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Localization of absorbed aluminium in pea root and its binding to nucleic acids

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Aluminium (Al^{3+}) considerably inhibited the root elongation of Alaska pea at concentrations higher than 10^{-4} M and pH 4.5. The absorbed Al^{3+} in roots was localized in the epidermis and regions where cells actively divided, such as root tip and differentiating lateral root. In cells, Al^{3+} accumulated mainly in the nuclei and walls. Al^{3+} in nucleic acids increased up to 24 hr after treatment with 10^{-3} M $AlCl_3$, and did not decrease markedly after transfer of 10-hr treated plants to water. Molecular sieving chromatography showed that Al^{3+} in nucleic acids associated preferentially with DNA. However, in vitro association of Al^{3+} with DNA and RNA was nearly the same. When Al^{3+} and nucleic acid were mixed in vitro, the molecules of Al^{3+} being adjusted to be equal to those of phosphorus in nucleic acid, only 25% of the Al^{3+} associated with nucleic acid. The presence of NaCl up to 0.4 M and $MgCl_2$ up to 0.05 M increased the association of Al^{3+} with DNA. When the phosphorus in DNA was masked by histone, the association of Al^{3+} with DNA was considerably reduced.

Aluminium (Al) is one of the major elements of soil constituents. The solubilized Al^{3+} in acid soil, often below pH 5.0, has long been known to have toxic influence on the growth of agriculturally important plant species (*10*); particularly, inhibition of root growth resulting in abnormal and undifferentiated tumor-like tissues (*1*, *3*, *13–15*). However, attempts to relate these observations with specific physiological processes are few. Clarkson and Sanderson (*7*), and Sivasubramaniam and Talibudeen (*21*) reported the inhibition by Al^{3+} of the uptake of calcium in barley plants and potassium in tea plants, respectively. Several reports indicate that various types of interaction between Al^{3+} and phosphorus may occur in the roots of plants. For instance, considerable quantities of absorbed phosphorus were fixed by Al^{3+} on the root surface (*5*). Also, phosphate metabolism was disturbed by Al^{3+} which resulted in a marked decrease in the rate of sugar phosphorylation, probably effected by the inhibition of hexokinase (*4*). In addition to these results, Clarkson reported that cell division was a primary site of disturbance by Al^{3+} (*6*, *20*). Al^{3+} also inhibits the genetic DNA synthesis in roots; this may be partly explained by an inhibition of cell division (*20*). It remains obscure, however, to what extent these phenomena are involved in the mechanism of Al^{3+} toxicity. One of the difficulties of clarifying the mechanism of Al^{3+} toxicity might arise from the fact that various plants respond unequally to Al^{3+} (*1*, *13*, *14*). In addition, investigation of the existing form of Al in vivo will be required to understand Al^{3+} toxicity. However, only little is known concerning the association between Al^{3+} and cell constituents in vivo (*1*, *24*, *25*). Several technical problems have made such an

approach difficult: 1) the inability to use suitable radioactive Al^{3+} as tracer, 2) the difficulty of micro assay of Al, because of the high Al atomization temperature. The latter problem was solved to a large extent by the recent development of a flameless atomic absorption spectrophotometer. Therefore, this work was conducted to find the localization of absorbed Al^{3+} in roots and the possible association of Al^{3+} with nucleic acids.

Materials and methods

Plant materials and Al^{3+} treatment

Pea (*Pisum sativum* cv. Alaska) was used throughout. The seeds were soaked in running water for 2 days. Next, they were germinated on moist absorbent cotton until the roots elongated to about 3 cm at 25°C in dark. Seedling roots were immersed in Al solution ($AlCl_3$) adjusted to pH 4.5 with diluted NaOH for the time indicated in each experiment. When the treatment period was longer than 24 hr, Al solutions were renewed every day. In the control experiment, distilled water adjusted to pH 4.5 with diluted HCl was used.

Measurement of Al

A flameless atomic absorption spectrophotometer (Hitachi 518) was used. When the Al in tissues was analyzed, the tissues were completely degraded with H_2SO_4 by heating. The Al^{3+} associated with nucleic acids was directly analyzed. From 20 to 50 μl of sample containing Al was taken by Eppendorf pipette very carefully and introduced into the graphite chamber of the flameless atomizer. The operating conditions were: 30 sec at 100°C for drying, 1 min at 1000°C for charring and 15 sec at 3000°C for atomizing. N_2 gas flowed at 0.5 liter/min during atomizing. The assay of each sample was done at least twice.

Observation of Al by staining

Staining of Al was done by aluminon (ammonium aurintricarboxylate) method used by Aimi and Murakami (*1*). This pigment is water soluble and gives a orange color by making a chelate complex with Al^{3+}.

Electron microprobe X-ray analysis

The roots immersed in Al^{3+} solution were treated by freeze-substitution method with acetone and embedded in epoxy resin. The operating conditions of EMX (Shimadzu-ARL) were: element, Al K_α; wave length, 8.338 Å; accelerating voltage, 25 KV; sample current, 0.01 μA; and sweep length, 40 μ/cm. The Al X-ray image was taken on Polaroid Land pack film Type 107.

Preparation and fractionation of nucleic acids

The roots were cut from Al^{3+}-treated seedlings and washed throughly with distilled water. The nucleic acids were prepared by the phenol method used previously (*16*). They were fractionated by Sephadex G-200 column (2.5 × 85 cm) and Bio-Gel A50 column (2.5 × 100 cm) which were equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The elution was done using the same buffer and each 3.5-ml fraction was collected. The determinations of RNA and DNA were performed by orcinol (*8*) and diphenylamine reactions (*2*), respectively. Enzymatic

digestions of nucleic acids were done at 37°C for 2 hr using heat-treated (90°C, 10 min) RNase (ribonuclease A, bovine pancrease, 5X crystallized type 1-A Sigma) for RNA and DNase (deoxyribonuclease I, beef pancreas Sigma) for DNA. After enzymatic digestion, samples were directly applied to Bio-Gel A50 column and eluted as before.

In vitro binding of Al^{3+} to nucleic acids

DNA and rRNA of pea roots were prepared and fractionated by MAK column as before (*16*). They were recovered with ethanol and dialyzed against 0.01 M Tris-HCl buffer (pH 7.0). Appropriate amounts of nucleic acids and $AlCl_3$ solution were mixed in a small test tube and incubated for 14 hr at 4°C. Next, the samples were completely dialyzed against distilled water (2 liter × 6) and unbound Al^{3+} was eliminated. DNA and calf thymus histone (Sigma) were mixed for 2 hr at 4°C, then Al^{3+} was added and the mixture was incubated at 4°C and pH 6.0 for 14 hr. Next, the samples were dialyzed against distilled water as before. In the control, Al^{3+} was added to DNA or histone solutions and incubated at 4°C for 14 hr, followed by complete dialysis against distilled water.

Results and discussion

Effect of Al^{3+} on the elongation of pea seedlings

As shown in Fig. 1, root elongation was not influenced by 10^{-5} M $AlCl_3$, but almost complete inhibition was observed at more than 10^{-4} M. The rate of inhibition was nearly the same as that of onion roots (*3*). Unlike the roots, elongation of shoots was much less affected by Al^{3+}. Even at 10^{-3} M $AlCl_3$, the rate of inhibition was less than 50% (data not presented here). About 2000 ppm Al was found in roots treated with 10^{-4} M $AlCl_3$. However, Al in shoots did not increase significantly with up to 10^{-3} M. The reversibility of Al^{3+} toxicity was investigated by transferring the Al^{3+}-treated samples to distilled water for 3 days. Pretreatment with Al^{3+} for only 1 hr reduced root elongation to up to 50% of the control (data not presented here). This high sensitivity of Al^{3+} toxicity was also observed in onion roots and distinct inhibition was found after 2-hr treatment with 10^{-3} M $Al_2(SO_4)_3$ (*3*). The remarkable feature of these results can be said to be the rapidity with which Al^{3+} toxicity takes effect. Clarkson (*3, 6*) suggested that some mechanisms associated with cell division were highly sensitive to Al^{3+}. Indeed, he observed the disappearance of mitotic figures (*3*).

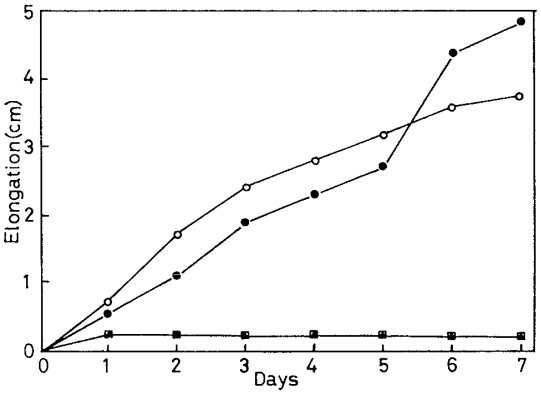

Fig. 1. *Al^{3+} inhibition of the elongation of Alaska pea roots.* Seedlings were treated with various concentrations of $AlCl_3$ adjusted to pH 4.5 in the dark. (○): control, (●): 10^{-5} M $AlCl_3$, (▲): 10^{-4} M, (□): 10^{-3} M. The data are average values of 10 samples.

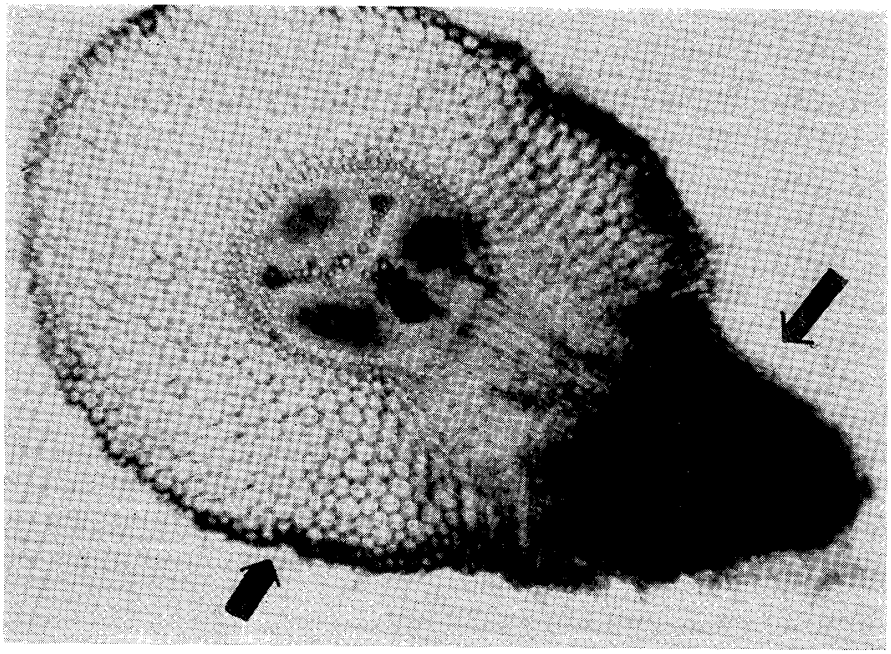

Fig. 2. *Distribution of absorbed Al^{3+} in Alaska pea roots showing the drastic accumulation of Al^{3+} at the differentiating lateral root.* ×150. The seedling was treated with 10^{-3} M $AlCl_3$ for 1 day and stained with aluminon. Arrows indicate pink color formed by chelation with Al.

Localization of absorbed Al^{3+}

Al^{3+} strongly inhibits the root elongation of Alaska pea (Fig. 1) as has been observed in other plants (*1*, *3*, *13–15*). The localization of absorbed Al^{3+} was investigated in the following experiments. It was not distributed uniformly in the roots according to microscopic observations of aluminon-stained transverse and

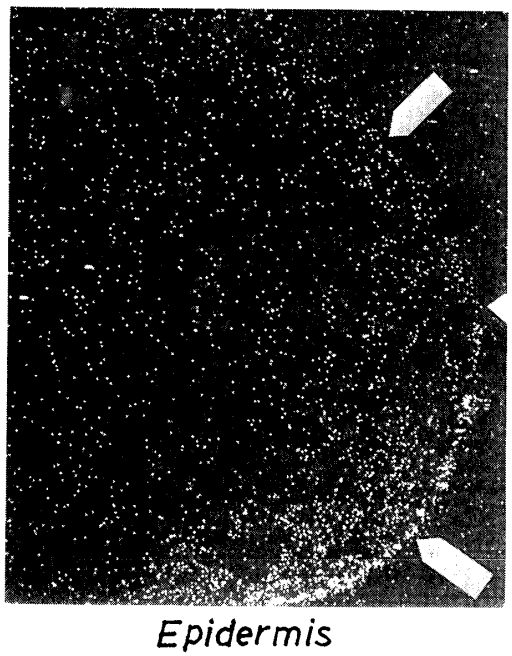

Fig. 3. *Electron microprobe X-ray image of Al in Alaska pea root.* The seedling was treated with 10^{-3} M $AlCl_3$ for 1 day. Spots indicate that Al is localized mainly at the epidermis.

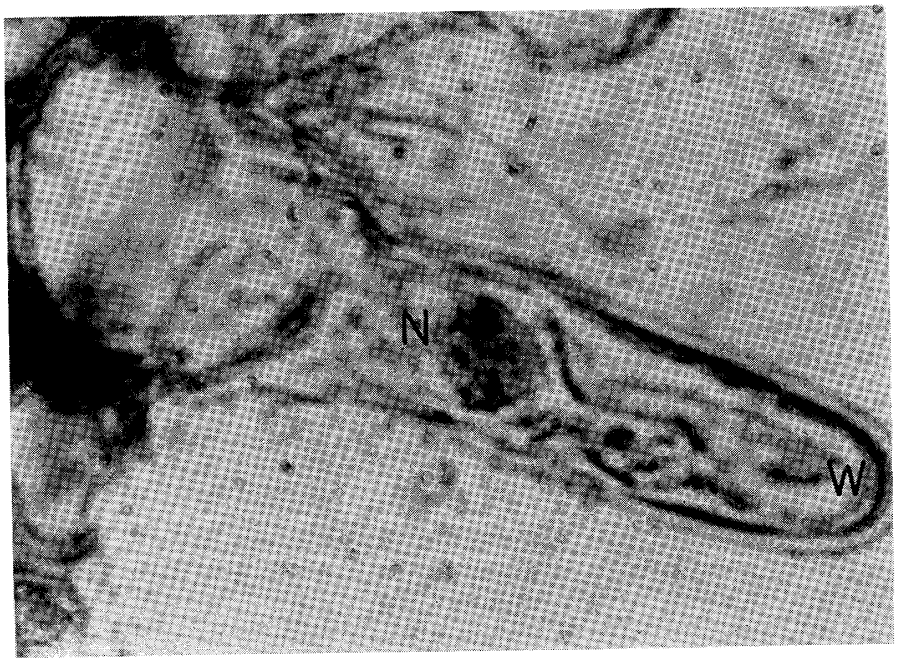

Fig. 4. *Localization of Al in Alaska pea root cell.* ×1500. Note the distinct localization of Al in nucleus and wall. N: nucleus, W: wall. For other details, see Fig. 2.

longitudinal root sections of various thicknesses. Al^{3+} was localized in actively dividing regions such as the root tip and differentiating lateral roots (Fig. 2). Besides these regions, it was localized to a limited extent in the epidermis of roots (Fig. 2 and 3). This appeared in the image of EMX-analysis (Fig. 3), which shows the half-round slice of the root which absorbed Al^{3+}. Spots indicating the localization of Al^{3+} were observed mainly around the epidermis. Similar localization has also been found in *Zea mays* root (*18*). As to Al^{3+} localization in the cells, it accumulated in the nucleus (*1*) and cell wall of root hair (Fig. 4), as well as in root tip cells.

In vivo binding of Al^{3+} to nucleic acids

Our results as well as those of other reports on inhibition of cell division (*3, 6*), DNA synthesis (*20*) and phosphate metabolism (*4*) lead us to investigate the possible association of Al^{3+} with nucleic acids. First, the levels of nucleic acids were compared between control and Al^{3+}-treated roots. As shown in Table 1, higher amounts of nucleic acids were found in the treated roots, indicating that nucleic

Table 1 *Changes of Al^{3+} associated with nucleic acids in Alaska pea roots*

Sample	Treatment time (hr)			
	5	24	48	72
Control	14.86	19.10	13.58	14.42
Al-treated	18.86	22.76	19.47	21.39

Pea seedlings were treated with 10^{-3} M $AlCl_3$ at 25°C and the nucleic acids were prepared by the phenol method. Values are expressed as the optical density at 260 nm per gram fresh weight.

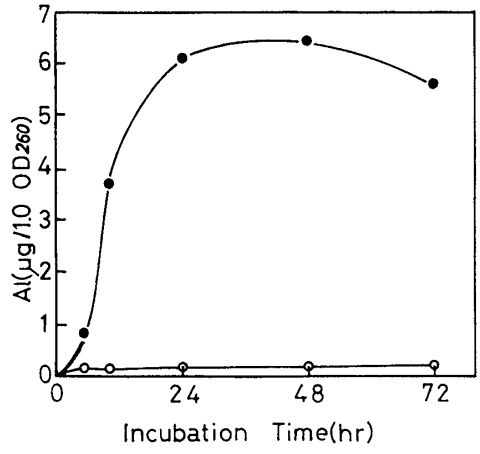

Fig. 5.

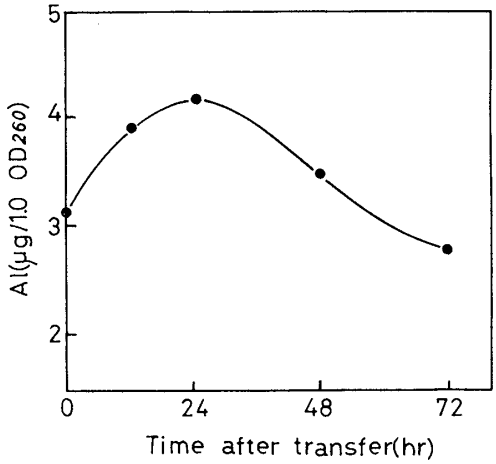

Fig. 6.

Fig. 5. *Al^{3+} contents in the nucleic acids of Alaska pea roots.* Seedlings were treated with 10^{-3} M $AlCl_3$. (●): Al^{3+}-treated, (○): control.

Fig. 6. *Effect of transfer to water on the contents of Al^{3+} in the nucleic acids.* Seedlings were treated with 10^{-3} M $AlCl_3$ for 10 hr then transfered to water.

acid metabolism was not markedly inhibited at least quantitatively in Al^{3+}-treated roots. Al was found in animal nucleic acids of various tissues: RNA of horse kidney and rat liver contained quite a lot of Al (*22*). The binding of metals to nucleic acids in some cases affects the basic metabolism, including enzyme induction, by controlling tempelate activity (*9*, *11*). Therefore, the amount of Al in nucleic acids of pea roots was estimated. As shown in Fig. 5, Al was not detected in control roots. However, Al in treated roots increased until 24 hr of treatment and maintained that level to the end of the experiment. No increase in Al after 24 hr is related to the complete stopping of root elongation (Fig. 1).

Next, the in vivo stability of Al^{3+} bound to nucleic acids was investigated by transferring the sample to distilled water after pretreatment with 10^{-3} M $AlCl_3$ for 10 hr (Fig. 6). Al^{3+} in nucleic acids increased for 24 hr after transfer. This might be due to the movement of absorbed Al^{3+} to the site, probably nuclei, where nucleic acids are synthesized in cells (*1* and Fig. 4). However, Al^{3+} in nucleic acids decreased gradually after 24 hr, because the external supply of Al^{3+} was stopped. This results also indicates that Al^{3+} once bound to nucleic acids is not easily dissociated in vivo.

In the following experiment, the preferential binding of Al^{3+} to DNA or RNA was investigated. Nucleic acids were fractionated for this purpose. There are several points which had to be considered in the present experiment. A large amount of phosphate could not be used because of its strong hindrance of Al atomization. Therefore, use of phosphate buffer was avoided. Also, ion-exchangers could not be used because of possible association of Al^{3+} with them during chromatography. Accordingly, we chose molecular sieving chromatography using Sephadex G-200 and Bio-Gel A50. The phenol-extracted nucleic acids from the roots treated with 10^{-3} M $AlCl_3$ for 1 day were loaded on a Sephadex G-200 column equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). As shown in Fig. 7, two UV-absorbing peaks were eluted. DNA and rRNA were eluted in the first peak and mainly sRNA

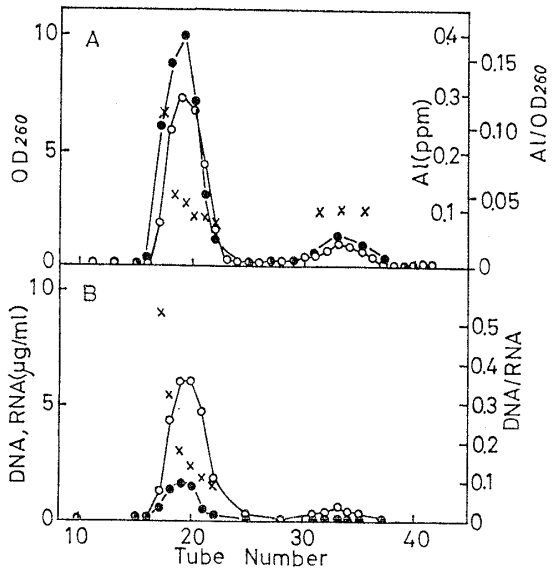

Fig. 7.

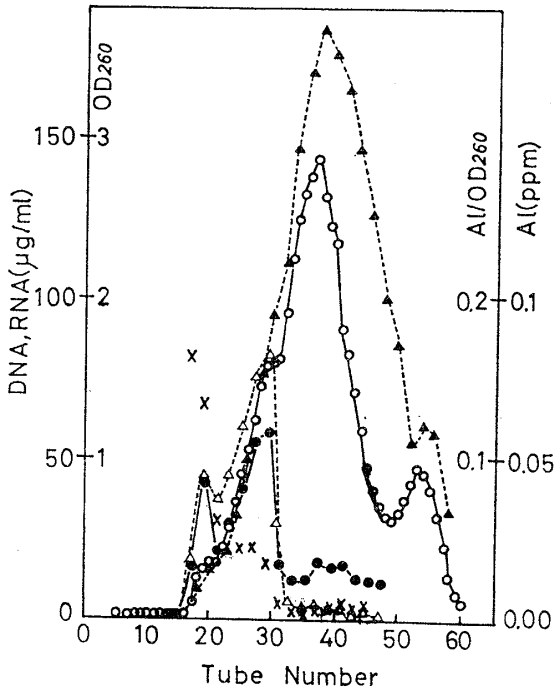

Fig. 8.

Fig. 7. *Fractionation of nucleic acids associated with Al^{3+} of Alaska pea roots by Sephadex G-200.* Seedlings were treated with 10^{-3} M $AlCl_3$ for 1 day and nucleic acids were prepared by the phenol method. A. (○): optical density (OD) at 260 nm, (●): Al, (×): Al/OD_{260}. B. (○): RNA, (●): DNA, (×): DNA/RNA.

Fig. 8. *Fractionation of nucleic acids associated with Al^{3+} of Alaska pea roots by Bio-Gel A50.* (○): optical density (OD) at 260 nm, (●): Al, (▲): RNA, (△): DNA, (×): Al/OD_{260}. For other details, see Fig. 7.

in the second one. The elution profile of Al coincided well with that of UV-absorbancy. DNA could not be completely separated from rRNA by Sephadex G-200. The molecular absorption coefficients of DNA and RNA are nearly the same. Therefore, an Al ratio resembling that of UV absorbancy in the first peak would appear if Al^{3+} binds to DNA and RNA equally. If the ratio is not similar, these should be specificity in the binding of Al^{3+} to DNA or RNA. The result clearly supports the latter case. In the first peak, the ratio of Al^{3+} to UV absorbancy was not uniform and declined sharply. This indicates that the substance which eluted earlier, probably DNA according to colorimetric determination of ribose and deoxyribose (Fig. 7B), associated with Al^{3+} more than the rRNA eluted later. Unlike the first peak, the second one was composed of mainly sRNA and the ratio of Al to UV absorbancy showed a uniform value. This means that sRNA associated with Al^{3+} equally and was eluted in the second peak. Thus, we used Bio-Gel A50 to fractionate DNA and rRNA more clearly. As shown in Fig. 8, two DNAs were eluted at around tubes 20 and 30. There are two physiologically distinct fractions of double-stranded DNA in wheat roots which can be separated by chromatography because they have different molecular weights and base compositions (*19*). Most of the Al was eluted in the DNA regions and a little of it was found in the rRNA

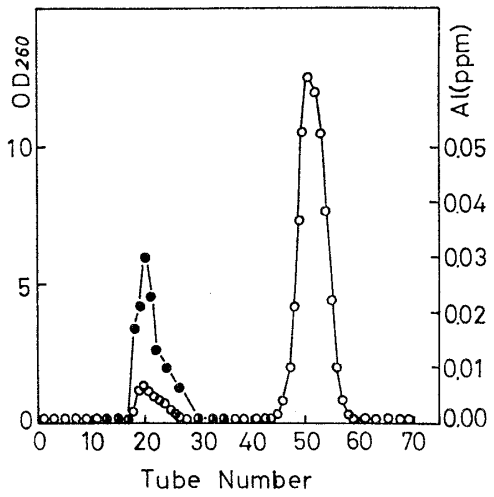

Fig. 9. *Fractionation of nucleic acids associated with Al^{3+} of Alaska pea roots after digestion with RNase.* Seedlings were treated with 10^{-3} M $AlCl_3$ for 1 day. The prepared nucleic acids were digested with RNase as mentioned in **Materials and methods** and fractionated by Bio-Gel A50. (○): optical density (OD) at 260 nm. (●): Al.

region. The ratio of Al amount to UV absorbancy indicated that Al^{3+} bound to DNA preferentially in vivo. To clarify this point further, the sample was digested with RNase and applied to Bio-Gel A50 column (Fig. 9). UV absorbancy due to rRNA was completely lost and DNA was eluted together with Al. Furthermore, the peaks of Al and UV absorbancy were completely lost after digestion of the RNase-treated sample by DNase (data not presented here). The preferential binding of Al^{3+} to DNA might be related to the localization of Al in roots; heavy accumulation was found at actively dividing regions and nuclei (Fig. 2 and 4). What physiological effect results from the binding of Al^{3+} to DNA? According to Einchhorn and Shin (*9*), metal ions can be readily placed into two categories, those that bind to phosphate and those that bind to bases. Magnesium (Mg) increases the melting temperature (Tm) of DNA by binding phosphate and stabilizes the the double helix, whereas copper(Cu) decreases Tm by binding to the bases and destabilizes the double helix. Thus Mg and Cu ions can be said to have opposite effects on DNA. Stabilizing the double helix leads to limited template activity. Due to the ease of binding between Al^{3+} and phosphorus, Al^{3+} will act to stabilize DNA unlike cadmium (Cd) which induces enzyme activity in pea tissues possibly by the increasing template activity (*11*).

In vitro binding of Al^{3+} to nucleic acids

Some binding properties of Al^{3+} to nucleic acids were investigated in vitro. DNA and rRNA purified by MAK column were used. The amounts of Al^{3+} which bind to DNA or rRNA were compared and there was essentially no difference (Table 2). When equimoles of Al^{3+} and phosphorus in DNA or RNA were mixed, the binding ratio (bound Al/P in nucleic acids) was not unity and Al^{3+} molecules bound to nucleic acids amounted to 25% of that of phosphorus molecules. However, when an excess of Al^{3+} was present, twice the number of Al^{3+} as compared to phosphorus molecules bound to nucleic acids. This probably indicates that another binding of Al^{3+} to bases occurred as in the case of manganese (*9*).

Effect of NaCl and $MgCl_2$ *on the in vitro binding of Al^{3+} to DNA*

With both metals, the positive effect on binding was observed at low concentrations, with the opposite seen at high concentrations (Table 3). $MgCl_2$ has a

Table 2 *In vitro binding of Al^{3+} to RNA and DNA*

RNA (μg)	Phosphorus in RNA (μmoles)	Al^{3+} (μmoles)	Al^{3+} bound to RNA (μmoles)	Al^{3+} bound to RNA / Phosphorus in RNA (%)
14.05	0.032	0.032	0.008	25
14.05	0.032	0.320	0.074	231
DNA (μg)	**Phosphorus in DNA (μmoles)**	**Al^{3+} (μmoles)**	**Al^{3+} bound to DNA (μmoles)**	**Al^{3+} bound to DNA / Phosphorus in DNA (%)**
14.05	0.032	0.032	0.010	31
14.05	0.032	0.320	0.071	222

RNA and DNA of Alaska pea roots in 0.01 M Tris-HCl buffer (pH 7.0) were mixed with appropriate amounts of $AlCl_3$ for 14 hr at 4°C. Samples were completely dialyzed against water to eliminate unbound Al^{3+}. The Al^{3+} bound to nucleic acid was measured. See details in **Materials and methods.**

Table 3 *Effect of NaCl and $MgCl_2$ on the in vitro binding of Al^{3+} to DNA*

DNA (μg)	Phosphorus in DNA (μmoles)	NaCl (M)	Al bound to DNA (μmoles)	%
11.24	0.029	0	0.0115	100
11.24	0.029	0.05	0.0111	96.5
11.24	0.029	0.1	0.0167	145.2
11.24	0.029	0.4	0.0219	190.4
11.24	0.029	1.0	0.0081	70.4
DNA (μg)	**Phosphorus in DNA (μmoles)**	**$MgCl_2$ (M)**	**Albound to DNA (μmoles)**	**%**
11.24	0.029	0	0.0133	100
11.24	0.029	0.01	0.0152	114.3
11.24	0.029	0.05	0.0193	145.1
11.24	0.029	0.1	0.0107	75.2

Equimoles of phosphorus in DNA of Alaska pea roots and $AlCl_3$ were mixed in the presence of various concentrations of NaCl or $MgCl_2$. The samples were then treated as shown in Table 2.

Table 4 *Effect on histone on in vitro binding of Al^{3+} to DNA*

DNA [a] (μg)	Histone (μg)	Al^{3+} bound to DNA (μmoles)	Inhibition (%)
11.7	0	0.0135	0
11.7	5.85	0.0089	34.1
11.7	11.7	0.0041	70.0
11.7	23.4	0.0028	79.3

[a] 0.030 μmoles of phosphorus in DNA.

DNA was mixed with various amounts of calf thymus histone, to which 0.030 μmoles of $AlCl_3$ was added. For other details, see Table 2.

much stronger effect than NaCl. The binding of Al^{3+} to DNA could occur in vivo judging from the ionic strength used in this experiment and the in vivo results (Fig. 5, 6, 7 and 8).

Effect of histone on the in vitro binding of Al^{3+} to DNA

The primal binding site of Al^{3+} is phosphorus in DNA. This was ascertained by masking phosphorus with histone. Most of the DNA in animal and plant cells is known to be masked by histone. Many reports show that the template activity of DNA is controlled through the interaction between DNA and chromosomal proteins (*12*, *17*, *23*). Therefore, it is interesting to know how the binding of Al^{3+} to DNA is influenced by histone. Results in Table 4 show that it is distinctly inhibited by the masking of DNA with histone. This indicates also that the primal binding site of Al^{3+} is the phosphorus group in DNA and the binding rate of Al^{3+} to DNA in vivo might be changed by the chromosomal situation which is partly affected by chromosomal proteins. This is related to the vast accumulation of Al in actively dividing regions of root tissue, where accessibility of Al^{3+} to DNA is high. Also, the binding of Al^{3+} to DNA altered by histone can be speculated to influence the template activity and be concerned with part of the injurious Al^{3+} mechanism of cell division reported by Clarkson (*3*, *6*). The biological activities affected by the Al^{3+}-DNA binding should be investigated. We should also examine the possibility that the binding of Al^{3+} to the cell wall lowers the cell wall extensibility and thereby inhibits root elongation.

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