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Impact of aluminium, fluoride and fluoroaluminate complex on ATPase activity of *Nostoc linckia* and *Chlorella vulgaris*

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This study demonstrates a pH-dependent inhibition of Mg^{2+}- and Ca^{2+}-ATPase activities of *Nostoc linckia* and *Chlorella vulgaris* exposed to $AlCl_3$, AlF_3, NaF and $AlCl_3$+NaF together. AlF_3 and the combination of $AlCl_3$+NaF were more inhibitory to both the enzymes as compared with $AlCl_3$ and NaF. Toxicity of the test compounds increased with increasing acidity. Interaction of $AlCl_3$+NaF was additive on *N. linckia* and *C. vulgaris*, respectively, at pH 7.5 and 6.8, and synergistic at pH 6.0 and 4.5. In the presence of 60 and 100 μM PO_4^{3-} an increased NaF concentration (in the $AlCl_3$+NaF combination) was required to produce the same degree of inhibition in ATP synthesis and ATPase activity. Toxicity of fluoroaluminate was reduced in the presence of EDTA and citrate. Except for beryllium to some extent, combinations of cadmium, cobalt, iron, manganese, tin and zinc with fluoride were not as effective as aluminium in inhibiting the ATPase activity. The presence of a 100 kDa protein band in SDS–PAGE of both control as well as $AlCl_3$+NaF-treated samples suggested that AlF_4^- inhibits the ATPase activity by acting as a functional barrier without affecting the structure of the enzyme.

Keywords: AlF_4^-, ATPase, *Chlorella vulgaris*, *Nostoc linckia*, SDS–PAGE

Introduction

Recent years have witnessed the emergence of much concern about the toxicity of aluminium, which is a major component of the Earth's crust. Aluminium is unique because it exists in different ionic forms at different pH, hence a minor shift in pH can affect its speciation and toxicity profoundly (Martin 1986). Nonetheless, aluminium toxicity to living organisms is greatly influenced by the presence of complexing ligands. Of the various compounds known to interact with aluminium, fluoride forms a relatively strong complex, which upon acidification yields a tetracoordinated fluoroaluminate complex, AlF_4^- (Martin 1986). It is worth mentioning that AlF_4^- acts as a structural analogue of PO_4^{3-} with a bond length equal to the P–O bond of PO_4^{3-} (Bigay *et al.* 1987).

It has been convincingly demonstrated that acidification of natural ecosystems is a problem of global concern (Olaveson & Nalewjko 1994) and survival of organisms in such acidic environments largely depends on the operation of a super-active plasma membrane H^+-ATPase responsible for maintaining cytoplasmic pH at neutrality (Rai *et al.* 1995). This H^+-ATPase is a phosphate-linked enzyme in both procaryotes and eucaryotes, and plays a significant role in regulating the movement not only of H^+ but also other vital ions across the cell membrane. Likewise, Ca^{2+}-ATPase also regulates the cell function by maintaining the cytoplasmic Ca^{2+} concentration.

The adverse effect of aluminium on various physiological and biochemical processes of algae, cyanobacteria and higher plants is well known (Pettersson & Bergman 1989, Greger *et al.* 1992, Husaini & Rai 1992, Huang *et al.* 1993). Fluoride has long been recognized as an inhibitor of a wide variety of enzymes like phosphatase, phosphorylase and kinase (Yang & Miller 1963, Lange *et al.* 1986). Research conducted recently (Jackson 1988, Rai *et al.* 1996) has vividly demonstrated the formation of the AlF_4^- complex in systems having $AlCl_3$, NaF and acidic pH. Catherine *et al.* (1994) demonstrated inhibition of H^+-ATPase of *Schizosaccharomyces pombe* by AlF_4^- through formation of an E_2–Mg–AlF_4^- complex.

In the light of the increasing contamination of the aquatic environment with aluminium and fluoride (Hornstrom *et al.* 1985, Zingde & Madalia 1988), accelerated formation of AlF_4^- at acidic pH, structural similarity between AlF_4^- and PO_4^{3-}, and the pivotal role of H^+-ATPase in survival of organisms in acidic environments, it is reasonable to postulate that AlF_4^- might inhibit ATPase activity at acidic pH by substituting at the phosphate binding site. It

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is also pertinent to mention that nothing is known about the mechanism of inhibition of algal processes by the fluoroaluminate complex. We, therefore, endeavoured to study: (i) the relative toxicity and inhibition kinetics of membrane-bound Mg^{2+}- and Ca^{2+}-ATPase of the procaryotic cyanobacterium *Nostoc linckia* and the eucaryotic green alga *Chlorella vulgaris* subjected to $AlCl_3$, AlF_3, NaF and $AlCl_3$ + NaF at different pH; (ii) the effect of increasing concentration of $AlCl_3$ in the presence of a fixed amount of NaF on the *in vitro* and *in vivo* Mg^{2+}- and Ca^{2+}-ATPase activities; and (iii) the effect of $AlCl_3$ + NaF on the protein profile of both test algae. Since contaminated waters are endowed with high concentrations of phosphate, ligands and toxic metals, effort has also been made to find out if phosphate, EDTA, citrate, beryllium, cadmium, cobalt, iron, manganese, tin and zinc can affect fluoride-induced inhibition of ATPase.

Materials and methods

Stock cultures of *N. linckia* and *C. vulgaris* were maintained in modified Chu-10 medium (Gerloff *et al.* 1950), at pH 7.5 and 6.8, respectively, under 75 μmol photon m^{-2} s^{-1} PAR light intensity and 14:10 h photo-period at 26 ± 2°C. *N. linckia* was grown in nitrate-free medium. Cultures from the logarithmic phase were used for toxicity testing. Stock solutions of $AlCl_3$, AlF_3 and NaF were filter sterilized by passing through Millipore membrane filters (0.22 μm) before supplementing to the culture medium. Survival was scored by the plate/colony count method (Rai & Raizada 1985). A 0.03 ml aliquot of exponentially grown culture containing 4.1×10^4 c.f.u. was inoculated onto the agar plates supplemented with varying concentrations of the test chemicals ($AlCl_3$, AlF_3 and NaF). For each concentration of test chemicals, three separate plates were inoculated and kept randomly in a sterile culture room. Colonies so developed were counted after 15 days and the per cent survival was scored with respect to untreated control. The LC_{50} values for AlF_3, $AlCl_3$ and NaF were 1.5, 4.0 and 20 mM, respectively, at pH 6.8 in the case of *C. vulgaris*. For *N. linckia* these concentrations were 0.5, 0.6 and 10 mM, respectively, at pH 7.5. LC_{50} concentrations were selected for further study. All experiments were performed in triplicate ($n = 3$) and repeated at least twice for ascertaining the reproducibility of the results.

For measurement of ATPase activity metal-treated algal cells were harvested, ruptured by an ultrasonicator (MSE, MK-2, USA), centrifuged at 10000 *g* for 30 min and the supernatant was dialysed for 3 h against 10 mM Tris–HCl buffer at pH 8.1. All these operations were carried out at 4°C. Mg^{2+}-ATPase activity was estimated following the method of Lockau & Pfeffer (1982) by measuring the phosphate liberated from ATP. For the Ca^{2+}-ATPase assay the method of Hicks (1986) was followed. For *in vitro* studies, solutions of the above compounds were prepared in 10 mM MES buffer (pH 6.0) and algal cells were homogenized in this buffer. Homogenate was dialysed against 4 mM MES buffer. Since no difference in inhibition by $AlCl_3$ + NaF was noticed before and after dialysis, the dialysis was therefore done after incubation. A 0.5 ml preincubated (90 min in light at 4°C) homogenate was transferred to the assay medium containing 6 mM ATP. Assay of Mg^{2+}- and Ca^{2+}-ATPase was carried out according to the above-mentioned method. For studying the effect of PO_4^{3-} on the toxicity of $AlCl_3$ + NaF to Mg^{2+}- and Ca^{2+}-ATPases, the test algae were grown in $AlCl_3$ + NaF and PO_4^{3-} (60 and 100 μM)-supplemented medium for 4 h and activity was assayed.

Citrate and EDTA were used at 1 mM for *N. linckia* and 4 mM for *C. vulgaris* to study their potential for amelioration of fluoroaluminate toxicity. In order to study the effect of other metal ions on fluoride-induced inhibition of ATPases, algal cells were treated with similar concentrations of all the metals (60 and 220 μM, respectively, for *N. linckia* and *C. vulgaris*) together with a fixed concentration of NaF (5 and 14 mM, respectively, for *N. linckia* and *C. vulgaris*) for 4 h.

ATP was extracted by using 4% TCA supplemented with 2 mM EDTA (Larsson & Olsson 1979) and total ATP content was measured after 4 h of treatment by a luciferin–luciferase assay using an LKB-1250 Luminometer.

SDS–PAGE of protein was done in a discontinuous system according to Laemmli (1970). Crude enzyme extract was prepared by sonication and protein was denatured in SDS–gel loading buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.1% bromophenol blue) prior to electrophoresis; a 20 μg ml^{-1} protein per lane was loaded. The stacking gel (5%) contained 1.0 M Tris–HCl (pH 6.8), whereas the resolving gel (8 and 10%) contained 1.5 M Tris–HCl (pH 8.8). The buffer reservoirs contained Tris–glycine buffer (pH 8.3). All the components of the system contained 0.1% SDS (Laemmli 1970). A 15 V cm^{-1} voltage was applied for separating the protein. The bands were visualized by Coomassie brilliant blue staining. Marker proteins ranging from 205 to 29 kDa (α_2-macroglobin, 205000; β-galactosidase, 116000; phosphorylase b, 97000; bovine serum albumin, 66000; fumarase, 48500; carbonic anhydrase, 29000) were used for molecular weight determination.

The results were verified by Student's *t*-test, χ^2-test, Duncan's new multiple range test and correlation co-efficient (*r*).

Results

A pH-dependent inhibition of Mg^{2+}- and Ca^{2+}-ATPase activity of *C. vulgaris* can be found from Table 1. AlF_3 (1.5 mM) was found to be more toxic than $AlCl_3$ (4.0 mM) and NaF (20.0 mM). However, a combination of 4.0 mM $AlCl_3$ + 20 mM NaF inhibited the ATPase activity additively (χ^2 not significant $P > 0.05$) at pH 6.8 and synergistically (χ^2 significant $P < 0.05$) at pH 6.0 and 4.5. Toxic potential of all the test compounds increased with an increase in acidity (from pH 6.8 to 4.5). It is interesting to note that Mg^{2+}-ATPase showed greater sensitivity than Ca^{2+}-ATPase with a per cent inhibition of 22, 33, 45 and 58 respectively in $AlCl_3$, NaF, AlF_3 and $AlCl_3$ + NaF-treated cells as compared with 3, 15, 18 and 20% for Ca^{2+}-ATPase

Table 1. Effect of $AlCl_3$, AlF_3, NaF and $AlCl_3$+NaF on inhibition kinetics of Mg^{2+}- and Ca^{2+}-ATPase of *C. vulgaris* at different pH

pH	Treatment	Apparent kinetic constants			
		K_m (mM ATP)		V_{max} (nmole P_i mg^{-1} protein min^{-1})	
		Mg^{2+}-ATPase	Ca^{2+}-ATPase	Mg^{2+}-ATPase	Ca^{2+}-ATPase
6.8	control	2.2	2.8	21.3	19.5
	4 mM $AlCl_3$	2.2	2.8	16.7 (22)	18.9 (03)
	1.5 mM AlF_3	2.2	2.8	11.9 (45)	15.9 (18)
	20 mM NaF	2.2	2.8	14.3 (33)	16.5 (15)
	20 mM NaF + 4 mM $AlCl_3$	2.2	2.8	**9.1 (58)	**15.6 (20)
6.0	control	2.4	2.8	18.5	17.5
	4 mM $AlCl_3$	2.4	2.8	14.1 (24)	13.7 (22)
	1.5 mM AlF_3	2.4	2.8	9.3 (50)	8.5 (51)
	20 mM NaF	2.4	2.8	11.7 (36)	10.4 (41)
	20 mM NaF + 4 mM $AlCl_3$	2.4	2.8	*3.1 (84)	*4.5 (74)
4.5	control	2.4	2.8	15.3	14.2
	4 mM $AlCl_3$	2.4	2.8	10.1 (34)	9.9 (30)
	1.5 mM AlF_3	2.4	2.8	6.9 (55)	7.1 (50)
	20 mM NaF	2.4	2.8	8.6 (44)	8.1 (43)
	20 mM NaF + 4 mM $AlCl_3$	2.4	2.8	*1.1 (93)	*2.1 (85)

The values are means of three ($n = 3$) independent variables. Data in parentheses denote % inhibition.
**Not significant: *$P < 0.05$ (χ^2-test).

at pH 6.8. Both the ATPases of *N. linckia* were inhibited in the same manner as *C. vulgaris* (data not shown).

A non-competitive inhibition of Mg^{2+}- and Ca^{2+}-ATPase (Table 1) of *C. vulgaris* was observed with $AlCl_3$, AlF_3, NaF and $AlCl_3$ + NaF. The K_m of both the ATPases remained unchanged, while V_{max} decreased. The K_m for Mg^{2+}-ATPase was 2.2 mM ATP at pH 6.8, and 2.4 mM ATP at pH 6.0 and 4.5; while for Ca^{2+}-ATPase it was 2.8 mM ATP at all the pH values studied. In *N. linckia*, K_m for Mg^{2+}-ATPase was 1.64 mM ATP at pH 7.5 and 6.0, and 1.7 mM ATP at pH 4.5. Ca^{2+}-ATPase showed a K_m of 2.0 mM ATP at all the three pH values studied (data not shown).

Figure 1 shows the effect of increasing concentration of $AlCl_3$ on both *in vivo* and *in vitro* Mg^{2+}- and Ca^{2+}-ATPase activity. At a fixed concentration of NaF (10 mM for *N. linckia* and 20 mM for *C. vulgaris*) the ATPase activity decreased with an increase in $AlCl_3$ concentration (significant positive correlation, $P < 0.01$). A concentration of 0.6 mM $AlCl_3$ was required for 75% inhibition of *in vivo* Mg^{2+}-ATPase of *N. linckia*. In contrast to this, 0.6 mM $AlCl_3$ inhibited 90% *in vivo* Ca^{2+}-ATPase activity. However, only 0.05–0.1 mM $AlCl_3$ produced complete inhibition of Mg^{2+}- and Ca^{2+}-ATPases of *N. linckia in vitro*. Likewise, 4 mM $AlCl_3$ inhibited 75% Mg^{2+}- and Ca^{2+}-ATPase activity of *C. vulgaris in vivo*, while at 0.1 mM it caused complete inhibition of *in vitro* activities of Ca^{2+}- and Mg^{2+}-ATPases.

Figure 2 illustrates that by increasing the PO_4^{3-} concentration from 60 to 100 μM and keeping the $AlCl_3$ concentration constant, an increased amount of NaF was required to produce the same level of inhibition of ATPase activity (Fig. 2A and B) and ATP synthesis (Fig. 2C and D) of the test organisms. In order to produce 50% inhibition of Ca^{2+}-ATPase activity of *N. linckia* at pH 6.0 the NaF concentration was increased from 5 to 8.3 mM ($P < 0.01$, Student's *t*-test) and for Mg^{2+}-ATPase from 6 to 10 mM ($P < 0.01$ Student's *t*-test) when the PO_4^{3-} concentration was raised from 60 to 100 μM (Fig. 2A). Likewise, to obtain 50% inhibition of Ca^{2+}- and Mg^{2+}-ATPase activities in *C. vulgaris* the NaF concentration was increased, respectively, from 13.4 to 21.4 mM ($P < 0.01$, Student's *t*-test) and from 10.8 to 18.6 μM ($P < 0.005$, Student's *t*-test) when the PO_4^{3-} concentration was increased from 60 to 100 μM (Fig. 2B). A similar trend was noticed for ATP synthesis, where the NaF concentration was increased from 5.5 to 9 mM ($P < 0.005$, Student's *t*-test) to produce the same level of inhibition of *N. linckia* grown in the medium with increased PO_4^{3-} concentration from 60 to 100 μM (Fig. 2C). To obtain the same level of inhibition in *C. vulgaris*, a 2-fold rise in NaF concentration (12.3 to 25 mM) was required in 100 μM PO_4^{3-} supplemented medium as compared with the 60 μM one ($P < 0.005$, Student's *t*-test).

Table 2 shows the ameliorative effect of EDTA and citrate on the toxicity of $AlCl_3$ + NaF to Mg^{2+}- and Ca^{2+}-ATPase of both the test organisms. Addition of 1 mM of EDTA and citrate to 10 mM NaF + 0.6 mM $AlCl_3$-treated *N. linckia* increased the Mg^{2+}-ATPase activity by approximately 20 and 15%, and Ca^{2+}-ATPase by 33 and 30%, respectively, at pH 6.0. Likewise, in *C. vulgaris*, about 21 and 17% increases in Mg^{2+}-ATPase and 21 and 15% increases in Ca^{2+}-ATPase activities were registered following addition of 4 mM each of citrate and EDTA to the medium supplemented with 20 mM NaF + 4 mM $AlCl_3$. Duncan's new multiple range test depicted a significant amelioration ($P < 0.05$) of $AlCl_3$ + NaF toxicity following spiking of EDTA

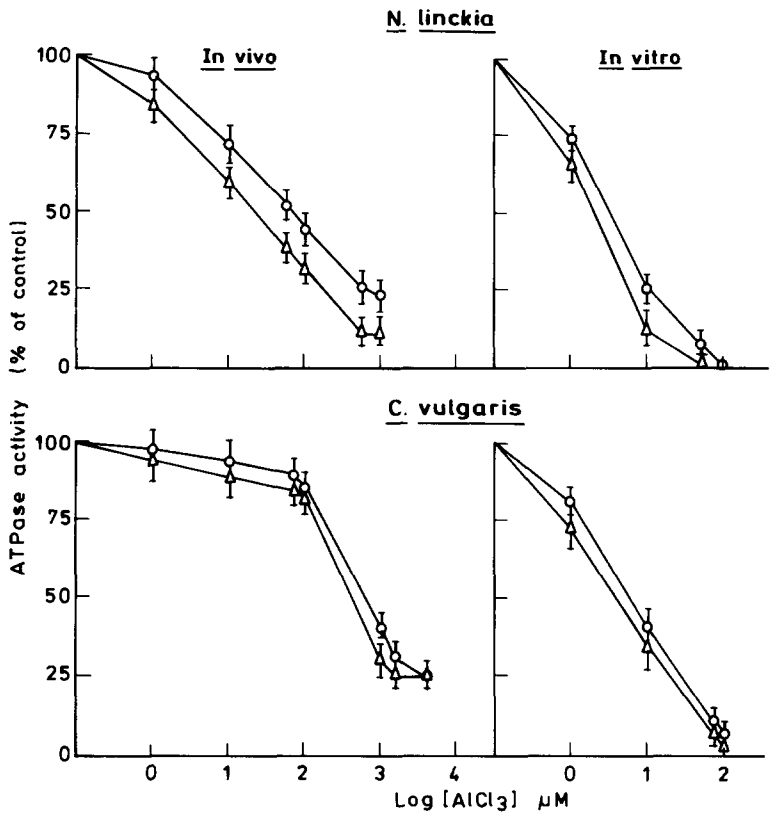

Figure 1. Inhibition of Mg^{2+}- (○) and Ca^{2+} (△)-ATPase activities of test organisms at increasing $AlCl_3$ concentration in the presence of a fixed NaF concentration (10 mM for *N. linckia* and 20 mM for *C. vulgaris*) at pH 6.0.

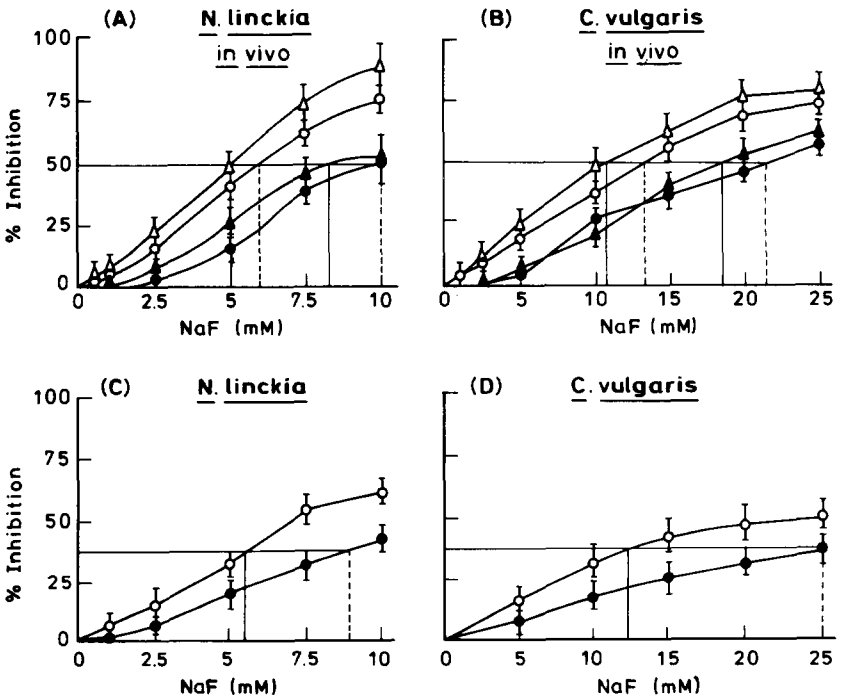

Figure 2. Effect of different concentrations of phosphate on NaF + $AlCl_3$-induced inhibition of ATPase activities (A and B) and ATP content (C and D) of *N. linckia* and *C. vulgaris* after 4 h of incubation. *N. linckia* and *C. vulgaris* were treated respectively with 0.6 and 4 mM $AlCl_3$. Open symbols, 60 μM PO_4^{3-}, closed symbols, 100 μM PO_4^{3-}.

Table 2. Amelioration of NaF + $AlCl_3$-induced inhibition of the Mg^{2+}- and Ca^{2+}-ATPase activity of *N. linckia* and *C. vulgaris* by citrate and EDTA after 2 h of incubation at pH 6.0

Treatment	Mg^{2+}-ATPase activity (nmol P_i released mg^{-1} protein min^{-1})	Ca^{2+}-ATPase activity (nmol P_i released mg^{-1} protein min^{-1})
N. linckia		
control	21.00 ± 1.26^a	25.00 ± 1.32^a
1 mM citrate	21.50 ± 1.29^a	26.00 ± 1.36^a
1 mM EDTA	21.08 ± 1.25^a	25.80 ± 1.29^a
10 mM NaF + 0.6 mM $AlCl_3$	5.67 ± 0.31	2.50 ± 0.13
10 mM NaF + 0.6 mM $AlCl_3$ + 1 mM citrate	9.96 ± 0.44^b	10.80 ± 0.58^b
10 mM NaF + 0.6 mM $AlCl_3$ + 1 mM EDTA	8.90 ± 0.42^b	9.90 ± 0.52^b
C. vulgaris		
control	35.00 ± 1.96^c	38.13 ± 2.28^c
4 mM citrate	40.80 ± 2.50^d	40.10 ± 2.50^c
4 mM EDTA	$38.50 \pm 2.20^{c,d}$	38.50 ± 2.30^c
20 mM NaF + 4.0 mM $AlCl_3$	9.80 ± 0.45	9.38 ± 0.55
20 mM NaF + 4.0 mM $AlCl_3$ + 4 mM citrate	17.15 ± 0.84^e	17.39 ± 1.02^d
20 mM NaF + 4.0 mM $AlCl_3$ + 4 mM EDTA	15.75 ± 0.91^e	15.25 ± 1.00^d

Values are mean ± SD.

Values superscripted by same letters are not significantly ($P < 0.05$) different from each other (Duncan's new multiple range test). Separate analysis was done for each column. $n = 3$.

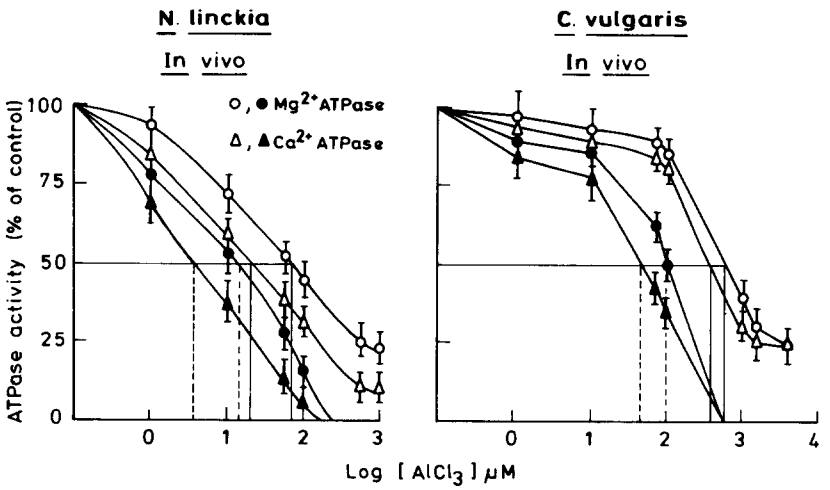

Figure 3. $AlCl_3$-induced inhibition of *in vivo* ATPase activities in the absence and presence of citrate in *N. linckia* (10 mM NaF) and in *C. vulgaris* (20 mM NaF) at pH 6.0.

and citrate. It is interesting to pin-point that in the absence of citrate in growth medium, 4- to 9-fold less $AlCl_3$ was required for 50% inhibition of ATPase activity at a fixed concentration of NaF ($P < 0.005$, Student's *t*-test) compared with that required in the presence of citrate (Fig. 3).

The data presented in Table 3 show the interactive effect of various metal salts on the Ca^{2+}- and Mg^{2+}-ATPase activities of both the test organisms. $AlCl_3$ when combined with NaF was found to inhibit Ca^{2+}- and Mg^{2+}-ATPase by 81, 76, 88 and 70%, respectively, in *N. linckia* and *C. vulgaris*. The intensity of inhibition was decreased to 50, 47, 45 and 40%, in the above order, when $AlCl_3$ was substituted by $BeCl_2$. The toxicity was further lowered when $CdCl_2$, $CoCl_2$, $FeCl_3$, $MnCl_2$ and $SnCl_2$ were combined with NaF. Interestingly, the combination of $ZnSO_4$ with NaF registered no inhibitory effect of Mg^{2+}-ATPase of both algae and Ca^{2+}-ATPase of *C. vulgaris*. Only 5% inhibition of Ca^{2+}-ATPase of *N. linckia* was noticed in the above combination (the inhibition was not significant at $P < 0.05$, Duncan's new multiple range test).

Results obtained from SDS–PAGE of *N. linckia* and *C. vulgaris* failed to demonstrate any change in the 100 kDa

Table 3. Effects of metal salts on fluoride-induced inhibition of Mg^{2+}- and Ca^{2+}-ATPase of *N. linckia* and *C. vulgaris* at pH 6.0 after 4 h of treatment

Metal salt + NaF	Mg^{2+}-ATPase activity (% of control)		Ca^{2+}-ATPase activity (% of control)	
	N. linckia	*C. vulgaris*	*N. linckia*	*C. vulgaris*
Control	100[a]	100[a]	100[a]	100[a]
$AlCl_3$	12	30	9	24
$BeCl_2$	55	60	50	63
$CdCl_2$	72	71	67	68
$CoCl_2$	86[b]	86[b]	83[b]	80[b]
$FeCl_3$	85[b]	85[b]	80[b]	78[b]
$MnCl_2$	96[a,c]	95[a]	94[a]	92
$SnCl_2$	89[b,c]	85[b]	80[b]	81[b]
$ZnSO_4$	100[a]	100[a]	95[a]	100[a]

The metal salts and NaF used were, respectively, 60 μM and 5 mM for *N. linckia* and 220 μM and 14 mM for *C. vulgaris*. The 100% value for Mg^{2+}-ATPase was 19.23 ± 1.11 and 21.0 ± 1.26, respectively, for *N. linckia* and *C. vulgaris*. These values were 23.21 ± 1.08 and 26.23 ± for Ca^{2+}-ATPase.
Values superscripted by same letters were not significantly ($P < 0.05$) different from each other (Duncan's new multiple range test). Separate analysis was done for each column. $n = 3$.

protein band after $AlCl_3$ + NaF treatment. Even increasing the incubation time from 4 to 24 h even failed to produce any difference in the protein band (data not shown).

Discussion

This study demonstrates a pH-dependent inhibition of Mg^{2+}- and Ca^{2+}-ATPase of *N. linckia* and *C. vulgaris* in the presence of $AlCl_3$, NaF, AlF_3 and NaF + $AlCl_3$ (Table 1). A higher toxicity of AlF_3 as compared with $AlCl_3$ may be due to the higher electrophilicity of Al^{3+} in AlF_3 than that in $AlCl_3$ (Fajan's rule). A synergistic effect produced by the $AlCl_3$ + NaF combination at pH 6.0 and 4.5 and additive effect at pH 7.5 and 6.8 suggests the formation of AlF_4^- at acidic pH. It is known that AlF_4^- has a tetrahedral geometry like that of PO_4^{3-} (Martin 1988); therefore, it can inhibit ATPase activity by interfering at the phosphate-binding sites.

The non-competitive inhibition of Mg^{2+}- and Ca^{2+}-ATPase by $AlCl_3$, AlF_3, NaF and NaF + $AlCl_3$ in *N. linckia* (data not shown) and *C. vulgaris* (Table 1) shows an irreversible binding of the test compounds to both the enzymes. A reduced toxicity of $AlCl_3$ + NaF at increasing PO_4^{3-} concentration suggests that PO_4^{3-} competes with AlF_4^- for binding sites of ATPase. In addition, the requirement of an increased dose of NaF for 50% ATPase inhibition at increasing PO_4^{3-} concentrations (Fig. 2) further testifies that AlF_4^- probably competes for the phosphate-binding site of ATPase. Following an increase in the NaF concentration, the formation of AlF_4^- will increase and any shift to a higher NaF concentration in the presence of higher PO_4^{3-} levels will require an increased AlF_4^- concentration for the inhibition of ATPase. Since p-type ATPase possesses a phosphorylated intermediate state (Pedersson & Carifoli 1987), it is possible that AlF_4^- could directly bind with the Asp residue of the enzyme during phosphorylation or form an ADP–AlF_4^- complex which substitutes ATP, thereby preventing hydrolysis of ATP and release of energy. Missiaen *et al.* (1988) for (Na^+, K^+)-ATPase of pig kidney and (Ca^{2+}, Mg^{2+})-ATPase from stomach smooth muscle, and Lunardi *et al.* (1988) for F_1-ATPase from beef heart mitochondria and *Escherichia coli*, reported the formation of ADP–AlF_4^- at the enzyme catalytic site. This may also be the reason for inhibition of ATP synthesis by AlF_4^- in the test algae.

A protective role of chelators like EDTA and citrate on the toxicity of $AlCl_3$ + NaF as observed in the present study (Table 2 and Fig. 3), agrees well with the findings of Lange *et al.* (1986) who demonstrated amelioration of aluminium-NaF-induced inhibition of hepatic microsomal glucose-6-phosphate by EDTA. Citrate, a well known chelator of aluminium (Martin 1986), is responsible for aluminium tolerance in higher plants (Miyasaka *et al.* 1991). Results presented in Table 2 are supported by the findings of Missiaen *et al.* (1988) and Sturr & Marquis (1990) who observed amelioration of NaF + $AlCl_3$-induced inhibition of (Na^+, K^+)-ATPase by an aluminium chelator deferoxamine. Differences in the Al^{3+} requirement for inhibition of *in vitro* and *in vivo* ATPase activity (low for *in vitro* conditions, Fig. 1) may be due to the binding/interference of aluminium with constituents of the growth medium like citrate, silicate, phosphate, etc. It is quite likely that similar binding between chelator and aluminium may occur in natural waters, thus decreasing the toxicity of aluminium and fluoride.

Be^{2+}, to some extent, but not the other metal cations (Co^{2+}, Cd^{2+}, Fe^{3+}, Mn^{2+}, Sn^{2+} and Zn^{2+}), inhibited the enzymes significantly when present in combination with fluoride, as observed for Al^{3+} in both *N. linckia* and *C. vulgaris* (Table 3). Like aluminium, beryllium is also known to form a tetrahedral complex with F^- (Martin 1988). Murphy & Coll (1993) demonstrated the formation of a stable fluoroberillium which binds with Ca^{2+}-ATPase of the sarcoplasmic reticulum. The failure of cadmium, cobalt, iron, manganese, tin and zinc to sensitize fluoride offers further support to the hypothesis that AlF_4^- formed in the presence

of $AlCl_3$ and NaF and inhibits ATPase by interfering at the PO_4^{3-} binding sites.

SDS–PAGE of *N. linckia* and *C. vulgaris* cell extracts failed to produce any alteration in the 100 kDa protein band in the presence of $AlCl_3$ + NaF. Even an increase in incubation time from 4 to 24 h did not produce any difference in the protein band. This suggests that AlF_4^- acts as a functional barrier for ATPases without affecting their structure. Inhibition of ATPase will lead to inhibition of ion translocation, which is vital for algal growth and metabolism. Further, the inhibition of H^+-ATPase activity will lead to acidification of the cytoplasm and disruption of the H^+ gradient across the plasma as well as the thylakoid membrane, leading to the failure of the photosynthetic machinery and other cellular processes. Notwithstanding, a rise in the Ca^{2+} concentration in the cytoplasm due to a failure of Ca^{2+}-ATPase may trigger the action potentials (Trebacz *et al.* 1994) which disrupt intracellular signaling and regulation of different physiological processes, leading finally to the death of algae.

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