. \* $p < 0.05$ ; experimental vs control. (b) Distribution of aluminum 48 h after addition of aluminum nitrate (500 µg/L) to SSW in the presence (gray, experimental) and absence (white, control) of *L. stagnalis*. Data are mean  $\pm$  SD of three experiments.

Al content was determined by ICPOES using individual sample based standards as previously described (22).

**Statistics.** Results are expressed as mean  $\pm$  S.D., and comparisons were by Student's *t*-test.

## Results

**Experiment 1.** At time zero, a mean concentration of 550 µg/L Al (500 µg/L added + contaminant Al, mainly from the salts in the SSW) was measured in control and experimental tanks. The concentration of Al then declined over the 48-h experimental period (Figure 1a). In the presence of *L. stagnalis*, the Al concentration fell more quickly than in the controls, being significantly different at 24 and 48 h ( $p < 0.05$ ; Figure 1a). Aluminum lost from solution by 48 h in the presence of snails was  $477 \pm 42$  µg/L (87  $\pm$  8% of the original concentration) as compared with  $275 \pm 66$  µg/L (49  $\pm$  9%) in the absence of snails (due to hydroxyprecipitation). From the experimental tanks, the concentration of Al measured in snail tissue at 48 h was  $3720 \pm 217$  µg/g dry wt (22  $\pm$  1% of the added Al) and, therefore, did not fully account for the difference observed in Al lost from solution between experimental and control tanks. Snails exposed to SSW without added Al had low tissue Al concentrations of  $226 \pm 16$  µg/g dry wt. Furthermore in the control tanks,  $258 \pm 73$  µg/L Al was recovered by acidification of the water, while  $353 \pm 42$  µg/L was recovered in the experimental tanks, confirming

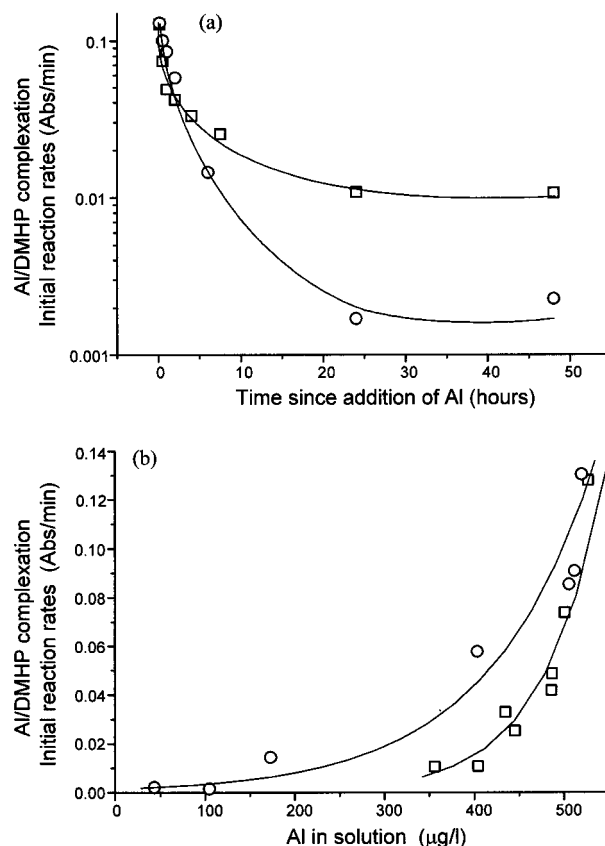


FIGURE 2. Measurement of lability of aluminum in SSW alone (square, control) and SSW containing *Lymnaea stagnalis* (circle, experimental). Initial reaction rate of Al:DMHP complexation, used as a measure of lability, is compared with both (a) time since addition of Al and (b) aluminum concentration in solution.

that additional Al was lost from solution in the presence of snails (Figure 1b).

In fresh control solutions (i.e., without snails), Al was readily available for chelation with DMHP (Figure 2a). However as the solutions aged, nonlabile polyhydroxy aluminum species are formed, from which the metal was less available for chelation (Figure 2a) and the concentrations of Al in the water column (in solution) also fell (Figure 1a) as polyhydroxy aluminum species precipitated out of solution. Thus, Figure 2 shows the initial rate of complexation of Al (in solution) with DMHP (lability) against both time and Al concentration in solution. Clearly the more rapid rate of Al complexation from control water (Figure 2a), compared to experimental water, is a function of the different Al concentrations in solution, since at the same concentrations the Al was *more* labile in experimental solutions (i.e., in the presence of snails; Figure 2b).

Fresh solutions of SSW contained no measurable EPS. However measurable EPS (1.0–1.5 mg/L) was found in solutions in both control and experimental tanks (Figure 3), probably because the solutions were prepared and aerated 26 h in advance allowing some bacterial colonisation. In control tanks the addition of aluminum nitrate markedly reduced the concentration of EPS with time to the limit of detectability (<0.5 mg/L) at 48 h (Figure 3). In contrast, the addition of *L. stagnalis* and then Al to these solutions (experimental) maintained and even marginally increased the levels of EPS in solution (Figure 3). By 4 h, EPS in experimental tanks was significantly greater than in controls ( $p < 0.05$ ). Furthermore, in experimental tanks, EPS concentration measured from surface samples ( $4.7 \pm 0.4$  mg/L)



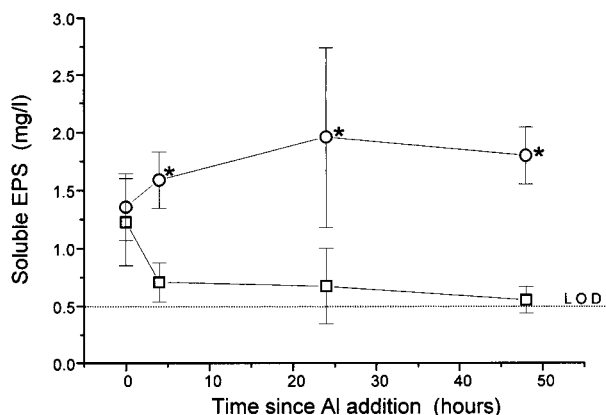


FIGURE 3. Effect on soluble EPS concentrations of the addition (at  $t = 0$ ) of aluminum nitrate ( $500 \mu\text{g/L}$ ) to aged, aerated SSW with (circle, experimental) or without (square, control) *Lymnaea stagnalis*. LOD, limit of detection for EPS. Data are mean  $\pm$  SD of three experiments. \* $p < 0.05$ ; experimental vs control.

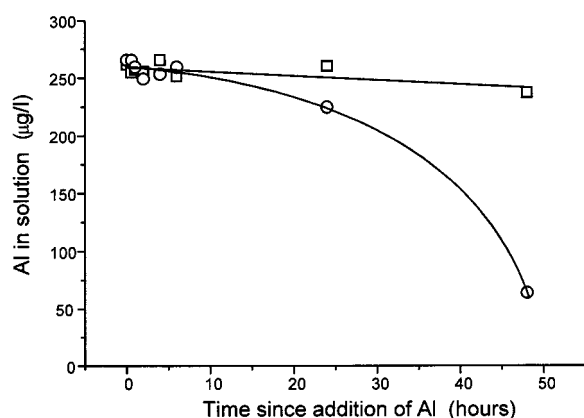


FIGURE 4. Aluminum in solution as a function of time, following the addition of aluminum nitrate ( $250 \mu\text{g/L}$ ) to SSW alone (square, control) or to SSW previously exposed to *Lymnaea stagnalis* for 16 h (circle, experimental).

was significantly greater ( $p < 0.02$ ) than those from bulk water samples ( $1.8 \pm 0.2 \mu\text{g/L}$ ).

**Experiment 2.** In the experimental SSW (preexposed to snails), a marked decrease in Al concentration from  $250$  to  $55 \mu\text{g/L}$  was observed over 48 h (Figure 4). In contrast, since the Al was added just below its boundary concentration for rapid precipitation of aluminum hydroxide ( $250 \mu\text{g/L}$ ), control solutions showed a negligible decrease in Al concentration over the same time period (Figure 4). In both solutions, acidification at 48 h completely recovered Al back into the soluble phase.

**Experiment 3.** Following isolation of the pedal mucopolysaccharide from *L. stagnalis*, it was confirmed that this bound Al and was responsible for a marked reduction of Al in solution (Figure 5). Aluminum remaining in solution in the presence of mucopolysaccharide was  $296.19 \pm 7.52 \mu\text{g/L}$ , which was significantly less ( $p < 0.05$ ) than that recovered from controls, waters, containing plates with no mucopolysaccharide ( $352.56 \pm 31.24 \mu\text{g/L}$ ). The mucopolysaccharide plates bound  $29.78 \pm 8.19 \mu\text{g}$  of Al as compared with  $4.15 \pm 3.40 \mu\text{g}$  of Al on plates with no mucopolysaccharide. There were negligible levels of Al ( $0.125 \pm 0.055 \mu\text{g}$ , equivalent to  $0.07 \pm 0.03\%$ ) in pedal mucopolysaccharide not exposed to Al-containing solutions.

## Discussion

The effect of extracellular polysaccharides on metal availability has been noted previously in the mammalian gas-

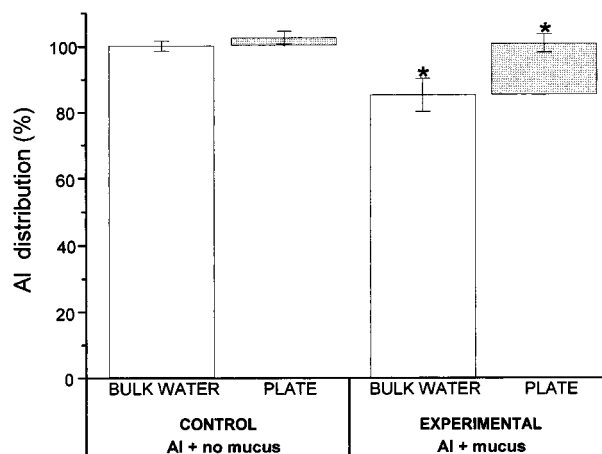


FIGURE 5. Aluminum binding by solid-phase pedal mucopolysaccharide (EPS) film from *Lymnaea stagnalis* (experimental plate) compared to plate with no snail mucopolysaccharide (control), 48 h after addition of aluminum nitrate ( $350 \mu\text{g/L}$ ) to the water. The percentage distribution of aluminum in the beaker (white) and on the plate (gray) is shown. Data are mean  $\pm$  SD of three experiments. \* $p < 0.05$ ; bulk water and plate vs control.

trointestinal tract (13, 27). Indeed, EPS is probably the primary regulating factor in the intestinal absorption of ingested metals in mammals (13, 27, 28). Two complementary mechanisms appear to exist (13); (a) soluble EPS in the lumen of the gut slows and stabilizes the hydroxypolymerization of hydrolytic metals, thus maintaining these elements in a bioavailable form; (b) the solid EPS layer traps metal/metal-hydroxy species on the surface of the mucosa, regulating their subsequent transport and uptake (13).

The situation is less clear in invertebrates. *L. stagnalis* actually accumulate significant amount of metals, including aluminum (20), suggesting that regulation of uptake of metals from the gut is less important (19). Furthermore, these experiments suggest that it is the solid-phase EPS layer that is secreted external to the animal that may significantly trap metals from their environment. The production of secretory EPS by *L. stagnalis* and other gastropods is a high energy-dependent process, and hence subsequent grazing of the mucopolysaccharide film is common (29). It is likely that uptake by the snails of metals trapped on the EPS film occurs during such grazing. This may represent a mechanism by which some aquatic invertebrates, such as *L. stagnalis*, assimilate essential metal nutrients from the environment (8). Recent work suggests that internal regulation of cations may then occur through specific metalloproteins in the snail (19).

It is also probable that solid-phase EPS films influence the geochemical cycling of some metals. Extracellular polysaccharide is secreted not only by snails but chiefly by algae and bacteria in the water column and on the substrate. In this study, the mucopolysaccharide secreted by *L. stagnalis* significantly reduced Al concentration in solution. Since Al is toxic to aquatic organisms, its reduced concentration in solution would similarly reduce its bioavailability and toxicity. Thus in natural waters the presence of EPS in the water column could be advantageous in counteracting increases in soluble Al levels, such as may result from acid drainage or deposition.

Finally, EPS from *L. stagnalis* is poorly soluble, but the little that did dissolve appeared to have an effect on the lability of Al, making it more available for chelation than in control solutions. This soluble EPS probably binds polyhydroxides of Al (28), which occur in the absence of ligands around neutral pH, and limits their natural growth, hence slightly increasing the lability of the metal ion (28). This is unlikely

to be of benefit to *L. stagnalis* but again may have some effect on the natural water chemistry (hence toxicity) of Al.

Thus EPS, in addition to humic and fulvic acids, may represent a significant environmental factor controlling the mobility and availability of metals in the environment. The distribution of EPS in the environment and its role in metal cycling deserves further attention.

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