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Aluminum Effects on Uptake and Metabolism of Phosphorus by the Cyanobacterium *Anabaena cylindrica*

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ABSTRACT

Aluminum severely affects the growth of the cyanobacterium *Anabaena cylindrica* and induces symptoms indicating phosphorus starvation. Pre- or post-treating the cells with high (90 micromolar) phosphorus reduces the toxicity of aluminum compared to cells receiving a lower orthophosphate concentration. In this study aluminum (ranging from 9 to 36 micromolar) and phosphorus concentrations were chosen so that the precipitation of insoluble $AlPO_4$ never exceeded 10% of the total phosphate concentration. The uptake of ^{32}P-phosphorus is not disturbed by aluminum either at high (100 micromolar) or low (10 micromolar) concentrations of phosphate. Also, the rapid accumulation of polyphosphate granules in cells exposed to aluminum indicates that the incorporation of phosphate is not disturbed. However, a significant decrease in the mobilization of the polyphosphates is observed, as is a lowered activity of the enzyme acid phosphatase, in aluminum treated cells. We conclude that aluminum acts on the intracellular metabolism of phosphate, which eventually leads to phosphorus starvation rather than on its uptake in the cyanobacterium *A. cylindrica*.

The detrimental effects of aluminum on organisms in acidified soils and waters are well established (6, 8). In Swedish lakes, the aluminum toxicity is most pronounced at pH 5 to 6, *i.e.* the pH interval where the level of aluminum rapidly increases in water subjected to acidification (11, 24). Negative effects of aluminum on phosphorus nutrition of plants and microorganisms have been shown in several studies. Nalewajko and Paul (16) showed that additions of aluminum (250 $\mu g \cdot L^{-1}$) significantly decreased microbial phosphate uptake and photosynthesis in water samples from two Canadian lakes. Dickson (3) suggested that apart from the pure toxic effects, an increase in the aluminum concentration produces oligotrophic waters as simultaneously a precipitation of phosphorus occurs, especially in the pH interval 5 to 6 (3).

A consequence of the lowered phosphorus availability is the extremely high phosphatase activities in organisms of aluminum rich lakes (12). In many plants, growing on acidified soils, aluminum tolerance appears to be associated with the efficiency in usage of phosphorus or the ability to tolerate low phosphorus levels (6). Two reactions between phosphorus and aluminum in barley seedlings have been suggested (2). One adsorption-precipitation reaction at the cell surface or in the free space and another reaction within the cell, probably mediated by an inhibition of hexokinase.

We have earlier reported on the inhibitory effects of aluminum on growth, photosynthesis and nitrogen fixation of the cyanobacterium *Anabaena cylindrica* (17). Evidence has also been presented for the rapid uptake of aluminum into cyanobacterial cells (18) and its accumulation into phosphorus rich cellular structures; polyphosphate granules and cell walls (19). In cyanobacteria, phosphorus limitation is known to cause accumulation of cyanophycin granules, a N-storage compartment composed of arginin-aspartate polymers (23). As we observed such a phenomenon, as well as other ultrastructural changes in *A. cylindrica* cells exposed to aluminum, we assumed that phosphorus limitation may be one mechanism behind the aluminum toxicity we noted earlier. This paper presents and discusses data from studies on effects of aluminum on uptake and metabolism of phosphorus in *A. cylindrica*.

MATERIALS AND METHODS

Organism and Cultivation. *Anabaena cylindrica*, strain 1403/2a, was obtained from the Cambridge Culture Collection of Algae and Protozoa, UK. Cells were grown in pure culture in N-free BG 11 medium (22) and sparged continuously with sterile air. Cultivation and cell harvesting were carried out as detailed earlier (17).

Experimental Design. The harvested cells were resuspended in autoclaved half-strength growth medium, buffered to pH 6.0 with 10 mM MES. To such cyanobacterial suspension, phosphate, supplied as $K_2HPO_4 \cdot 3\ H_2O$, was added to the desired final concentration up to 100 μM. Aluminum (9–36 μM) was added as $AlCl_3 \cdot 6\ H_2O$ from freshly prepared stock solutions. The experimental flasks were placed in continuous light (70 μmol photons $m^{-2}\ s^{-1}$) at 25°C and shaken gently. All experiments were run in triplicate and repeated at least twice.

Growth and Culture Density. Growth was followed spectrophotometrically by measuring the absorbance at 650 nm (A_{650}). Chl *a* was determined by the method of Harborne (7) and protein content according to Bradford (1).

Phosphate Uptake. Volumes of 10 ml algal suspension were preincubated 4 h in 50 ml flasks before adding aluminum, immediately followed by the addition of ^{32}P-labeled inorganic phosphate. Phosphorus-32, as orthophosphate (carrier free) in diluted HCl, was diluted with K_2HPO_4 to the desired specific activity, 0.2 μCi μmol^{-1} for high phosphate experiments (100 μM) or 2.0 μCi μmol^{-1} for low phosphate incubation (10 μM). Uptake was terminated by the addition of excess (1 mM) nonradioactive phosphate, followed by centrifugation at 1500*g* for 5 min. Supernatant (2 ml) was diluted to 10 ml with redistilled H_2O and the ^{32}Pi remaining in the growth medium determined by counting the Cerenkov radiation. This centrifugation procedure was compared with the more commonly employed filtration on 0.45 μm pore size filters (16). Both methods gave essentially the same result. Centrifugation was chosen because of its higher reproducibility and simplicity. All samples were corrected for background radioactivity and color quenching, and calculated to 1% counting error or less (confidence limit 95%). In some

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experiments cells were preincubated with aluminum (36 μM) for 4 h, washed, and resuspended in fresh growth medium before ^{32}P-phosphate uptake studies were carried out as described above. Also the precipitation of insoluble $AlPO_4$ was determined by incubating flasks without cyanobacteria for 15 min with additions of aluminum and ^{32}Pi at appropriate concentrations.

Polyphosphate Granule Staining. Cells were harvested after appropriate incubation times with or without aluminum, washed once in distilled H_2O before fixation with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 1 h. The staining procedure given by DuBois (4) was followed, using 10% $Pb(NO_3)_2$ and precipitating the lead with 1% $(NH_4)_2S$. The stained cells were observed in light microscope and the frequency of polyphosphate granules calculated using the grading system of Peverly *et al.* (20). In the grading system, ranging from 0 to 5 grade 0 indicates no polyphosphate granules present, grade 3 is equivalent with 50% of the cells having several granules and grade 5 indicates that all cells are filled with polyphosphate granules. At least 1000 cells were counted in each treatment.

Acid Phosphatase Activity. The method of DuBois (4) was followed, with the exception that 10 mM MES (pH 6.0) was used as the incubation buffer. ^{32}P-Orthophosphate was obtained from Amersham International, *p*-nitrophenol phosphate and *p*-nitrophenol from Sigma.

RESULTS

Effect of Phosphorus Availability on Aluminum Toxicity. The toxic effects of aluminum on *A. cylindrica* were markedly dependent on the applied concentration of Pi in the growth medium. Figure 1 shows a typical experiment where a Pi concentration of (90 μM) increases growth and survival of aluminum treated cells compared to a lower Pi concentration (18 μM). Notably, the 5-fold lower Pi concentration did not *per se* decrease the growth rate of the samples *i.e.* in absence of aluminum.

Another approach is shown in Figure 2. Cells were preincubated in growth medium with (90 μM Pi) or without phosphate for 24 h then washed and resuspended in fresh medium containing 90 μM Pi and aluminum. As seen, the cells pretreated with phosphorus were much less affected when exposed to aluminum than previously P-starved cells. Such results can not be explained by interactions between aluminum and phosphate in the growth medium but rather by a dependence on the phosphorus status of the cyanobacterial cells. The significance of the intracellular phosphorus status was also observed when cells exposed earlier to aluminum were posttreated with phosphate (Table I). In general, an incubation time of 1 to 2 h in 18 μM aluminum followed by washing and resuspension in fresh medium was enough to induce toxic effects such as decreased growth rate and inhibited photosynthesis and nitrogen fixation. These toxic effects could totally be reversed more or less immediately by postincubating the cells at high concentrations of Pi in absence

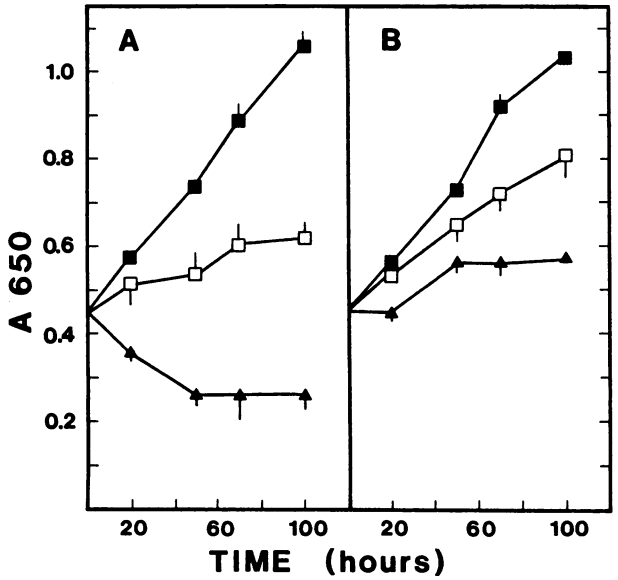

FIG. 1. Effect of phosphate concentration on the growth, expressed as increase in absorbance at 650 nm (A_{650}), of *A. cylindrica* in presence of aluminum at pH 6.0. A, Growth in medium containing 18 μM K_2HPO_4. (■), control; (□), 9 μM $AlCl_3$; (▲), 18 μM $AlCl_3$. B, Growth in medium containing 90 μM K_2HPO_4. (■), control; (□), 18 μM $AlCl_3$; (▲), 36 μM $AlCl_3$. *Bars* indicate SD of triplicate samples where this exceeds the size of the symbols.

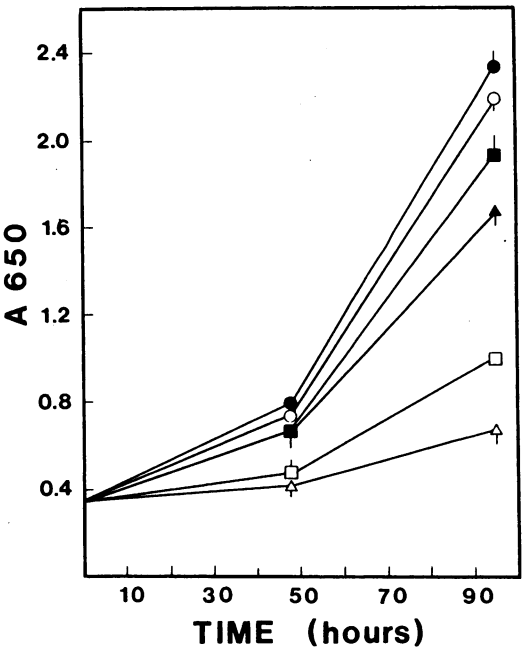

FIG. 2. Growth, expressed as increase in absorbance at 650 nm (A_{650}), of *A. cylindrica* in presence of aluminum after preincubation for 24 h with or without phosphate. After preincubation the cells were harvested, washed and resuspended in fresh medium containing 90 μM K_2HPO_4 and aluminum was added (pH 6.0). *Solid symbols* represent cells preincubated in 90 μM K_2HPO_4 and *open symbols* represent preincubation without phosphate. (●,○) controls; (■,□), 18 μM $AlCl_3$ (▲,△), 36 μM $AlCl_3$. *Bars* indicate SD of triplicate samples where this exceeds the size of the symbols.

Table I. *Effect of Preincubation with Aluminum on Growth of A. cylindrica*

Preincubation with $AlCl_3$ for 2 h in medium containing 90 μM K_2HPO_4 (pH 6.0). After preincubation cells were harvested, washed and resuspended in medium without phosphate. The samples were divided in two parts. To one half, 90 μM K_2HPO_4 was added, the other was grown without phosphate. The culture density, expressed as absorbance at 650 nm, was set to 0.528 ± 0.02 in all experimental flasks at the initiation of postincubation and again determined after 48 h. Means ± SD are given, $n = 3$.

Preincubation with $AlCl_3$	Postincubation	
	Without K_2HPO_4	With K_2HPO_4
μM	A_{650nm}	
0	0.670 ± 0.05	0.730 ± 0.05
18	0.517 ± 0.06	0.625 ± 0.05
36	0.357 ± 0.04	0.660 ± 0.05

of aluminum.

^{32}P-Phosphate Uptake in Presence of Aluminum. In order to be able to establish the amount of phosphorus actually taken up by the cyanobacteria the precipitation of insolbule $AlPO_4$ was assayed by using flasks without cyanobacteria and different combinations of aluminum and phosphate. Precipitation of $AlPO_4$ was depending on both the aluminum and the phosphate concentrations. The aluminum concentration was chosen so that the precipitation without cyanobacteria never exceeded 10% of the total ^{32}Pi added in the uptake studies. At 100 μM K_2HPO_4 and 36 μM $AlCl_3$ the precipitation of ^{32}Pi was 9.8 ± 2.0 μmol L^{-1} and at the lower concentration of phosphate 10 μM and 9.0 μM $AlCl_3$, 1.0 ± 0.3 μmol L^{-1}. The values of precipitated ^{32}Pi obtained in this manner were subtracted from the values obtained when studying the uptake by the cyanobacteria. Cyanobacteria are known to use different carrier mediated systems for phosphate depending on the concentration of Pi in the medium (10). Such carriers could be the site for the aluminum toxicity observed. We therefore conducted ^{32}Pi uptake studies at two different concentrations of Pi (10 and 100 μM), and adjusted the aluminum concentration to severely inhibit the growth of *A. cylindrica* without causing lysis of the cells. Figure 3 shows the uptake of ^{32}Pi from 10 μM phosphate medium in presence or absence of aluminum. No effect of aluminum on the uptake could be detected. The effect of pretreating the cells with aluminum for 4 h prior to performing the uptake experiment was then examined (Fig. 4). Again, no inhibition of the ^{32}Pi uptake could be seen from 100 μM phosphate medium.

Several studies have also shown a stimulatory effect on Pi uptake by divalent cations, usually Ca^{2+} and Mg^{2+} (5, 9, 14, 21). Our experiments showed that Ca^{2+} stimulated the uptake of Pi at 100 μM phosphate (Fig. 4) but not at 10 μM phosphate (data not shown) under the growth conditions used. A Ca^{2+} concentration of 120 μM (half-strength growth medium) saturated the stimulatory effect. However, as seen in Figure 4, presence of aluminum in the medium together with Ca^{2+} did not affect the uptake of ^{32}Pi.

Accumulation and Degradation of Polyphosphate Granules in Presence of Aluminum. Figure 5 shows the growth of *A. cylindrica* and the accumulation of Pi into polyphosphate granules in cells grown in batch cultures. It took about 24 h before the accumulation of Pi in polyphosphate granules reached its maximum, *i.e.* almost all cells contained several polyphosphate granules. This time schedule was not changed in aluminum exposed cells. Degradation of the granules was apparent after 48 h and within 100 h almost no polyphosphate granules could be observed in the control cells. The cells exposed to aluminum, however, still contained approximately one large polyphosphate granule per cell, corresponding to grade 3 in the grading system

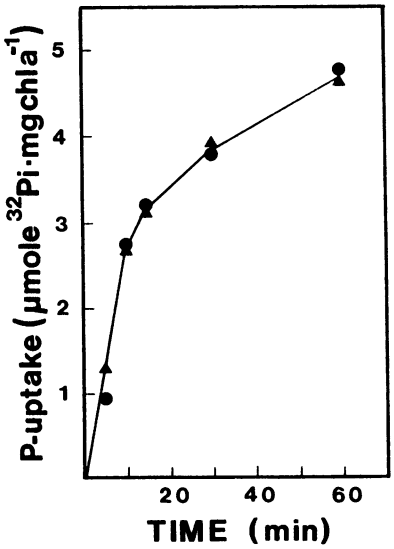

FIG. 3. Uptake of ^{32}Pi by *A. cylindrica* from 10 μM ^{32}P-labeled phosphate (pH 6.0). (●) control; (▲) 9 μM $AlCl_3$ added 10 min before uptake studies started.

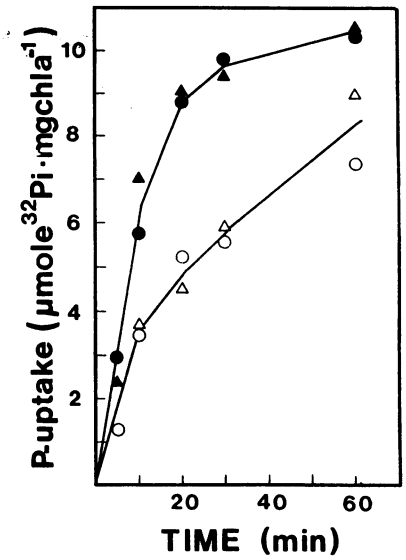

FIG. 4. Uptake of ^{32}Pi by *A. cylindrica* from 100 μM ^{32}P-labeled phosphate (pH 6.0). *Solid symbols* represent incubation in medium containing 120 μM Ca^{2+} and *open symbols* represent incubation without Ca^{2+}. (●,○) controls; (▲,△) cells preincubated with 36 μM $AlCl_3$ for 4 h before uptake studies started.

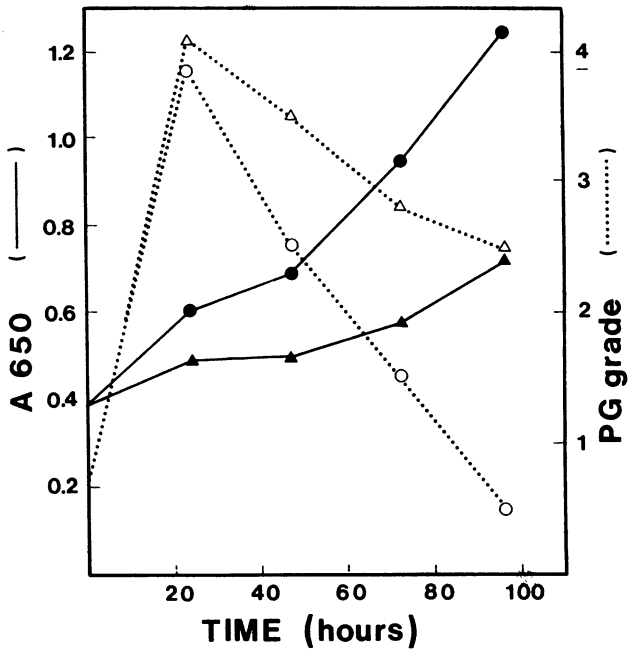

FIG. 5. Growth of *A. cylindrica*, expressed as increase in absorbance at 650 nm (A_{650}), and accumulation of polyphosphate granules (PG) in presence of aluminum. The frequency of polyphosphate granules was calculated using the grading system of Peverly *et al.* (20). Grade 3 is equivalent with 50% of the cells having several granules. Incubation in medium containing 90 μM K_2HPO_4 at pH 6.0. (●,○) controls; (▲,△) 36 μM $AlCl_3$. *Bars* indicate SD of triplicate samples where this exceeds the size of the symbols.

used (20).

As acid phosphatase (EC 3.1.3.2.) is proposed as the principal enzyme responsible for hydrolysis of the polyphosphates (4), the activity of this enzyme was assayed. Table II shows the acid phosphatase activity in *A. cylindrica* after incubation with aluminum for 24 and 70 h. There is a significant decrease in the activity after treatment with aluminum, *e.g.* a 20% decrease in activity is noted after a 24 h treatment.

DISCUSSION

It has been shown that many cyanobacteria together with other plankton organisms disappear in the pH range 5 to 6 in lakes suffering from acidification (11). In some cases the disappearance was closely associated with an increased aluminum concentration. Although it is difficult to relate data obtained in the laboratory to what is actually occurring under field conditions, it is obvious from this study that cyanobacteria could well suffer from aluminum toxicity at the early stages of acidification when the aluminum concentration is still rather moderate, particularly as the phosphorus concentration in most acid lakes is as low as 5 to 10 $\mu g\ L^{-1}$ (11). The importance of the phosphorus status of *A. cylindrica* in this context was established in the present study. Although an increased precipitation of insoluble $AlPO_4$ at high phosphate concentrations in the medium could partly reduce the aluminum toxicity (Fig. 1), such a precipitation could not explain the detoxification of aluminum when pre- and post-treating the cells with high phosphorus supplements (Fig. 2). In fact cells pretreated with high phosphate concentration for 24 h grew better when exposed to aluminum than cells that had been harvested after 7 or 8 d of exponential growth in normal culture medium (compare Fig. 1B and Fig. 2), although the growth conditions were the same during the experiments.

It has earlier been shown that the uptake of phosphate by some cyanobacteria is facilitated by a carrier activated by Ca^{2+} and Mg^{2+} (5). As we observed a stimulation by Ca^{2+} on the uptake of phosphate in *A. cylindrica* we postulated that such a carrier mediated transport could be a site for the aluminum inhibition observed. However, as no inhibitory effect of aluminum on the uptake of ^{32}Pi in presence of Ca^{2+} could be found either at low or at high concentrations of phosphate in the medium, such a mechanism is excluded. Phosphorus, rapidly taken up in excess of immediate needs (luxury consumption), is stored as polyphosphates in special granules in most cyanobacteria (10, 20). Polyphosphates are highly charged anions and therefore strongly associate with cations. In a previous study we observed an accumulation of aluminum in polyphosphate granules of *A. cylindrica* (19). In these experiments the rapid accumulation of polyphosphate granules both in control cells and in cells exposed to aluminum indicated that the uptake and incorporation of phosphate by *A. cylindrica* was not disturbed even after 24 h although growth was strongly affected (Fig. 5). These results contradict the work of Nalewajko and Paul (16). In their study on microbial samples from two Canadian lakes, aluminum depressed the phosphate uptake significantly at 50 $\mu g \cdot L^{-1}$ (18 μM).

Table II. *Effect of Aluminum on Activity of Acid Phosphatase in A. cylindrica*

Incubation in medium containing 90 μM K_2HPO_4 (pH 6.0). Means ± SD are given, $n = 3$.

$AlCl_3$	Protein Content		Acid Phosphatase Activity	
	24 h	70 h	24 h	70 h
μM	$\mu g \cdot ml^{-1}$		*mg PNP*[a] mg^{-1} *protein* h^{-1}	
0	24.4 ± 1.2	40.2 ± 1.7	0.91 ± 0.09	1.28 ± 0.12
36	24.1 ± 2.1	41.9 ± 2.2	0.73 ± 0.04	0.97 ± 0.07

[a] *p*-Nitrophenol phosphate.

Also phosphate uptake appeared more susceptible than photosynthesis to inhibition by aluminum. The contradictory results may depend on several reasons. First Nalewajko and Paul reported a high formation of particulate phosphate by aluminum. This means that less phosphate was available for the microorganisms. Secondly, their samples contained a mixture of phytoplankton species with different susceptibility to aluminum.

As the major difference observed between the control and the aluminum treated cells was a reduced mobilization of polyphosphate granules, we conclude that one mechanism for the aluminum toxicity in *A. cylindrica* could be in the utilization of the polyphosphates which eventually leads to the phosphorus starvation that was indicated in a previous study (17). This conclusion is sustained by the reduction in activity of acid phosphatase by aluminum (Table I). McCain and Davies (15) showed that in phosphorus limited *Agrostis* plants, with an increased activity of the acid phosphatase, aluminum inhibits the activity. However, this was not observed in phosphorus sufficient plants. In contrast, Woolhouse (25) found an increased activity of the enzyme in plant roots exposed to aluminum. This was attributed to an increased membrane permeability and release of the enzyme. It may be premature to conclude from our data whether the reduced activity of acid phosphatase is the sole cause for the lowered rate of breakdown of polyphosphate granules in cells exposed to aluminum. Other explanations may be binding of aluminum to the polyphosphates making them less available for enzymatic breakdown, or a lowered activity of other enzyme(s) involved in the metabolism of phosphate of the cyanobacterial cell.

One reason for the sensitivity of the cyanobacterium *A. cylindrica* to aluminum could be its ability to rapidly accumulate high concentrations of aluminum into the cell (18), or rather, the inability to prevent such an accumulation. A rapid uptake pattern by cyanobacteria has also been observed on exposure to heavy metals such as Cd, Cu, Hg, and Pb (13). We have earlier shown that in phosphorus rich *Anabaena* cells aluminum is mainly found in polyphosphate granules and cell walls, a compartmentalization which possibly makes it less available. This may function as a "detoxification" mechanism and last until the onset of mobilization of the polyphosphate granules (18). In contrast, phosphate starved cells apparently have a reduced capacity to accumulate aluminum in polyphosphate granules and cell walls (19), although the uptake *per se* is independent of the phosphate concentration (18). Thus, phosphorus starvation may lead to intracellular aluminum being free to act on enzyme(s) and membrane system and may explain the severe effects observed under such conditions.

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