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The interaction of aluminium with silicic acid in the presence and absence of a phosphorylated protein

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Contents

Abstract

(1) Silicic acid alone does not bind Al^{3+} (10^{-3} M) at pH values below 6.8, but above this an association is formed which is not broken by addition of the dye chrome azurol S. The precipitate which forms slowly between silicic acid and Al^{3+} can be removed by centrifugation. At this concentration (10^{-3} M), Al^{3+} alone precipitates close to pH 7.

(2) Phosvitin binds Al^{3+} at pH 5.0, with a K_D of 10^{-8} M (E. Rowatt and R.J.P. Williams, J. Inorg. Biochem., 55 (1994) 249; T.P. Geladopoulos and T.G. Sotiroudis, J. Inorg. Biochem., 54 (1994) 247) with a ratio of Al^{3+} to phosvitin of 70. The complex precipitates when about half the Al^{3+} is added but redissolves at high concentrations of phosvitin.

(3) Silicic acid does not remove Al^{3+} from phosvitin and phosvitin does not remove Al^{3+} from a mature precipitate of aluminosilicate, although it removes some Al^{3+} if it is added to aluminium newly associated with silicic acid.

(4) The relationships between aluminium, phosvitin and silicic acid can only be explained by taking into account the kinetics of aging of aluminium-silicic acid precipitates.

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1. Introduction

There is an outstanding question concerning the effect of aluminium ions, Al^{3+}, on a variety of organisms including land plants, aquatic animals and even humans. While all organisms are vulnerable to Al^{3+} if the ion reaches certain organs, e.g. gills of fish or the brain of humans, it is stated that considerable protection is provided by the presence of silicic acid in solution [1]. The question which has very important environmental implications is the degree to which this protection is indeed useful. The variable parameters which could control protection are the concentration of Al^{3+}, the pH, and the nature of the biological absorbing agent in the organism, under the conditions of saturation of waters with $Si(OH)_4$. We and others [2,3] have already studied the binding of Al^{3+} to a model target protein, phosvitin, chosen since it is a heavily phosphorylated protein and it is believed with good reason from stability constants of Al^{3+} complexes that organic phosphates are the most powerful sequestrants [4]. There are many protein phosphates in cells. The binding constant of Al^{3+} to phosvitin moves from 10^8 at pH 5 to above 10^{11} at pH 7.0. The binding of Al^{3+} to silicic acid at a variety of pH values is less well established since the final reaction product is an "aged" precipitate. There are no direct studies of the competition between silicic acid and a phosphated protein like phosvitin for Al^{3+}. It is this gap in knowledge which we fill in this article.

2. Experimental details

2.1. Materials and methods

$Al(NO_3)_3 \cdot 9H_2O$ (99.997%) was obtained from Aldrich. It was dissolved in 0.5 M Tris–0.6 M acetate buffer pH 5.0 and kept at +4 °C. The stock solution was diluted in 25 mM Tris–30 mM acetate buffer pH 5. Na_4SiO_4 from Alfa Products was a kind gift from Dr. C. Exley, University of Keele. It was dissolved in water at 2.5 mM giving a pH > 10 and taken to pH 5 or pH 7 with 1 N HCl. Phosvitin was supplied by Sigma Chemicals and was freed from polyvalent cations by passing through a Bio-Rex Ion Exchange Membrane (Bio-Rad). Tris, HCl, acetic acid and NaOH were Aristar grade chemicals from BDH. Tris buffer was taken to the appropriate pH with acetic acid, giving for 2 mM Tris a concentration of approximately 1.5 mM acetate at pH 7 and 3 mM acetate at pH 5. Chrome azurol S was supplied by Sigma Chemicals and kept frozen in 1 mM solution. Coomassie Plus Protein Assay Reagent was supplied by Pierce, IL, US. Plastic equipment and water of low conductance were used to reduce contamination with Ca^{2+} and other cations.

Absorption values were measured on a Cecil 505 double-beam spectrophotometer with a Cecil 500 control record module. Al^{3+} precipitates were deposited by centrifug-

ing at 7800*g* on a Beckmann Minifuge. pH was measured using a Philips PW9418 pH meter with a BDH glass minielectrode.

2.2. Estimation of Al^{3+}

Al^{3+} was estimated spectrophotometrically at 546 nm in the range 0–5 μM using chrome azurol S (20 μM in experiments in Figs. 1–5 and Table 1 and 8 μM in Table 2) in aliquots buffered to pH 5 with 2 mM Tris acetate buffer as described by Rowatt and Williams [2], except in the experiments on the effect of pH. In such experiments, absorption was read against water and mean values for dye absorption at a given pH were subtracted from mean values for absorption of dye + Al^{3+} at the same pH. In other experiments, estimations were carried out on samples obtained according to the protocols below and after solutions had been made up to pH 5. Firstly Al^{3+} was estimated as free ion in the presence of Al^{3+} bound to one of the other components, phosvitin and silicic acid, directly at pH 5. Secondly the aluminosilicate complex formed at pH 7 was dissociated by leaving at pH 5 for several hours and then the Al^{3+} was measured as above. Finally, Al^{3+} bound to phosvitin phosphate groups was set free by incubating the phosvitin–Al^{3+} complex in 0.25 N NaOH at 37 °C overnight to dephosphorylate the phosvitin and to release the Al^{3+}. The solution was brought back to pH 5 for Al^{3+} measurement.

2.3. Estimation of silicic acid

Silicic acid in 1 ml aliquots containing up to 100 μM was estimated by the method of King and coworkers [5] using 0.1% NH_4 molybdate, adding 0.1% ascorbic acid after 4 min and measuring absorption at 700 nm after 5 min at room temperature. The timing of all operations had to be kept constant as absorption can increase by 0.001 absorption units s^{-1}.

2.4. Estimation of phosvitin

Phosvitin was estimated with Coomassie Plus Protein Assay Reagent using 0.5 ml in 1 ml assay solution containing 0.7 μM phosvitin with the addition of 2 mM $Fe_2(SO_4)_3$ [2]. The Protein Assay Reagent was added 4 min after the Fe^{3+} and absorption measured at 595 nm after 5 min incubation.

Each estimation used a standard curve calibrated with pure compounds.

2.5. Protocols

In order to investigate the effect of pH on the measured absorption due to Al^{3+}, the constituents, chrome azurol S ± Al^{3+} in the experiments of Fig. 1, were mixed at room temperature in Tris buffer at pH 4.5 and the pH raised to pH 7.2 by sequential addition of 6 μl aliquots of 0.05 M Tris base, measuring pH and A_{546} within 2 min of each addition of base.

In the experiments of Fig. 2 chrome azurol S + Al^{3+} ± silicic acid were mixed and the pH of four samples was raised to values between 5.1 and 7.1. After measurement

of the pH of each sample, A_{546} was recorded at constant pH for 2 h. The first measurements were made within 2 min of mixing.

In the experiments of Figs. 3 and 4 on the interaction of Al^{3+} and SiO_4^{4-} and of Al^{3+} and phosvitin, components were incubated at pH 7 in Tris buffer. (Since Al^{3+} and SiO_4^{4-} do not combine at pH 5 these ions were mixed at that pH and the pH was then raised to 7. Phosvitin and Al^{3+} do combine at pH 5 and Al^{3+} was taken to pH 7 before phosvitin was added.) In many cases precipitates formed. Analysis was therefore carried out as follows. After incubation as stated at room temperature for 30 min in the experiment of Fig. 3 and 1 h in the experiment of Fig. 4, the samples were centrifuged at 7800*g* for 1.5 min and supernatant and deposit, after separation, were returned to pH 5, the supernatant by addition of HCl and the deposit by resuspending in 20 mM Tris buffer pH 5.0, the pH best suited for Al^{3+} estimation. Al^{3+} bound to SiO_4^{4-} was estimated with chrome azurol S after incubation at pH 5 for 5–10 h to break down the aluminosilicate complex. Fig. 3 records experiments showing $[Al^{3+}]$ and $[SiO_4^{4-}]$ in the solution and the precipitate for a constant concentration of SiO_4^{4-} (2 mM) and variable concentrations of Al^{3+}. In the experiments of Fig. 4, Al^{3+} (3 mM) was held constant and [phosvitin] varied to find the concentrations of phosvitin which prevented precipitation of Al^{3+}. Al^{3+} bound to phosvitin was measured after dephosphorylation of the protein with 0.25 M NaOH.

In the experiments of Fig. 5, Al^{3+} (5 μM), SiO_4^{4-} (2 mM) and phosvitin (2.8 μM) were mixed in Tris buffer at pH 5, the pH was taken to 7 with Tris base and the absorption at 546 nm read at intervals. After 90 min the pH was taken back to 5 and absorption again recorded for 40 min.

In the experiments of Table 1, Al^{3+} was made up freshly in 20 mM Tris pH 5. An aliquot of the Al^{3+} solution was added to Tris base to take the pH to 7 (Al^{3+} final concentration, 0.5 mM) followed by phosvitin (17.5 μM final concentration) and silicic acid (2 mM), each mixture being made and the pH adjusted within 1 min. Solutions were incubated for 2 h with agitation to maintain sample homogeneity when precipitates were forming. Precipitates were collected after centrifugation at 7800*g* for 1.5 min and resuspended as in Figs. 3 and 4. Al^{3+} bound to phosvitin was estimated after dephosphorylation of the protein with 0.25 M NaOH and Al^{3+} bound to SiO_4^{4-} by leaving the complex with chrome azurol S in Tris buffer at pH 5 for 5–10 h to break down the aluminosilicate complex. SiO_4^{4-} and phosvitin were measured as before.

In the experiments shown in Table 2, Al^{3+} (3 mM) and silicic acid (2 mM) having been mixed in Tris buffer at pH 5 were incubated together at pH 7 for 5 min. Phosvitin (105 μM) was incubated with the mixtures with agitation to maintain sample homogeneity when precipitates were forming for a further 2 h before separation by centrifugation. The pH was returned to 5 as previously. The constituents were then estimated by the methods used in Figs. 2, 4 and 5.

3. Results

As shown previously the dye, chrome azurol S, can be used to estimate aluminium between pH 5 and 6.5 using absorption A at 546 nm. Above pH 6.5 the method is

insensitive but an estimate of free Al^{3+} can be made up to a value of pH 7 by mixing Al^{3+} and dye at a lower pH and raising the pH of the mixture to 6.9–7.0 with Tris base. Fig. 1 is a record of such experiments in which the pH of samples of dye + Al^{3+} (5 μM) was raised from 5 to 7 by sequential addition of 6 μl aliquots of Tris base, measuring pH and A_{546} within 2 min of each addition. The time between additions was 2 min. The stability of the solutions with time was checked. At pH values below 6.5, A_{546} of chrome azurol S + Al^{3+} is constant for 2 h but above pH 6.5 it falls, about 30% being lost over 2 h at pH 7.1.

On repeating the above experiment with silicic acid present the silicic acid does not reduce absorption up to pH 6.5 but above that the value for A_{546} falls relative to that in Fig. 1 (Fig. 2). The data given in Fig. 2 show that below pH 6.5 the presence of silicic acid in the solution does not affect the Al^{3+} binding to the dye. Above this pH it is equally clear that there is less free Al^{3+} in solution than in the absence of $Si(OH)_4$. Note that the Al^{3+} is precipitated by the silicic acid at pH above 6.8. The precipitation is complete above pH 7: no Al^{3+} remains detectable in solution (see below).

Interaction of Al^{3+} and silicic acid can also be shown by removing the aluminosilicate by centrifugation. In the experiments of Fig. 3, a fixed concentration of silicic acid has been incubated at pH 7 with increasing concentration of Al^{3+}. Al^{3+} is present in the deposit at all concentrations and silicic acid is precipitated equivalent to the [Al^{3+}] with a ratio of the order of 1:1.

In the presence of phosvitin the Al^{3+} in solution or in a precipitate at pH 7 is compared with the Al^{3+} available to the dye in the absence of protein. The data in Fig. 4 show that when phosvitin is in excess of the 70:1 Al^{3+}:phosvitin binding

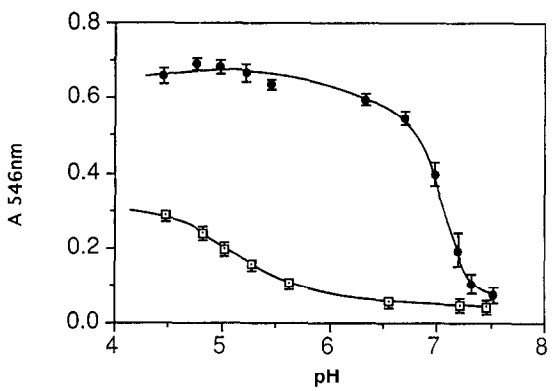

Fig. 1. Variation with pH in absorption at 546 nm of chrome azurol S and Al^{3+}. Chrome azurol S (20 μM) ± Al^{3+} (5 μM) in 1 ml 2 mM Tris acetate buffer pH 4.5 was incubated at room temperature in cuvettes. Aliquots of Tris base (approximately 6 μl aliquots of 0.05 M) were used to raise the pH of the solution in steps up to pH 7, and pH and A_{546} were measured initially and after each addition. Mean values for each pH were calculated from values (from four experiments) varying within 0.1 pH unit. Values in the graph without Al^{3+} are given after subtraction of a water blank; in those with Al^{3+}, the value of the dye blank at the appropriate pH has been subtracted: □, chrome azurol S; ●, chrome azurol S + Al^{3+}.

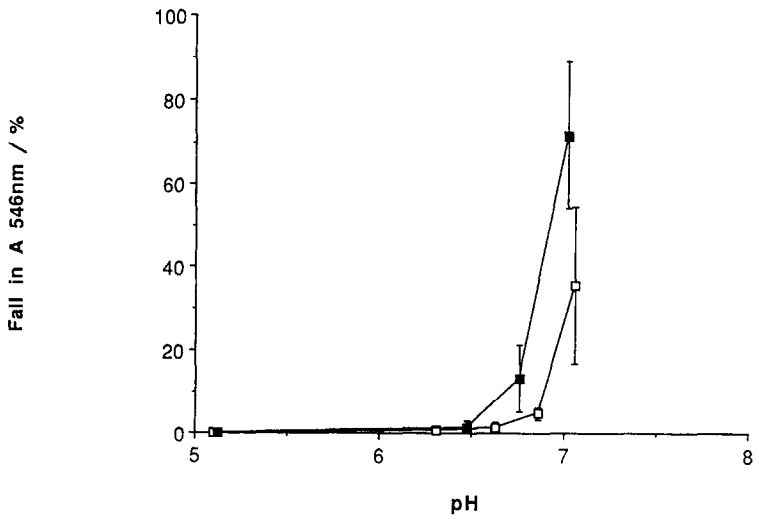

Fig. 2. Measurement of Al^{3+} binding by SiO_4^{4-} at various pH values. Chrome azurol S (20 μM) + Al^{3+} (5 μM) ± Na_4SiO_4 (2 mM) in 1 ml 2 mM Tris acetate buffer pH 5.0 in cuvettes were incubated at room temperature. One sample was kept at pH 5 and the pH of the rest was raised with Tris base to values between 6 and 7 (four cuvettes per experiment, six experiments). pH and A_{546} were measured immediately and at intervals for 2 h. The fall in A was calculated at 2 h (with dye blank) and mean values were calculated from results differing by ±0.2 pH unit: □, Al^{3+}; ■, Al^{3+} + silicic acid.

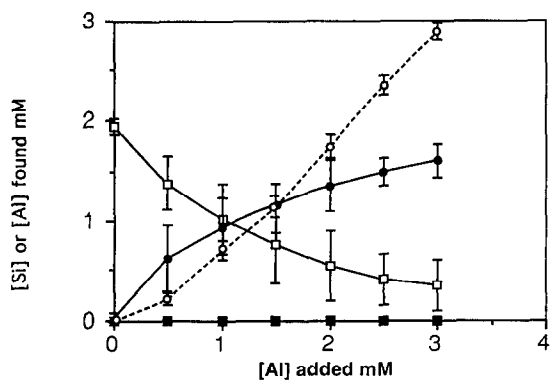

Fig. 3. Precipitation of Al^{3+} + SiO_4^{4-} at pH 7. SiO_4^{4-} (2 mM) and increasing concentrations of Al^{3+} were mixed in Tris buffer at pH 5 and the pH taken to 7 with Tris base. After 30 min at room temperature, the solutions were centrifuged at 7800*g* for 1.5 min and [SiO_4^{4-}] and [Al^{3+}] measured in supernatant and deposit at pH 5. Samples for Al^{3+} assay were left at pH 5 with chrome azurol S for 16 h so that the Al^{3+}–SiO_4^{4-} complex dissociated, the chrome azurol S keeping dissociated Al^{3+} in solution. Results are the mean of three experiments: □, SiO_4^{4-} in supernatant; ●, SiO_4^{4-} in deposit; ■, Al^{3+} in supernatant; ○, Al^{3+} in deposit.

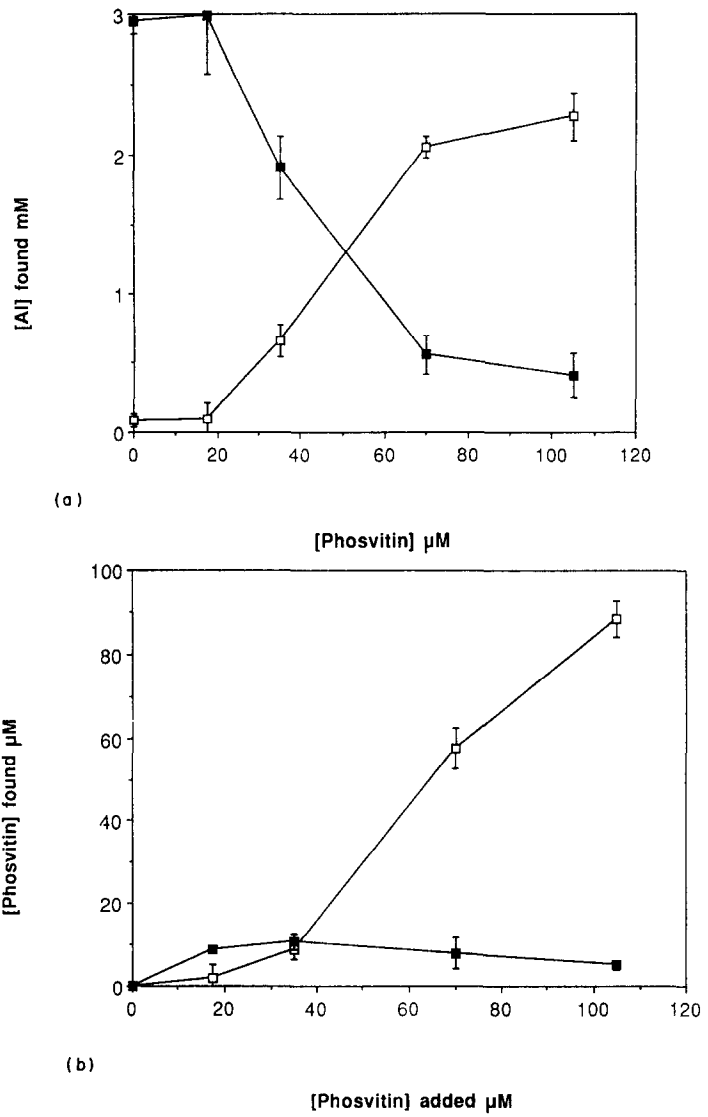

Fig. 4. Precipitation of phosvitin by Al^{3+}. Al^{3+} (3 mM) was added to 2 mM Tris buffer at pH 5, the pH taken to 7 with Tris base and increasing concentrations of phosvitin added. After 1 h at room temperature samples were centrifuged and the pH of fractions was taken to 5 with HCl. Phosvitin was measured directly in both fractions and Al^{3+} was measured after dephosphorylation of the protein with 0.25 M NaOH overnight at 37 °C. Recovery of phosvitin was not good, especially at low concentrations in the deposit. (a) Al^{3+} found in supernatant (□) and deposit (■). (b) Phosvitin found in supernatant (□) and deposit (■).

ratio the phosvitin keeps all the Al^{3+} in solution. At a higher concentration of aluminium (3 mM), Al^{3+} precipitates without additional anions and can be removed by centrifugation.

The next figure, Fig. 5, shows the distribution of Al^{3+}, silicic acid and phosvitin in solution at pH 7. Chrome azurol S is included so that free [Al^{3+}] can be followed with time. As stated before under these conditions the absorption caused by Al^{3+} alone (5 μM) falls but silicic acid (2 mM) and phosvitin (2.8 μM) increase the fall in absorption considerably with the higher concentration of silicic acid causing a slightly more rapid fall. Lowering the pH to 5 after 90 min incubation results in an increase in free Al^{3+} in both the solution of Al^{3+} alone and in that of Al^{3+} + silicic acid. There is no comparable dissociation of Al^{3+} from phosvitin even when silicic acid is present.

This effect was studied further in the experiments shown in Tables 1 and 2. In the experiments of Table 1, phosvitin (17.5 μM) was added to Al^{3+} in Tris buffer at pH 7.0 and silicic acid (2 mM) was added within 1 min. After 2 h incubation at room temperature with gentle agitation, any precipitate was separated by centrifugation and the components estimated in supernatant and deposit. Al^{3+} was found in the deposit after incubation with silicic acid but not in that from incubation of Al^{3+} + phosvitin or of all three components.

In the experiments of Table 2 higher concentrations of Al^{3+} and phosvitin were used. Silicic acid (2 mM) was incubated with Al^{3+} (3 mM) in Tris buffer pH 7.0 for 5 min before addition of phosvitin (105 μM). After 2 h incubation at room temperature with agitation, precipitates were removed as before and Al^{3+} was found in the deposit of Al^{3+} incubated alone, or with silicic acid. Al^{3+} was kept in solution by phosvitin and with phosvitin + silicic acid some Al^{3+} was held in solution by phosvitin but some was precipitated with silicic acid. Note that the silicic acid concentration was four times that of the Al^{3+} and only one-quarter of the silicic acid was

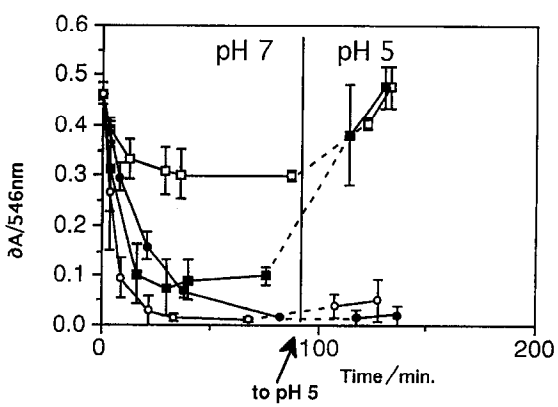

Fig. 5. Al^{3+} binding by phosvitin and silicic acid. Al^{3+}, SiO_4^{4-} and phosvitin were mixed in Tris buffer at pH 5 with chrome azurol S (20 μM), the pH was taken to 7 with Tris base and the absorption at 546 nm read at intervals. After 90 min the pH was taken back to 5 and absorption again recorded for 40 min: □, Al^{3+}; ●, Al^{3+} + phosvitin; ■, Al^{3+} + SiO_4^{4-}; ○, Al^{3+} + SiO_4^{4-} + phosvitin.

Table 1
Binding of Al^{3+} by phosvitin and SiO_4^{4-} at pH 7. Phosvitin added before SiO_4^{4-}.

Ions present			Concentration of ions found					
Al^{3+}	SiO_4^{4-}	Phosvitin	Al^{3+} (μM)		SiO_4^{4-} (mM)		Phosvitin (μM)	
			Supernatant	Deposit	Supernatant	Deposit	Supernatant	Deposit
+			347 ± 33	47 ± 16				
	+				2.1 ± 0.1			
		+					15.8 ± 2.4	
+	+			492 ± 25	1.6 ± 0.1	0.4 ± 0.6		
+		+	468 ± 12				17.4 ± 1.3	
+	+				2.1 ± 0.1		14.6 ± 1.4	
+	+	+	393 ± 10	42 ± 30	2.1 ± 0.1		14.8 ± 1.0	

Al^{3+} (final concentration 0.5 mM) in 0.01 M Tris buffer pH 5 was mixed with 2 mM Tris base to give pH 7.1. Phosvitin (final concentration 17.5 μM) was added followed within 1 min by silicic acid (final concentration 2 mM). The solutions were incubated with gentle agitation at room temperature for 2 h. Precipitates were then deposited by centrifugation at 7800*g* for 1.5 min and the precipitates resuspended in 20 mM Tris buffer pH 5.0 and the pH of the supernatants lowered to 5 with 0.1 N HCl. Phosvitin and silicic acid were estimated as before. Al^{3+} was estimated after dephosphorylation of phosvitin with 0.25 N NaOH at 37 °C overnight. The pH of these solutions with Al^{3+} and chrome azurol S (8 μM) in the Al^{3+} estimation system was taken to 5.0 with 0.1 N HCl and absorption at 546 nm read after 5–16 h. The concentration of an ion in a fraction is only recorded if it is more than 8% of the main fraction. Mean of three experiments.

precipitated. In similar experiments in which Al^{3+} and silicic acid were incubated for 1 h before the 2 h incubation with phosvitin, all Al^{3+} was precipitated with silicic acid and none was found in solution with phosvitin.

4. Discussion

The following points are clear.

(1) Al^{3+} on its own is only fully available to the dye below pH 6.5. This means that the solubility product of $Al(OH)_3$ exceeds that of the binding constant of the dye increasingly above that pH. The precipitation is a relatively slow process to reach equilibrium.

(2) Al^{3+} is precipitated to a greater degree by $Si(OH)_4$ than on its own but only at pH > 6.5. At pH 7 (Fig. 3) the precipitation is complete but again the precipitate ages.

(3) Al^{3+} is not precipitated by phosvitin if the ratio of protein to Al^{3+} is such that all phosvitin is in excess over that required to bind $70Al^{3+}$ per molecule. Al^{3+} is not available to the dye in the presence of phosvitin. The phosvitin binding constant is at least 10 times that of the dye.

(4) When phosvitin and silicic acid are given equal access to Al^{3+} or if Al^{3+} is bound to phosvitin first then silicic acid does not bind the Al^{3+}.

Table 2
Binding of Al^{3+} by phosvitin and SiO_4^{4-} at pH 7. $SiO_4^{4\pm}$ added before phosvitin

Ions present			Concentration of ions found					
Al^{3+}	SiO_4^{4-}	Phosvitin	Al^{3+} (mM)		SiO_4^{4-} (mM)		Phosvitin (μM)	
			Supernatant	Deposit	Supernatant	Deposit	Supernatant	Deposit
+				2.8±0.2				
	+				1.7±0.3			
		+					96.1±5.1	
+	+			2.8±0.2		1.8±0.1		
+		+	1.9±0.4	0.7±0.3			94.3±3.1	
	+	+			1.7±0.4		101.8±4.3	
+	+	+	1.3±0.5	1.9±0.58	0.4±0.3	2.0±0.1	90.5±2.1	

Al^{3+} (3 mM) and SiO_4^{4-} (2 mM) were mixed in a tube in 25 mM Tris buffer pH 5 and the pH was raised to 7.0 with Tris base (about 1/25 volume 0.5 M). After 5 min at room temperature, phosvitin (105 μM) was added for a further 2 h incubation during which time the solution and precipitates were gently agitated. Samples were then centrifuged to deposit $Al_4(SiO_4)_3$ and the pH of the supernatant was returned to 5 with HCl while the deposit was resuspended in 20 mM Tris buffer pH 5. Silicic acid, phosvitin and Al^{3+} were estimated as shown in Section 2.1 with Al^{3+} estimated after alkaline hydrolysis followed by incubation overnight with chrome azurol S before measurement of absorption at 546 nm. Concentrations of constituents in one fraction which constitute less than 8% of the concentration in the other fraction are not recorded.

(5) If Al^{3+} and silicic acid are mixed at pH 7 and allowed to age for 5 min then any removal of Al^{3+} from the SiO_4^{4-} is very slow and may never be complete. After long periods of aging of aluminium-silicate precipitates the aluminium is even less available.

We conclude that addition of silicic acid to water at pH values below 6.5 will not remove Al^{3+}. The phosphorylated protein combines strongly with Al^{3+} at these pH values. Al^{3+} toxicity in fish rarely occurs above pH 6 and, at this pH of the surrounding water, the pH of the water of the gill boundary layer which is made more alkaline by the fish gill [6] is still only 6.7. Provided that the pH range of binding by the phosphorylated proteins found in the fish gill is similar to that of phosvitin, it is unlikely that silicic acid would prove a satisfactory detoxicant for Al^{3+}. This does not imply that silica cannot act as a preventative under conditions such as those outlined in Ref. [1]. We have not carried out long-term studies of the effect of silicic acid on Al^{3+} concentrations in the absence or presence of other binding agents as yet. (Since this paper was submitted, we have seen the work of Farmer and Lumsdon [7] which shows that $Si(OH)_4$ is not well able to combine with Al^{3+} in acidic natural waters.)

Some other features of multicellular organisms are important. Obviously if a protein has phosphorylated groups close to one another as in phosvitin, then at pH 7 the binding constant for aluminium is 10^{10} or more, and Al^{3+} may well assist cross-linking of proteins and their precipitation. This occurs for phosvitin at Al^{3+} ratios lower than 70:1. There can be little protection from small molecules in the

cell or removal by $Si(OH)_4$. As far as we know aluminium silicates have not been seen in cells, but Al^{3+} is found in Alzheimer-related phosphoproteins. (No causal effect is implicated by our work but exacerbation is a clear risk).

Now, a pH of 7 is maintained inside the cytoplasm of animal cells and in extracellular fluids and the only regions of lower pH are some vesicles. In plant cells the vacuole and the outside of cells can be as low as pH 5.0 [8]. Many plants precipitate $Si(OH)_4$ at this pH but no aluminium is found [9,10]. It would appear that Al^{3+} is either not absorbed or rejected by plants since the total concentration of this element remains low.

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