Speciation of Al^{III} in Blood Serum − The Al^{III}−Citrate−Phosphate Ternary System

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Keywords: Aluminium / Citric acid / Inorganic phosphate / Speciation of blood serum / NMR spectroscopy / Bioinorganic chemistry

Time-dependent speciation studies have been carried out on the ternary Al^{III}–citrate (A)–phosphate (B) system in order to clarify the solution state of Al^{III} in blood serum. The potentiometric results indicate that ternary complexes predominate both in freshly prepared mixtures and at equilibrium. The ternary species AlAB and $AlABH_{-1}$ and the binary species $AlAH_{-1}$ and $AlBH_{-1}$ are present at physiological pH. As the solution ages the amount of the trinuclear species $[Al_3(AH_{-1})_3(OH)]^{4-}$ increases at the expense of the mononuclear binary and ternary complexes. ^{31}P NMR spectra measured at neutral pH provide corroborating evidence of the formation of the ternary complexes AlAB and $AlABH_{-1}$ and binary species $AlBH_{-1}$ and Al_2BH_{-3} found potentiometrically. Time-dependent ^1H NMR measurements show that monodentate phosphate can slowly displace citrate from the otherwise very stable structure of the trinuclear species $[Al_3(AH_{-1})_3(OH)]^{4-}$. At blood serum concentrations [$c(Al^{III})$ = 3 μM], Al^{III} is mostly bound to citrate either in binary species or in the ternary species formed with phosphate. However, with increasing Al^{III} concentrations binding to phosphate becomes more important.

Introduction

The toxicity of aluminium is recognized as the cause of various neurological disorders, including dialysis dementia and other skeletal and haematological disorders.[1,2] Its involvement in Alzheimer's disease (AD) is rather controversial, although it is largely accepted that Al^{III} is not a causative agent but may serve as a risk factor by inducing conformational changes in neuropeptides, leading to the enhanced formation of plaques and tangles, the common markers of AD.[3,4] To understand the toxic effects of Al^{III}, it is important to know the chemical forms in which Al^{III} can enter the organism or in which it is present in natural waters, soil waters, biological fluids, and tissues. The absorption, excretion, tissue retention, and deposition of Al^{III} depend on the properties of the Al^{III} complexes formed with exogenous and endogenous biological ligands. After absorption in the body, Al^{III} is transported via the blood stream to the target organs. The main carrier of Al^{III} in blood serum is transferrin;[3−5] albumin, the other high molecular mass (h.m.m.) protein, is not an efficient aluminium binder.[6] Transferrin binds about 80% of the total Al^{III}; the remaining 20% is complexed by the low molecular mass (l.m.m.) bioligands present in blood serum.[7] As regards the most important l.m.m. Al^{III} binders, the picture is rather controversial. Dayde et al.,[8] Jackson,[9] and Harris[7] propose inorganic phosphate, while Clevette and Orvig,[10] Duffield et al.,[11] and Öhman and Martin[12,13] suggest that citrate is the main Al^{III} binder. The main reasons for the divergent results are the kinetically rather sluggish equilibration of the Al^{III}−citrate system and the very slow ligand-exchange processes of the citrate complexes of Al^{III}.[14−16] In freshly prepared solutions of Al^{III} and citrate, 1:1 mononuclear species are present; these polymerize to the trinuclear complex $[Al_3(AH_{-1})_3OH]^{4-}$ in rather slow processes. In the presence of a large excess of the ligand, 1:2 complexes predominate and the trinuclear species is of less importance. At pH 7.4 and a metal ion-to-ligand ratio of 1:1, besides the aforementioned trinuclear complex, the mononuclear species $[Al(AH_{-1})]^-$ and $[Al(AH_{-1})OH]^{2-}$ are present in solution; at higher ligand excesses, the bis complexes $[Al(AH_{-1})(A)]^{4-}$ and $[Al(AH_{-1})_2]^{5-}$ are formed.[14−17]

The Al^{III}−phosphate system can only be studied directly in a fairly acidic pH range, because at pH > 4 $AlPO_4$, or, more precisely, a mixed hydroxo-phosphato complex precipitates. To estimate the binding constants of Al^{III}−phosphate species at physiological pH, linear free-energy relationships (LFER) have been used. Harris[7] and Atkari et al.[18] concluded that the predominant species at pH

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7.4 is the mixed hydroxo complex $Al(PO_4)(OH)^-$, but they obtained values differing by approximately an order of magnitude for the binding constant of this species.

All the models proposed for the biological speciation of Al^{III} only take into account binary species of Al^{III} with l.m.m. bioligands. However, biological systems invariably contain many potential binder ligands and hence the formation of ternary complexes may also be very important. Since the main potential l.m.m. Al^{III} binders in blood serum are citrate and phosphate, the formation of their ternary species would seem to be a very logical assumption. For this reason, we have studied the Al^{III}−citrate (A)−phosphate (B) ternary system. As a consequence of the slow oligomerization processes, time-dependent speciation measurements could be carried out. The time-dependent species distribution can give a more accurate description of the Al^{III} speciation in blood serum as Al^{III} does not generally attain thermodynamic equilibrium in biological fluids because of its very sluggish ligand-exchange reactions.

Results and Discussion

Time-Dependent Potentiometric Study

Potentiometric titrations were carried out in order to determine the protonation constants of the two ligands and the concentrations of their solutions. The proton complex formation constants obtained in this way are reported in Table 1.

For the Al^{III}-containing samples, as for the binary Al^{III}−citrate system,[16] very sluggish electrode responses were observed in the slightly acidic and neutral pH ranges. Thus, instead of carrying out normal titrations, the Al^{III}-containing samples were analysed using the batch method described in the Exp. Sect.. The time course of the pH changes proved to be dependent on the starting pH of the samples: starting at pH < 5.6, the hydrogen ion concentration increases with time, while above this pH it decreases. Similar behaviour was observed for the binary Al^{III}−citrate system[16] and was explained in terms of the slow oligomerization of the mononuclear complexes with formation of the trinuclear species $[Al_3(AH_{-1})_3OH]^{4-}$. While the oligomerization of the semi-protonated mononuclear complexes is accompanied by the release of protons in the acidic pH range, at basic pH the mixed hydroxo species oligomerize with the liberation of hydroxide ions.[16] This explanation seems to be applicable to the ternary system as well (vide supra). For each sample, the zero-time data (the pH values corresponding to the time of mixing of the components) were extrapolated from the first three minutes of the pH changes.

Through a combination with the potentiometric data obtained at the same time, a series of titration curves was created. Some selected curves obtained at an Al^{III}−citrate−phosphate ratio of 1:1:1 are depicted in Figure 1. The main differences between the titration curves relating to different times are seen in the pH range 3−5.6, the range of formation of the trinuclear citrato complex. At higher pH, in contrast to the results obtained for the Al^{III}−citrate system, no measurable time dependence was observed. The titration curve obtained after 30 h represents the equilibrium state. At an Al^{III}−citrate−phosphate ratio of 1:1:4, the time-induced changes are not so spectacular, due to the large excess of phosphate, which may partly suppress the oligomerization processes and also buffer the pH effect of the oligomerization.

In the evaluation of the titration curves corresponding to the equilibrium state, the stability constants of the binary complexes in the Al^{III}−citrate system were regarded as known values; we used the results obtained earlier in a time-dependent speciation study of the binary Al^{III}−citrate system.[16] For the binary Al^{III}−phosphate species, our basis was the speciation model proposed by Atkari.[19] For this system, pH equilibrium could be attained in less than 5 min for each point and thus normal titrations were carried out for speciation description. The applied model, however, gives practically no information on the species formed at pH > 4, since the formation of a precipitate of alumi-

Table 1. Stability constants of proton and Al^{III} binary complexes of citrate (A) and phosphate (B) and ternary complexes of citrate and phosphate at 25 °C and I = 0.2 M (KCl)

log K[a]/log β Al^{III}−citrate[16]		Al^{III}−phosphate[19]		Al^{III}−citrate−phosphate	
$K([HA]^{2-})$	5.57(2)	$K([HB]^{2-})$	11.52(1)		
$K([H_2A]^-)$	4.27(2)	$K([H_2B]^-)$	6.63(1)		
$K([H_3A])$	2.87(3)	$K([H_3B])$	1.86(1)		
$[AlAH]^+$	10.18	$[AlBH_2]^{2+}$	19.65	$[AlABH_2]^-$	28.46(4)
[AlA]	7.85	$[AlBH]^+$	17.60	$[AlABH]^{2-}$	25.02(7)
$[AlAH_{-1}]^-$	4.27	[AlB]	13.50	$[AlAB]^{3-}$	19.68(6)
$[AlAH_{-2}]^{2-}$	−1.77	$[AlBH_{-1}]^-$	8.37	$[AlABH_{-1}]^{4-}$	12.03(7)
$[AlA_2]^{3-}$	12.73	$[Al_2B]^{3+}$	17.42		
$[AlA_2H_{-1}]^{4-}$	7.81	$[Al_2BH_{-2}]^+$	11.05	fitting (Δ cm^3)[b]	0.0083
$[AlA_2H_{-2}]^{5-}$	0.4	$[Al_2BH_{-3}]$	6.9		
$[Al_3A_3H_{-4}]^{4-}$	16.34				

[a] log K values are the logarithms of the stepwise protonation constants, which are numerically the same as the negative logarithms of the acidity constants pK_a of the corresponding species; ±3 S.D. values are given in parentheses. − [b] Average difference between the experimental and calculated titration curves, expressed in cm^3 of titrant.

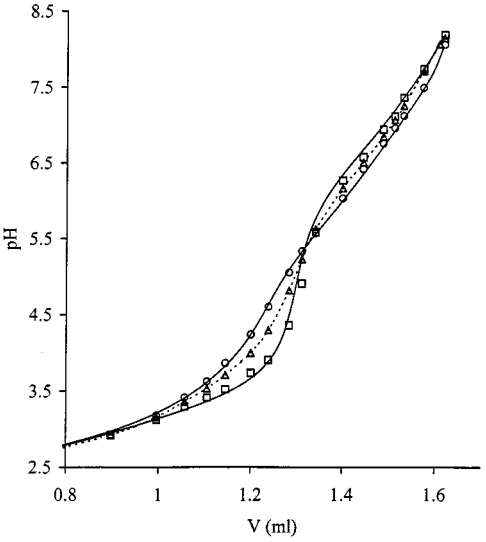

Figure 1. Titration curves (plotted as mL titrant KOH versus pH) of the Al^{III}−citrate−phosphate system at a ratio of 1:1:1, c_{Al} = 0.004 M and at different times after mixing of the reactants: (O) immediately after mixing (zero time), (Δ) 40 min after mixing, (□) at equilibrium (after 30 h); the solid curve was calculated using the stability constants reported in Table 1; the intermediate dashed curve serves only to join the titration points and does not represent any theoretical model

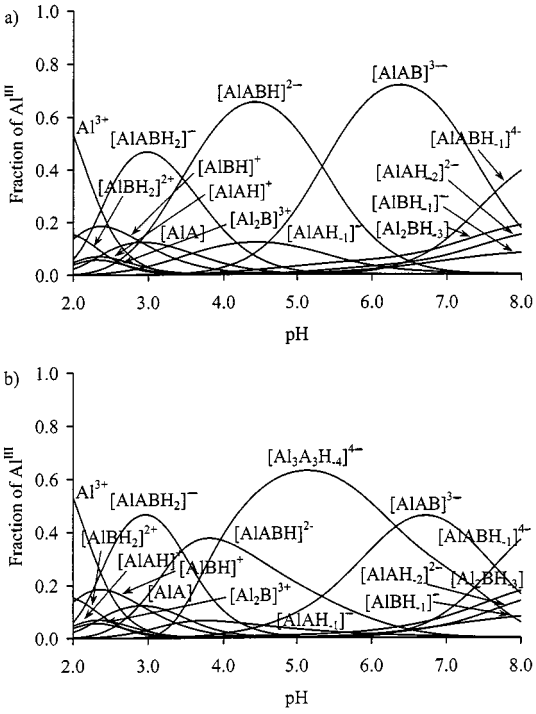

Figure 2. Species distribution curves as a function of pH for the Al^{III}−citrate−phosphate system at a 1:1:4 ratio, c_{Al} = 0.004 M and at different times after sample preparation: (a) immediately after mixing, (b) at equilibrium (after 30 h)

nium(III) phosphate does not allow potentiometric studies above this pH. For data evaluation in the neutral and slightly basic pH ranges, the stability constants of the Al^{III}−phosphate complexes [AlB] (log β = 13.50) and $[AlBH_{-1}]^-$ (log β = 7.20) estimated by LFER calculations[18] were used. In addition to the binary citrato and phosphato complexes, the formation of ternary Al^{III}−citrate−phosphate species was assumed in the speciation calculations. The species distribution calculated using the stability constants obtained for the ternary system at equilibrium showed that the amount of the Al^{III}−phosphate complex $[AlBH_{-1}]^-$ was negligible. In contrast, NMR spectroscopy indicated the presence of a considerable amount of this binary Al^{III}−phosphate complex at neutral pH (see later). This led to the conclusion that the stability constant of the complex $[AlBH_{-1}]^-$ (log β = 7.2) obtained by means of LFER calculations[18] may be underestimated. It should be noted here that Harris[7] suggested a stability constant one order of magnitude higher (log β = 8.37) for this species. When this value was used in the model calculations, the fitting of the experimental titration data were slightly improved. The speciation model obtained and the stability constants of the complexes are listed in Table 1. The titration curves corresponding to the zero-time data were fitted well if the sole formation of mononuclear complexes was assumed and the trinuclear complex $[Al_3(AH_{-1})_3OH]^{4-}$ was excluded from the speciation model. Speciation curves of the complexes formed on mixing of the components and in the equilibrium state are depicted in Figure 2.

Both immediately after mixing and at equilibrium, the ternary complexes $[AlABH_2]^-$, $[AlABH]^{2-}$, $[AlAB]^{3-}$, and $[AlABH_{-1}]^{4-}$ predominate in the system over the entire pH range. In addition to these complexes, as Figure 2 indicates, the concentration of the trinuclear species $[Al_3(AH_{-1})_3OH]^{4-}$ increases with time at the expense of the initially formed mononuclear binary and ternary complexes. In fresh solutions, the trinuclear species is hardly formed, while at equilibrium it is the predominant complex over a wide pH range. It can also be seen that at physiological pH (7−7.4) most of the Al^{III} is bound in the ternary complexes $[AlAB]^{3-}$ and $[AlABH_{-1}]^{4-}$, but that significant amounts of the binary phosphato complexes $[Al_2BH_{-3}]$ and $[AlBH_{-1}]^-$ are also present. Because of the excess of phosphate, the mononuclear and trinuclear binary citrato complexes are somewhat less important at an Al^{III}−citrate−phosphate ratio of 1:1:4. In the absence of citrate, Al^{III}−phosphate precipitates under neutral to alkaline conditions as a solid of variable composition, $Al(PO_4)_f(OH)_{3(1-f)}$, where the value of f varies from 1 to 0 as a function of the pH and concentration of the solution.[13] Detailed solubility measurements led Martin and Öhman[13] to conclude that $f = 0.75$ at pH 7.4; thus, the precipitate may be described as $Al(PO_4)_{0.75}(OH)_{0.75}$ and characterized by $\log K_{SO} = [Al^{3+}][PO_4^{3-}]^{0.75}/[H]^{0.75} = -11.1$. In the presence of citrate (at Al^{III}−citrate ratios of at least 1:1 and up to a maximum tenfold phosphate excess), the solutions are clear even at higher concentrations (0.04 and 0.1 M).[13] However, besides the proposed soluble Al^{III}−phosphate complexes $[Al_2BH_{-3}]$ and $[AlBH_{-1}]^-$, this Al^{III}−hydroxide−phosphate precipitate dissolves to form not only ternary Al^{III}−citrato−phosphato−hydroxo complexes, but probably also outer-sphere complexes with the

binary Al^{III}−citrate species through electrostatic and/or hydrogen-bonding interactions. This interpretation may explain our observation that potentiometry indicates the formation of a greater amount of ternary complexes than is suggested by NMR spectroscopy (vide infra).

As regards the ternary complexes, the complexation starts with the formation of $[AlABH_2]^-$ in acidic solution. In this complex, both ligands are coordinated to Al^{III}, probably each in a monoprotonated form. Thus, the complex should be written as $[AlCitH(HPO_4)]^-$. Here, the citrate binds to Al^{III} as a tridentate ligand through one terminal and the central carboxylate group and also the protonated hydroxy group (as in the binary species $[AlAH]^+$ [16]) and the hydrogen phosphate coordinates to Al^{III} as a monodentate ligand. The other terminal carboxylic group of the citrate ion is protonated and unbound. Further deprotonations occur on the coordinated hydroxy group and the unbound terminal carboxylate group with formation of the complexes $[Al(Cit)(HPO_4)]^{2-}$ and $[Al(CitH_{-1})(HPO_4)]^{3-}$. The ternary species $[AlABH_{-1}]^{4-}$ eventually formed can exist in two microforms: $[Al(CitH_{-1})(PO_4)]^{4-}$, with the phosphate coordinated to Al^{III} as a trivalent ligand with all hydroxy groups in deprotonated form, and $[Al(CitH_{-1})(HPO_4)(OH)]^{4-}$, which is a mixed hydroxo species.

NMR Spectroscopic Measurements

The formation of ternary Al^{III}−citrate−phosphate species was also unambiguously demonstrated by NMR spectroscopy. For correct NMR signal assignment, we first studied the Al^{III}−phosphate system. Figure 3, a, shows the ^{31}P NMR spectrum recorded at pH ≈ 2.2 and an Al^{III}−phosphate ratio of 1:2. The intense sharp signal at δ = 1.03 is due to free phosphate, while the other resonances can be attributed to the complexed ligand. Although a number of authors have investigated the Al^{III}−phosphate system, the assignment of the ^{31}P NMR signals remains uncertain. Akitt et al.[20,21] attributed these signals to differently protonated mononuclear 1:1 and 1:2 species and a polynuclear complex. Feng and Waki[22] assumed the presence of coordination isomers. The authors generally agree that, in the presence of excess ligand, the signal at δ = −6.37, which is the closest to the signal of the free ligand, is due to a bis-Al^{III}−phosphate complex. Our results for an Al^{III}−phosphate solution at a metal ion-to-ligand ratio of 1:4 and pH ≈ 2.2 support this assumption, since the intensity of the signal in question is increased compared to that in the spectrum recorded at a metal ion-to-ligand ratio of 1:2 and also relative to the other signals in the spectrum (not shown). For the ternary Al^{III}−citrate−phosphate system, the formation of binary Al^{III}−citrate species results in a higher phosphate excess than in the citrate-free solution under the same conditions, which favours formation of the bis-Al^{III}−phosphate complex. This is reflected in an increased intensity of the resonance at δ = −6.37 (Figure 3, b). Akitt et al.[21] also detected this signal at more acidic pH, and assigned it to a highly protonated 1:2 complex, AlB_2H_6. However, the formation of this species with the phosphate in fully protonated form is highly questionable, especially at pH ≈ 2. No similar bis complex has been found at acidic pH in any potentiometric study. As regards the other signals in the spectrum (see Figure 3, a), by analogy with the Al^{III}−phosphinate system, Feng and Waki[22] established that the coordination mode of the ligand (proceeding from the signal of the free ligand to higher fields) varies in the following order: monodentate, bidentate, and bridged coordination. To summarize the literature results and those from our own experiments, and additionally taking into account our potentiometric findings, the signals in the spectrum depicted in Figure 3, a, could be assigned as follows: free phosphate: δ = 1.03; highly protonated bis complex with monodentate phosphate coordination: δ = −6.37; monodentate phosphate-coordinated species $[AlBH_2]^{2+}$ and $[AlBH]^+$: δ = −6.91; the complex $[AlBH]^+$ with the ligand coordinated in a bidentate fashion and the binuclear phosphate and/or hydroxide-bridged complex $[Al_2B]^{3+}$: δ = −11.42 (the shape of the resonance suggests the presence of two overlapping signals belonging to two different species). The signal at δ = −16.42 was assigned by Akitt et al.[21] to another polynuclear (most probably trinuclear) complex. Feng and Waki[22] could not detect this latter species in less concentrated solution (0.025 M). At the millimolar concentrations employed in our potentiometric study, the formation of this polynuclear complex is probably negligible.

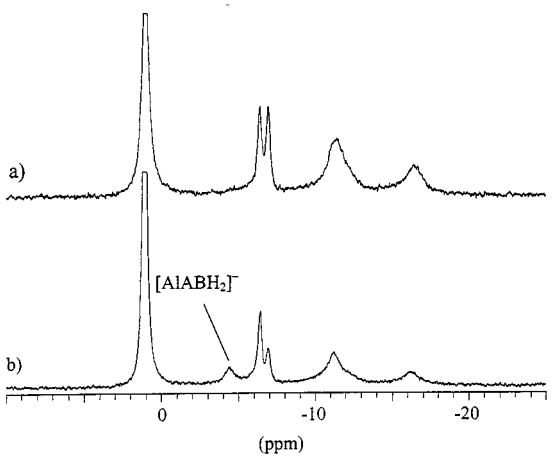

Figure 3. ^{31}P NMR spectra measured at pH ≈ 2.2 in a solution of (a) Al^{III}−phosphate at a 1:2 ratio and (b) Al^{III}−citrate−phosphate at a 1:1:2 ratio; c_{Al} = 0.04 M

Besides the aforementioned signals, in the spectrum of the citrate-containing solution at pH ≈ 2.2 a new ^{31}P resonance is seen at δ = −4.41; this could clearly be assigned to the ternary complex $[AlABH_2]^-$. On the basis of the chemical shift of this signal in the complex $[AlABH_2]^-$, the phosphate group must be coordinated in a monodentate fashion. On increasing the pH of the samples, the resonance of the ternary complexes is shifted downfield, which indicates that there is a rapid proton-exchange between the differently protonated ternary species. At pH ≈ 3, the resonances corresponding to the ternary complexes $[AlABH_2]^-$ and $[AlABH]^{2-}$ (δ = −4.2) and the monodentate phosphate-

coordinated binary complex $[AlBH]^+$ ($\delta = -5.64$) can be assigned unambiguously, but a small amount of the complex $[Al_2B]^{3+}$ is also present, giving a broad signal centred at around $\delta = -11$. At higher pH, due to the poor resolution of the spectra (the signals are broad and merged) the signals are extremely difficult to assign. This is well-illustrated by the spectrum recorded at pH ≈ 7.4 (Figure 4), where the very broad signals at $\delta = -3.7$, -9.3, and -13.9 were attributed to the ternary complexes $[AlAB]^{3-}$ and $[AlABH_{-1}]^{4-}$, the mononuclear 1:1 complex $[AlBH_{-1}]^-$, and the dinuclear complex $[Al_2BH_{-3}]$, respectively. Since no spectra of the binary Al^{III}−phosphate system could be recorded because of precipitation at this pH, signals were assigned on the basis of their relative intensities, taking into account the potentiometric speciation results and the spectra measured in the acidic pH range. The pattern of the signals, however, may suggest the presence of coordination isomers, possibly including the bidentate phosphate-coordinated isomer of the ternary complex and the proposed outer-sphere complexes of the Al^{III}−hydroxide−phosphate precipitate formed with the binary Al^{III}−citrate species (vide supra).

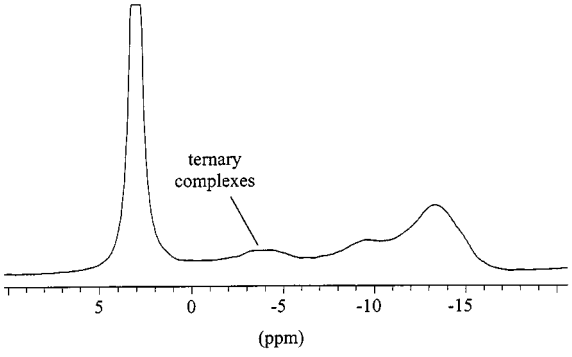

Figure 4. ^{31}P NMR spectrum recorded for the Al^{III}−citrate−phosphate system at a metal ion-to-ligand ratio of 1:1:2 and pH ≈ 7.4; $c_{Al} = 0.04$ M

In the ^1H NMR spectra of the same solutions, only the AB quadruplet of free citrate can be unambiguously assigned. The resonances of the other complexes present are merged into one very broad signal in the chemical shift range $\delta = 3.0-2.4$, probably as a consequence of the quadrupole effect of ^{27}Al nuclei and the relatively fast exchange reactions between these complexes. The resolution of the spectra was not improved even at low temperatures (5−10 °C). In the pH range of formation of the trinuclear complex $[Al_3(AH_{-1})_3OH]^{4-}$, its signals are well-separated and easily discerned as a result of its rigid structure and slow proton- and ligand-exchange reactions.[23] Figure 5 shows the ^1H NMR spectrum obtained at pH ≈ 7.4, which clearly indicates the slow formation of the trinuclear complex.

Since the primary aim of this work was to characterize the behaviour of the Al^{III}−citrate−phosphate system under physiological conditions, we attempted to obtain quantitative information from ^1H and ^{31}P NMR measurements. For a quantitative evaluation of the spectrum, a known amount of dioxane was added to a solution of the

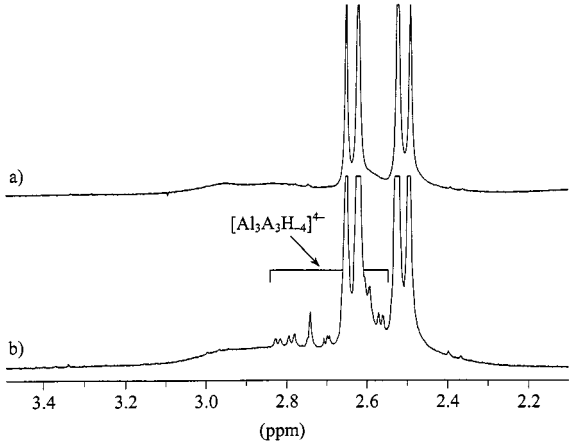

Figure 5. ^1H NMR spectra of the Al^{III}−citrate−phosphate system at a metal ion-to-ligand ratio of 1:1:2 and pH ≈ 7.4: (a) in freshly prepared solution, (b) at equilibrium; $c_{Al} = 0.04$ M

Al^{III}−citrate−phosphate 1:1:2 system under thermodynamic equilibrium at pH ≈ 7.4 and the amount of free citrate was calculated from the ^1H NMR spectrum using the deconvolution method. About 43% of the citrate was found to be free, while 57% was complexed in binary and ternary species. Since the ratio of Al^{III}−citrate in these solutions was 1:1, and since potentiometry indicates that only 1:1 Al^{III}−citrato species exist in the system, an amount of Al^{III} equivalent to the free citrate (43%) should be complexed in the binary Al^{III}−phosphato species. We should mention here that the ^{27}Al NMR spectrum of this solution featured one very broad resonance centred at $\delta \approx 3$, suggesting the formation of complexes containing Al^{III} in an octahedral environment, and gave no indication of the presence of free $[Al(OH)_4]^-$ (no signal at $\delta = -80$), i.e. no uncomplexed Al^{III} in the sample. Integration of the signals due to the free and the complexed phosphate in the ^{31}P NMR spectrum of the same solution revealed that ca. 65% of the phosphate was free and ca. 35% was in complexed form. This means that about 70% of the Al^{III} was bound in phosphate-containing complexes (including the ternary species), as the ratio of Al^{III}−phosphate in the sample was 1:2. The ^1H and ^{31}P NMR spectra revealed that ca. 43% of the total Al^{III} was bound in binary Al^{III}−phosphato species, ca. 30% in binary Al^{III}−citrato complexes, and ca. 27% in ternary complexes. In contrast, the speciation calculations indicate 58% in ternary complexes. This difference in the results of NMR and potentiometry may be explained partly by the relatively high degree of error in the percentage values calculated from the NMR measurements due to the rather poor quality of the spectra, and partly by the assumption of the formation of outer-sphere complexes (vide supra), which cannot be clearly observed by NMR spectroscopy.

These results indicate that (although a full quantitative speciation description of the Al^{III}−citrate−phosphate system could not be given) under the conditions (concentrations and metal ion-to-ligand ratios) of our NMR measurements, the binding abilities of the ligands citrate and phos-

phate to complexed Al^{III} are practically the same at physiological pH.

Reaction of the Trinuclear Species in the Presence of Inorganic Phosphate

The most characteristic species of the Al^{III}–citrate speciation is the trinuclear complex $[Al_3(AH_{-1})_3OH]^{4-}$. This is the predominant complex in equimolar Al^{III}–citrate solution between pH 4 and 9, i.e. in the range including physiological pH. The solid-state structure of the complex has been determined by X-ray crystallography[24] and the same rather asymmetric binding mode was confirmed by solution ^1H and ^{13}C NMR studies.[23] This raises the question as to what happens to this very stable and kinetically rather inert species in the presence of phosphate. Is phosphate able to decompose the trinuclear species? To answer this question, a twofold excess of phosphate (pH 7.4) was added to a 0.1 M solution of the trinuclear complex adjusted to pH 7.4. A slight increase ($\approx$ 0.2 pH unit) in the pH of the solution was observed and the spectral changes over time were monitored by ^1H, ^{31}P, and ^{13}C NMR. The ^1H NMR spectrum shows that the very characteristic proton spectrum (Figure 6, a) of the trinuclear complex collapses in the presence of phosphate (Figure 6, b), and that citrate is slowly partially displaced by phosphate as the AB quadruplet of free citrate clearly appears in the spectrum recorded after five days (Figure 6, c).

In the ^{31}P NMR spectrum of the same sample, a sharp signal initially appeared at $\delta = -1.8$, besides the resonance of the free phosphate. The intensity of this signal slowly decreased and it disappeared completely at equilibrium.

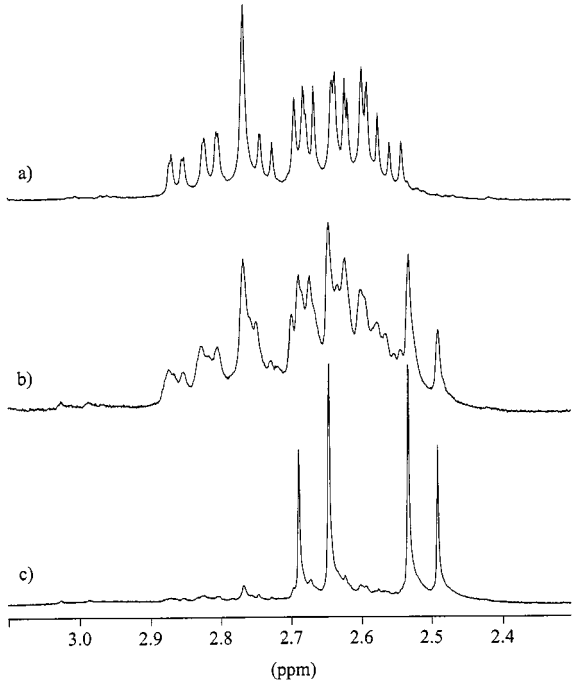

Figure 6. Decomposition of the trinuclear Al^{III}–citrate complex in the presence of a twofold excess of phosphate as monitored by ^1H NMR spectroscopy: (a) spectrum of the trinuclear species, (b) 10 h and (c) 5 days after the addition of phosphate; $c_{complex}$ = 0.1 M

The pattern of the equilibrium spectrum was the same as that obtained when Al^{III} was added to a solution of a mixture of citrate and phosphate (see Figure 4). The signal at $\delta = -1.8$ may presumably be assigned to a mixed-ligand complex formed as an intermediate species during decomposition of the trinuclear complex. The signals of this intermediate complex can easily be recognized in the ^{13}C NMR spectrum of the above solution. Besides the signals of the trinuclear complex (δ = 41.80, 42.90, 43.67, 44.98, 45.95, 46.24, 73.37, 75.51, 75.68, 177.18, 177.64, 177.89, 177.92, 178.19, 178.45, 180.78, 182.75, and 182.89),[16] 14 new resonances appear at δ = 41.83, 43.77, 45.51, 46.10, 46.46, 73.10, 75.25, 177.43, 177.73, 177.91, 178.88, 179.44, 180.85, and 183.29, which suggests that the intermediate species is most probably an oligonuclear mixed-ligand complex with a relatively asymmetric structure. During the formation of this complex, phosphate may be incorporated into the structure of the trinuclear complex $[Al_3(AH_{-1})_3OH]^{4-}$, replacing one or more of the coordinated donor groups of one of the citrate molecules. The ligand exchange between the trinuclear complex $[Al_3(AH_{-1})_3OH]^{4-}$ and the intermediate species is slow on the NMR time scale.

Modelling of Blood Serum

As one of the aims of the work was to determine whether citrate or phosphate is the primary l.m.m. Al^{III} binder in blood serum, we constructed the species distribution at plasma concentrations by using the speciation model and the stability constants obtained from the potentiometric measurements (Figure 7). For the calculations, the serum model suggested by Harris[7] was used: c(phosphate) = 1.1 mM, c(citrate) = 99 μM; the other l.m.m. constituents of blood serum, such as lactate, oxalate, amino acids, etc.,[7] have significantly lower affinities for Al^{III} and their participation in Al^{III} binding can therefore be neglected. This was assumed in all previous modelling calculations.[8–13] The total Al^{III} concentration was taken as 3 μM, which is in the range of serum concentrations observed for patients on haemodialysis.[7] In the calculations, we also took into account the fact that only ca. 20% of the total Al^{III} in the serum is bound to l.m.m. biomolecules while ca. 80% is bound to the h.m.m. components, mostly to transferrin[7] (vide supra). Figure 7 shows that at biologically relevant Al^{III} concentrations ($c_{Al}^{III} \approx$ 3 μM, of which only 0.6 μM is bound to l.m.m. components) the mononuclear complexes predominate and ternary species are formed in significant amounts. The oligomerization processes are strongly suppressed. At physiological pH, 51% of the Al^{III} is bound to citrate in the complex $[AlAH_{-2}]^{2-}$, ca. 28% is bound in the ternary species $[AlAB]^{3-}$ and $[AlABH_{-1}]^{4-}$, and ca. 21% is bound in the binary species with phosphate, $[AlBH_{-1}]^{-}$. If, however, we envisage an Al overload case, when the serum Al^{III} level can increase up to 100 μM, assuming a complete saturation of transferrin the distribution of Al^{III} between the h.m.m. and l.m.m. fractions of serum is 26:74%. The distribution of Al^{III} between citrate, phosphate binary, and citrate–phosphate ternary complexes becomes ca. 32%, 51%, and 17%, respectively. Interestingly, at increasing Al^{III}

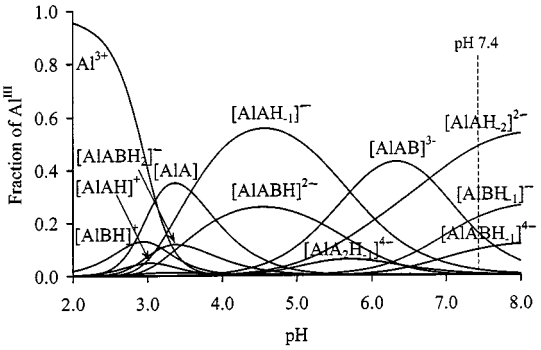

Figure 7. Species distribution curves for complexes formed in the Al^{III}−citrate−phosphate system at serum concentrations ($c_{Al} = 6 \times 10^{-7}$ M, $c_{citrate} = 99 \times 10^{-6}$ M, $c_{phosphate} = 1.1 \times 10^{-3}$ M, see text)

concentrations, phosphate becomes the more important binder.

Citrate-bound Al^{III} (either in binary or in ternary species) has been observed directly in blood plasma by Sadler et al. by means of ^1H NMR spectroscopy.[25] In a more recent study, Bantan et al.[26] investigated the speciation of l.m.m. Al^{III} complexes in human serum taken from eight healthy volunteers by means of fast liquid chromatography and electrospray techniques. In agreement with our results, they found that the main l.m.m. Al^{III} species present in the serum were binary Al^{III}−citrate and Al^{III}−phosphate and ternary Al^{III}−citrate−phosphate complexes. The distribution of these complexes varied from individual to individual.

Conclusions

This work has clearly demonstrated that the assumption of ternary complex formation may be of great importance in the modelling of biological systems. In the ternary Al^{III}−citrate−phosphate system under model conditions, the ternary species dominate, especially in freshly prepared solutions. Gradual increases in the concentrations of the trinuclear species over time are observed. This has been confirmed by time-dependent NMR spectral measurements. At physiological pH and millimolar concentrations, most of the Al^{III} is bound in the ternary complexes $[AlAB]^{3-}$ and $[AlABH_{-1}]^{4-}$, but some Al^{III} is also bound in the binary species involving citrate or phosphate. The trinuclear species $[Al_3(AH_{-1})_3OH]^{4-}$ slowly decomposes in the presence of excess phosphate, indicating that monodentate phosphate is not a negligible Al^{III} binder and that the trinuclear citrato species is not completely stable under these conditions. The latter is nevertheless relatively inert as the partial citrate replacement by excess phosphate reaches equilibrium only after several days.

At normal blood serum concentrations and physiological pH, Al^{III} is bound partly in mononuclear ternary complexes but mostly in the mixed Al^{III}−citrate−hydroxo complex $[AlAH_{-2}]^{2-}$.

Experimental Section

Reagents: Citric acid of the highest available purity was purchased from Sigma; the phosphate used was the highest quality monopotassium salt from Fluka. The ligands were used without further purification. Their purities were checked and the precise concentrations of their solutions were determined by the Gran method.[27] An Al^{III} stock solution was prepared from recrystallized $AlCl_3 \cdot 6H_2O$; its metal concentration was determined gravimetrically following conversion into the oxinate. The stock solution contained 0.1 M HCl to prevent hydrolysis of the aluminium.

Potentiometric Measurements: The stability constants of the proton complexes of the ligands were determined by pH-potentiometric titrations of 10.0 cm^3 samples. The ligand concentration was 0.004 M. The titrations were performed using 0.2 M carbonate-free KOH solution of known concentration under a purified argon atmosphere. The ionic strength of all solutions for potentiometric measurements was adjusted to 0.2 M with KCl and the samples were thermostatted at 25.0 ± 0.1 °C.

In this work, only the Al^{III}−citrate−phosphate ternary system was analysed; stability constants for the binary systems were taken from our previous studies.[16,18,19] The time-dependent speciation measurements were performed using the batch technique, in a similar manner as described for the Al^{III}−citrate binary system.[16] After appropriate amounts of stock solutions of the ligand and base had been mixed with the Al^{III} solution, the pH of the resulting mixture was recorded at given times until thermodynamic equilibrium of the system had been obtained (ca. 30 h). The procedure was repeated for different starting pH values, whereby titration curves relating to different times were obtained. The measurements were carried out at Al^{III}−citrate−phosphate ratios of 1:1:1 and 1:1:4 in 10.0 cm^3 solutions with intense stirring under argon atmosphere. The pH range studied was 2−8. For each ratio, the time-dependent changes were monitored for 20 different samples. However, in order to increase the reliability of the speciation description of the system at thermodynamic equilibrium and to reduce the uncertainties in the calculated stability constants, twice as many samples were prepared, but every second one was only analysed 30 h after mixing, when pH equilibrium had been attained. The stability constants listed in Table 1 were calculated from the total number of batch points (40 points for each metal ion ligand composition). In the case of the Al^{III}−phosphate binary system,[18,19] pH equilibrium could be attained within 5 min for each point and the normal titration procedure was used to obtain the stability constants for the complexes formed. The pH was measured with an Orion 710A precision digital pH-meter with a Metrohm combined glass electrode, which was calibrated for hydrogen ion concentration according to Irving et al.[28]

The concentration stability constants $\beta_{pqrs} = [M_pA_qB_rH_s]/[M]^p[A]^q[B]^r[H]^s$ were calculated with the aid of the computer program PSEQUAD.[29] The stability constants used for the hydroxo species of Al^{III} were taken from ref.[30] and corrected to $I = 0.2$ M using the Davies equation: −5.49 for $[AlH_{-1}]^{2+}$, −13.54 for $[Al_3H_{-4}]^{5-}$, −97.2 for $[Al_{13}H_{-32}]^{7-}$, and −23.40 for $[AlH_{-4}]^{-}$.

NMR Spectroscopy: ^1H, ^{31}P, and ^{13}C NMR spectra were recorded at 25 °C on Bruker AM360 and DMX500 spectrometers. For the ^1H and ^{13}C NMR spectra, chemical shifts were referenced to the signal of TMS as an external standard, while for the ^{31}P NMR spectra 85% orthophosphoric acid was used as an external standard. The samples were prepared in D_2O and the pH was adjusted with concentrated NaOD and DCl, using the relationship pH =

pD + 0.4. The concentrations of the solutions were 0.04−0.1 M in Al^{III}; samples with Al^{III}−phosphate ratios of 1:1, 1:2, and 1:4 and Al^{III}−citrate−phosphate ratios of 1:1:1, 1:1:2, and 1:1:4 were investigated. Unless otherwise stated, all NMR measurements were made on thermodynamically equilibrated samples. − ^{27}Al NMR spectra were measured on a Bruker AM200 instrument at 25 °C. Chemical shifts were recorded with respect to $Al(H_2O)_6^{3+}$. Solutions containing 0.1 M Al^{III} and having a metal ion-to-ligand ratio of 1:1:2 were prepared in water, to which 10% D_2O was added to provide a lock signal.

Acknowledgments

This work was carried out within the framework of the COST D8 collaboration and was supported by the Hungarian Research Fund (OTKA 23776/97) and the Hungarian Ministry of Education (FKFP 0013/97).

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Received May 7, 2001
[I01160]