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Reactivity of Al(III) with membrane phospholipids: a NMR approach

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The complexes $Al(acac)_3$ (1) (acac = 2,4-pentanedionate) and $Al(malt)_3$ (malt = 3-hydroxy-2-methyl-4-pyronate) (2) react with DL-α-dipalmitoylphosphatidylcholine (DPPC) under a 1:1 molar ratio in $CDCl_3$ at 37 °C, as shown by the substantial release of ligands (20–50%) from the metal coordination sphere (^{1}H-NMR), by evident changes in the ^{1}H-NMR spectrum of DPPC in the reaction mixture and by the appearance of a ^{31}P-NMR signal due to metal-coordinated DPPC. ^{31}P-NMR spectra reveal that both 1 and 2 also react with DPPC in water, in the presence of 1% Triton X-100 and Tris buffer. Under these conditions, 1 and 2 do not react with ghosts from human erythrocytes. On the contrary, the far less hydrolytically stable complex $Al(lact)_3$ (lact = lactate) appears to be reactive under identical conditions, as shown by ^{31}P-NMR spectra.

Keywords: Al(III), complexes, phospholipids, membrane

Introduction

Al(III) is an established neurotoxin (Sturman & Wisniewski 1988, Nicolini *et al.* 1991) and an extensive literature illustrates its ability in inducing pathological events in the function of plasmatic membranes (Banks & Kastin 1989) and recent findings also reveal a strong influence of the metal center on the integrity of the blood–brain barrier (Favarato *et al.* 1992). In spite of extensive observations, the molecular bases of these observations are still unknown.

In recent years biophysical work (Deleers *et al.* 1985, 1986) has shown that Al(III) at micromolar concentrations induces phase separation, aggregation, dye release and membrane rigidification in phosphatidylserine- and phosphatdylethanolamine-containing lipid vesicles.

The ability of Al(III) to injure *biological* membranes was firstly recognized on the basis of physico-chemical evidence (Vierstra & Haug 1978) in 1978. Thus, $(Al(H_2O)_6)^{3+}$ was found to induce a significant reduction of membrane fluidity in *Thermoplasma acidophilim* at pH 3, as revealed by electron spin resonance measurements on bacterial cells ghosts exposed to the action of the metal center, after probing with 5-NSA (2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolyndinyloxyl).

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We recently reported that rabbit erythrocytes suspended in aqueous solutions of the hydrolytically stable and lipophilic (Corain *et al.* 1992a) complex $Al(acac)_3$ (**1**) (acac = 2,4-pentanedionate) undergo a prominent morphological effect (echinoachanthocytosis) accompanied by a significant increase of osmotic fragility at physiological pH values. $Al(malt)_3$ (**2**) (malt = 3-hydroxy-2-methyl-4-pyronate) (hydrolytically stable, hydrophilic, Finnegan *et al.* 1986, 1987) and $Al(lact)_3$ (**3**) (hydrolytically metastable, Corain *et al.* 1992b; and hydrophilic, Tapparo and Perazzolo, 1989) were found to be inactive in terms of morphological effect and slightly active in terms of osmotic fragility. Equally inactive were found to be Hacac and $Fe(acac)_3$ (Zatta *et al.* 1989), which may be safely considered as a suitable 'blank compound' for $Al(acac)_3$, being related to its aluminum analog in terms of hydrolytic stability and lipophilicity. Subsequently, we reported (Corain *et al.* 1991, Zatta *et al.* 1992) that the aggression of **2** to rabbit erythrocytes membrane is in fact accompanied by a strong reduction of membrane fluidity, as shown by experiments corresponding to those reported by other authors (Vierstra & Haug 1978). The major relevance of all these data appear to be the circumstance that these biological and biophysical effects caused by Al(III) have been recorded at *physiological* pH values, under *strict metal speciation control*. Quite recently, the aggressive character of Al(III) to plasmatic membranes, with an evident decrease of membrane fluidity, has been confirmed on human erythrocytes exposed to aluminum

hydroxide (at pH 7.4) at micromolar (analytical) concentrations (Van Rensburg *et al.* 1992).

We report here on the interaction of Al(III) with DL-α-dipalmitoylphosphatidylcholine (DPPC), a prominent component of the external leaflet of cell membranes, under controlled chemical conditions. We offer also clear evidence based on ^{31}P-NMR measurements on the binding interaction of Al(III) with ghosts from human erythrocytes in water suspensions, in the presence of Tris-buffer and Triton X-100.

Methods

Reagents

Unless otherwise stated, all chemicals were of reagent grade. $Al(acac)_3$ was purchased from Aldrich Chimica (Milan, Italy) and recrystallized from toluene–petroleum ether. $Al(malt)_3$ was synthesized and purified according to Finnegan *et al.* (1987). Both toxin were controlled by elemental analysis. $Al(lact)_3$ was purchased from ICN Biomedicals (Cleveland, OH) and, after analytical control, it was used as received. DPPC and standard phospholipid analytical reagents were obtained from Sigma Chimica (Milan, Italy).

Chloroform-*d* (atom 100.0%) and deuterium oxide (atom 99.9%) were used as solvents for all NMR determinations.

Protein assay was carried out by the BCA method (Pierce, Rockford, IL, USA).

Al(III) and phospholipid standard solutions

Al(III) and phospholipid standard organic solutions were prepared by dissolving the required amount of solid complex or synthetic phospholipid in 0.5 ml of $CDCl_3$ under a gentle nitrogen stream. The tube was quickly capped and ^{1}H- and ^{27}Al- or, alternatively, ^{1}H- and ^{31}P-NMR control spectra were immediately recorded. Al(III) and phospholipid standard aqueous solutions were prepared by suspending the required amount of toxin or phospholipid in 1% Triton X-100 Tris-buffered (0.1 M) (pH 7.4) deutero-aqueous solution. Under these conditions, ^{27}Al- and ^{31}P-NMR control spectra were recorded.

Al(III) and phospholipid mixture

Equimolar amounts of the relevant Al(III) species and of DPPC were placed in a 5 mm NMR sample tube and dissolved, under gentle nitrogen stream, in 0.5 ml of $CDCl_3$ or 0.5 ml of D_2O containing 1% Triton and Tris buffer (0.1 M) (pH 7.4). The tube was quickly capped, gently shaken and the spectra were recorded at 24 and 37 °C, and compared with those obtained for separate free Al(III) complexes and free DPPC.

Interaction of human ghosts treated with Al(III) species

Freshly heparinized blood was centrifuged at 2500 r.p.m. in an ALC 4236 centrifuge for 15 min at 4 °C. Plasma along with the buffy coat layer was discarded. Erythrocytes were then treated with a phosphate buffer saline (PBS) solution and centrifuged for 15 min at 4 °C. This operation was repeated four times. Compacted erytrocytes (0.8 ml) were placed in an ultracentrifugation tube, suspended in 8 ml of 5 mM Tris–HCl solution (pH 7.0) and ultracentrifuged for 30 min at 14500 r.p.m. at 4 °C. The supernatant was discarded and erytrocytes washed again with 8 ml of 50 mM Tris–HCl, 0.5 M NaCl solution (pH 7.0) and ultracentrifuged at 14500 r.p.m. for 10 min at 4 °C. Finally, the first ultracentrifugation procedure was repeated until the complete removal of hemoglobin. Aliquots of ghosts in 0.1 M Tris–HCl solution (pH 7.4) were stored at −30 °C.

Approximately 10^{-3} M solutions of ghosts were transferred into a 5 mm NMR sample tube and a ^{31}P-NMR control spectrum was recorded. An equal volume of 1% Triton X-100 Tris-buffered solution of $Al(acac)_3$ (5×10^{-3} M), $Al(malt)_3$(5×10^{-2} M) or $Al(lact)_3$ (10^{-1} M) was then added to the sample tube, which was gently shaken for 3 min. ^{31}P-NMR spectra were accumulated at 24, 37, 50 and back to 24 °C (see Figure 6 for further details).

NMR spectroscopy

All NMR measurements were carried out on a BRUKER-AC200 spectrometer equipped with a selective broad band probe, and operated at 81.015 MHz for ^{31}P and 52.148 MHz for ^{27}Al. All ^{31}P and ^{27}Al experiments were conducted under proton-decoupled conditions. The chemical shift reference ($\delta = 0.0$ p.p.m.) was tetramethylsilane (TMS), 85% H_3PO_4 and 0.1 M aqueous $AlCl_3 \cdot 6H_2O$ for ^{1}H, ^{31}P and ^{27}Al, respectively. Typical experimental parameters during the acquisition of ^{31}P spectra referring to the interaction of Al(III) species with ghosts were: pulse sequence, one pulse (with decoupler on); pulse width, 9.0 μs corresponding to a 90° spin-flip angle; acquisition time, 10.9 s; sweep width, 3000 Hz; cycling delay, 1 s; free induction decay size, 65536; number of acquisitions, 19000. In addition, a computer-generated filter time constant introducing 3 Hz line broadening was applied as needed to improve signal-to-noise ratios.

Results

^{1}H-and ^{31}P-NMR spectra of DPPC in $CDCl_3$

The ^{1}H spectrum exhibits two distinct sets of resonances in the $\delta = 0.5$–3 and 3–5.5 p.p.m. ranges. The signals in the first range are due to the lipid part of the molecules, while those in the lower fields range refer to the glyceryl skeleton and to the choline residue (Figure 1).

Unambiguous assignment of the resonances shown in Figure 1 was made possible by their relative chemical shifts, spin–spin splittings and integrations, and by two-dimensional homonuclear shift correlation experiments (COSY) (Ernst *et al.* 1987). The ^{31}P-NMR spectrum of DPPC in $CDCl_3$ exhibits a single sharp resonance at −2.5 p.p.m.

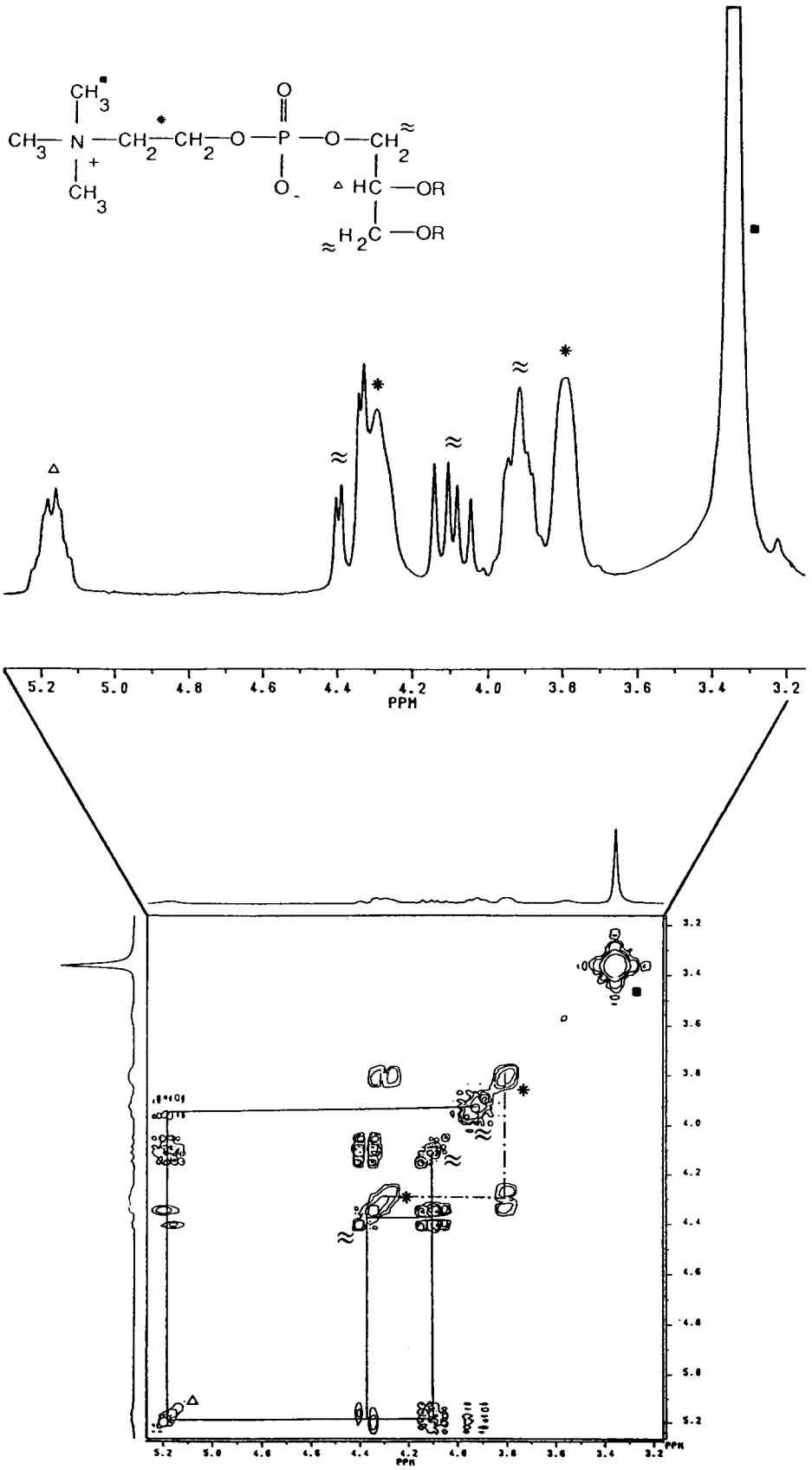


Figure 1. Two-dimensional COSY45 ^{1}H-NMR contour plot of 5×10^{-3} M DPPC solution in chloroform-*d* over the 5.2–3.2 p.p.m. region at 37 °C, including the glyceryl and the choline protons.

^1H- and ^{27}Al-NMR spectra of $Al(acac)_3$ (1) and $Al(malt)_3$ (2) in $CDCl_3$

The spectra of **1** and **2** appear to be unchanged for at least 2 h, at room temperature, at concentration $> 10^{-2}$ M. The observed data for **1** are in agreement with those reported in the literature (Benn *et al.* 1982); in particular, the methyl and the methine protons are seen at 1.97 and 5.45 p.p.m., respectively. The ^1H spectrum of **2** (Figure 2) exhibits a singlet at $\delta = 2.41$ and two doublets at $\delta = 6.62$ and 7.80 p.p.m. due to the methyl and methine protons (AB system) of the pyronate ring, respectively.

The ^{27}Al spectrum of a 10^{-2} M solution of **1** appears as a relatively sharp singlet (linewidth 10 Hz) at −2.4 p.p.m., while the spectrum of a 10^{-2} M solutions of **2** displays a relatively broad singlet (linewidth ~ 100 Hz) at 35.3 p.p.m. In contrast with these observation the spectra of more diluted solutions of both complexes exhibit further broad downfield absorptions attributable to the formation of less symmetric octahedral species (Karlik *et al.* 1983b) as the result of the partial hydrolysis of the $Al(O-O)_3$ complexes caused by trace amounts of water present in the solvent. The variation in the ^{27}Al spectra, which is associated with the dilution effect, is paralleled by changes in the proton spectra. In particular, a 5×10^{-3} M solution of **2**, just 5 min after dissolution, exhibits a set of

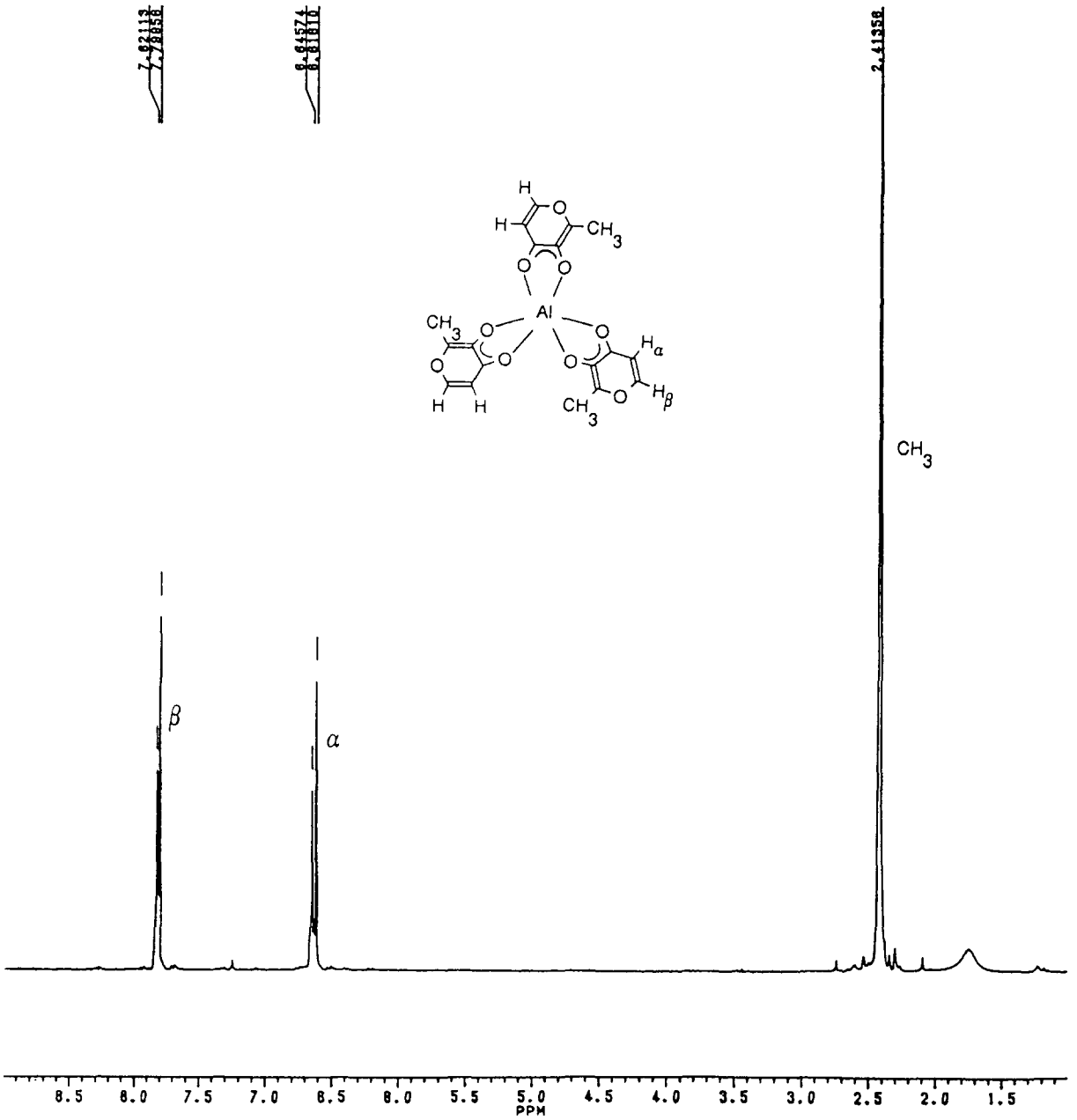


Figure 2. ^1H-NMR spectrum of 5×10^{-2} M $Al(malt)_3$ in chloroform-*d* at 24 °C.

resonances attributable to a partially hydrolyzed derivative (see Figure 3b). If this solution is left inside the NMR tube and left in contact with the open atmosphere, the spectrum gradually changes with the time to give a well defined final pattern, which is depicted in Figure 3(e) after 96 h, at room temperature. It has to be noticed that only minor amounts of free maltol (< 2%) appear to be present after this reaction time so that the reaction products have to be *maltolate* Al(III) *complexes*.

Reactivity of Al(acac)$_3$ (1) and Al(malt)$_3$ (2) with DPPC in CDCl$_3$

The ^{1}H spectrum of equimolar solutions of **1** or **2** (5×10^{-3} M) and the phospholipid, recorded after 5 min after mixing at 37 °C, show unambigous evidence of extensive release of free Hacac or Hmaltol paralleled by a marked broadening of the signals due to the protons of the glyceryl skeleton and of the choline residue of DPPC. The results are illustrated in Figure 4 for the case of Al(malt)$_3$. It is evident that the whole of the maltol observed in solution is present either as uncoordinated (~ 50%) or metal bound (~ 50%) in the Al(malt)$_3$ form.

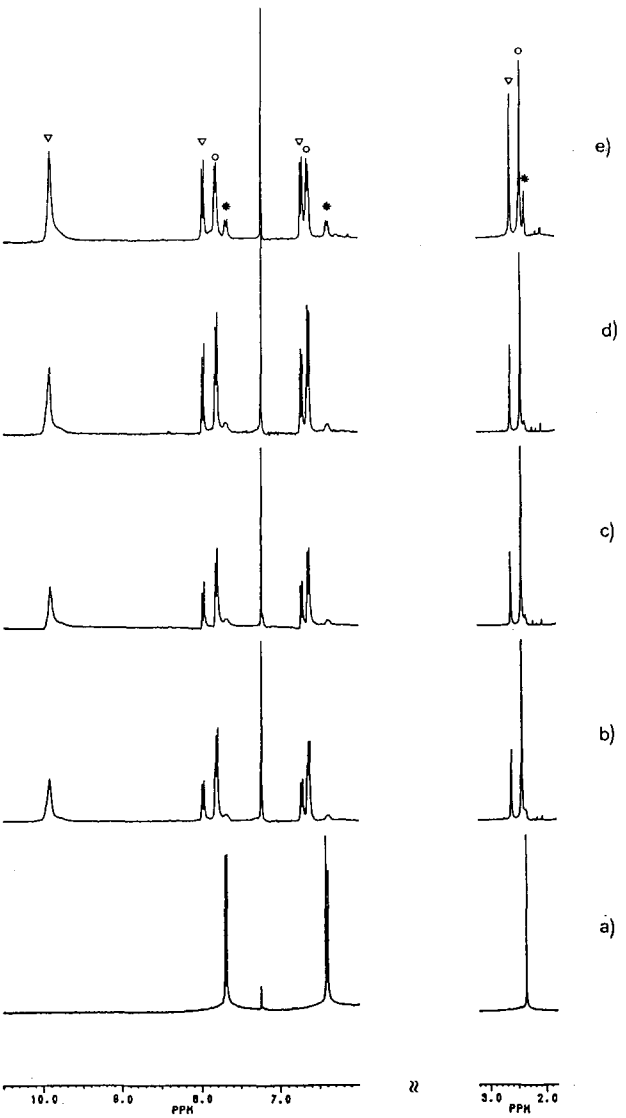


Figure 3. ^{1}H-NMR spectra in chloroform-*d* of: (**a**) 5×10^{-3} M free maltol, and 5×10^{-3} M Al(malt)$_3$ at (**b**) 5 min, (**c**) 2 h, (**d**) 4 h and (**e**) 96 h after dissolution. *, Signals refer to free maltol protons; ○, signals refer to Al(malt)$_3$ protons; ▽, signals refer to a hydrolized maltolate complex, tentatively Al(maltH)$_3$(OH)$_3$.

It is worth noting the splitting of the signal due to the methyl group of the choline residue, which appears to be a direct spectroscopic consequence of the involvement of the phospholipid in the coordination sphere of the metal centre. Quite similar, albeit less marked, spectroscopic results were observed in the case of **1**, for which the release of free Hacac is estimated to be equal to ~ 20%. When the reaction of both **1** and **2** is followed at room temperature, the results are quite similar to those just described, but the extent of ligand release is somewhat smaller (~ 20 and 3%, respectively).

Further and direct evidence of the entrance of the phospholipid into the metal coordination sphere is given by the relevant ^{31}P spectra for both **1** and **2**. Thus, again under a 1:1 molar ratio, the singlet due to uncoordinated DPPC is accompanied by a singlet evidently attributable (Karlik 1983a) to the metal-coordinated biomolecule (Figure 5). Also in this case, the extent of the reaction is less for **1**. The broader shape of the signal observed for **2** is clear evidence of a 'rapid' exchange between free and co-ordinated DPPC. On the contrary, the adduct between **1** and the biomolecule appears to be kinetically more stable on an NMR timescale.

Finally, quite in contrast with the useful information given by the ^{1}H and ^{31}P spectra, ^{27}Al-NMR measurements of these reaction mixtures proved to be practically useless, owing to the expected very large broadness of the signals due to the Al–biomolecule complex (Akitt, 1987).

Reactivity of AIL$_3$ complexes with DPPC and ghosts from human erythrocytes in aqueous solutions

To show a possible interaction of Al(III) with DPPC in water, experiments were performed with Tris buffer in the presence of 1% Triton X-100 that was able to solubilize the phospholipid (Fendler 1982). Preliminary experiments (G. Trovò, unpublished results) revealed that only ^{31}P spectra turn out to be a reliable analytical tool. In fact, a 5×10^{-3} M DPPC solution, after treatment with an equal volume of a $\sim 8 \times 10^{-3}$ M water solution of **1** at 37 °C displays a well defined, albeit rather broad signal centered at −11 p.p.m., to be compared with the sharp singlet at −0.35 p.p.m. due to free DPPC. When 1:1, 5×10^{-3} M solutions of DPPC and **1** or **2** (Al(III) = 5×10^{-3} M) were used, the ^{31}P spectrum reveals no change at 24 °C, but a marked reduction of the intensity of the −0.35 p.p.m. peak at 37 °C with no apparent concomitant growth of any other peak. The effect of Al(III) is partially reversible with the temperature.

Related results are obtained upon reacting **1** or **2** with

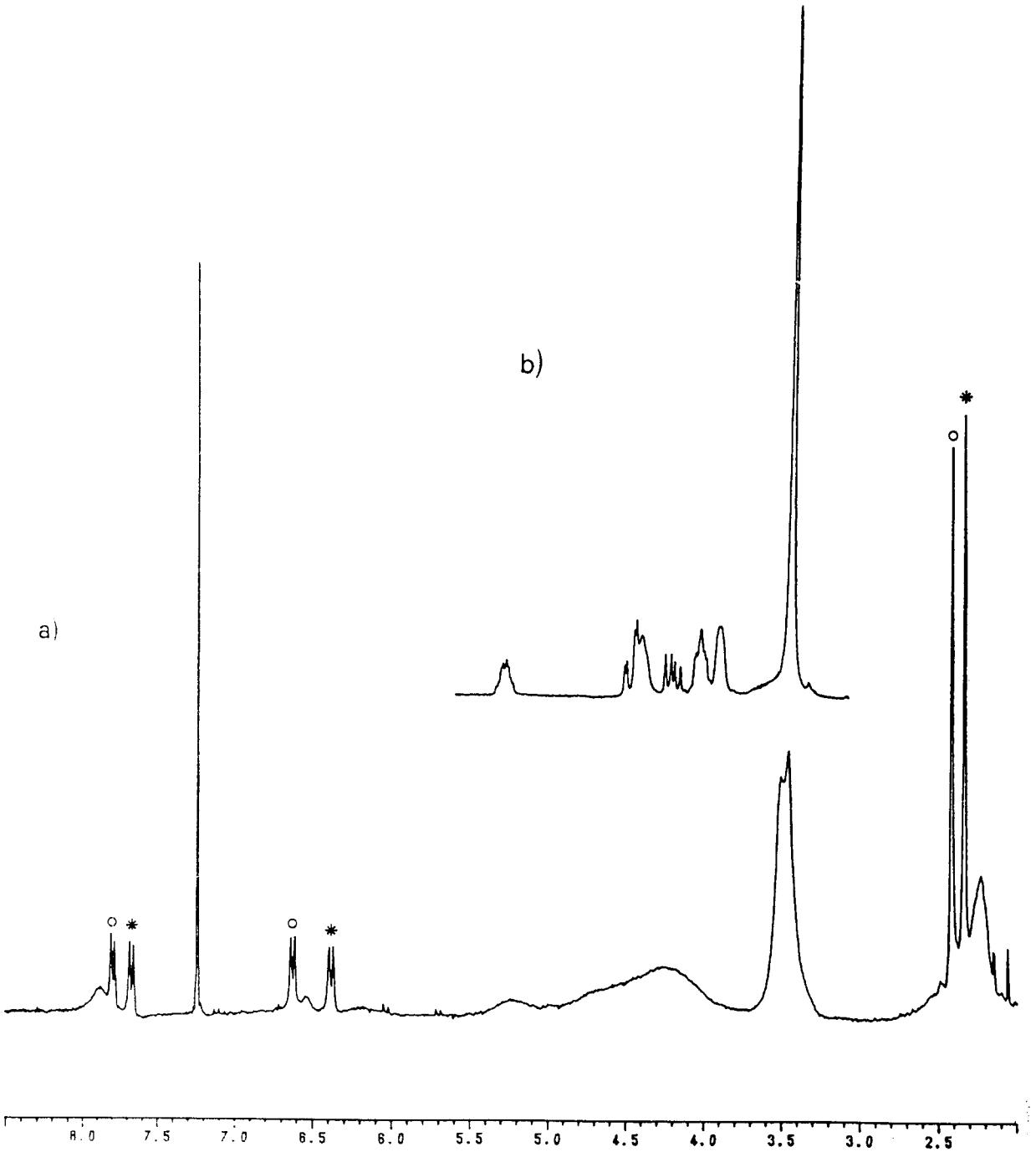
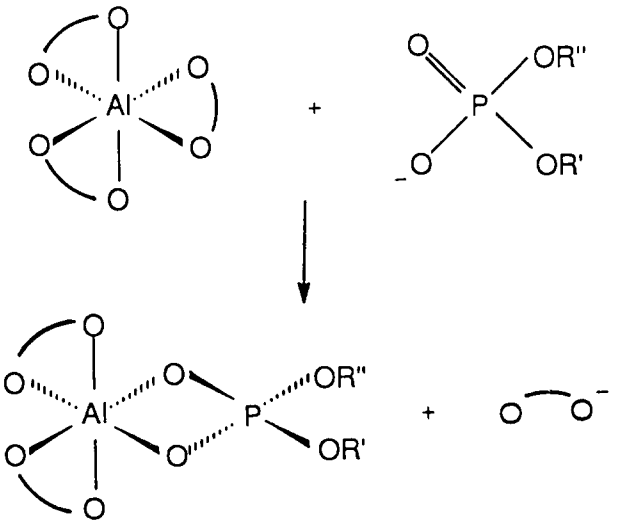


Figure 4. (**a**) ^{1}H-NMR spectrum of an equimolar reaction mixture (5×10^{-3} M) of DPPC and Al(malt)$_3$ in chloroform-*d* at 37 °C. *, Signals refer to protons of unbound maltol; ○, peaks refer to Al(malt)$_3$ protons. Note the splitting of the choline methyl group signal in the reaction mixture at ~ 3.5 p.p.m. and the overlap of several broad resonances in the 4.0–5.5 p.p.m. region compared with the signals of free DPPC (**b**).

human ghosts. Under the conditions of pH and detergent concentration utilized in the just described experiment, untreated ghost erythrocytes exhibit two ^{31}P peaks at $\delta = -0.35$ (DPPC) and $\delta = +0.30$ p.p.m. (two merged signals due to phosphatidylethanolamine and sphingomyeline). The actions of **1** (5×10^{-3} M) and **2** (5×10^{-2} M) do not cause any spectral change at 24 and 37 °C. On the contrary, 0.1 M Al(lact)$_3$ produces a marked broadening effect on both peaks at 37 °C, which appears to be more evident at 50 °C (Figure 6). Remarkably, no spectral change is observed induced in this case when the temperature is lowered to 24 °C.

Discussion

The data presented in this paper reveal that DPPC reacts extensively with $Al(acac)_3$ and $Al(malt)_3$ in chloroform. Thus, (i) the release of the β-enolate ligands, (ii) the concomitant development of ^{31}P signals attributable to metal-coordinated DPPC and (iii) the coexistence of two different sets of ^{1}H signals due to DPPC in equimolar Al(III)–DPPC solutions leave little doubts about the entrance of DPPC into the Al(III) coordination sphere through the phosphate ligating site:



$R' = CH_2–CH(OR''')–CH_2(OR''')$;
$R'' = CH_2–CH_2–N(CH_3)_3^+$;
$R''' = C(O)–(CH_2)_{14}–CH_3$.

The formation of Al(III)–biomolecule adducts through phosphatidic head-groups has been proposed in recent years on the basis of multinuclear NMR studies (Karlik *et al.* 1983a,b, Laussac *et al.* 1983) and potentiometric investigations (Sovago *et al.* 1990). Moreover, Al(III)–phospholipid adducts very likely related to that described herein have been recently proposed after an investigation centered in the reactivity of Al(III) with ATP and with mixtures of membrane phospholipids in a water/organic solvent medium (Panchalingam *et al.* 1991). These authors evaluated the reactivity of aqueous $Al(lact)_3$ with ATP and phospholipids at pH 7.2 on the basis of changes in the relevant ^{31}P-NMR signals at 24 °C. Remarkably, under these conditions, in which Al(III) is known to exist mainly as the metastable species $Al(OH)_3(H_2O)_3$ (Corain *et al.* 1992a), DPPC turned out to be the less reactive among nine membrane phospholipids investigated. In contrast with this last observation, we find that DPPC exhibits a marked reactivity with millimolar solutions of **1** and **2** in $CDCl_3$ together with an appreciable reactivity also in micellar aqueous solutions of DPPC. On the other hand, the lack of solubility of $Al(lact)_3$ in $CDCl_3$ prevents the study of the interaction of this complex with the other more reactive phospholipids in this solvent.

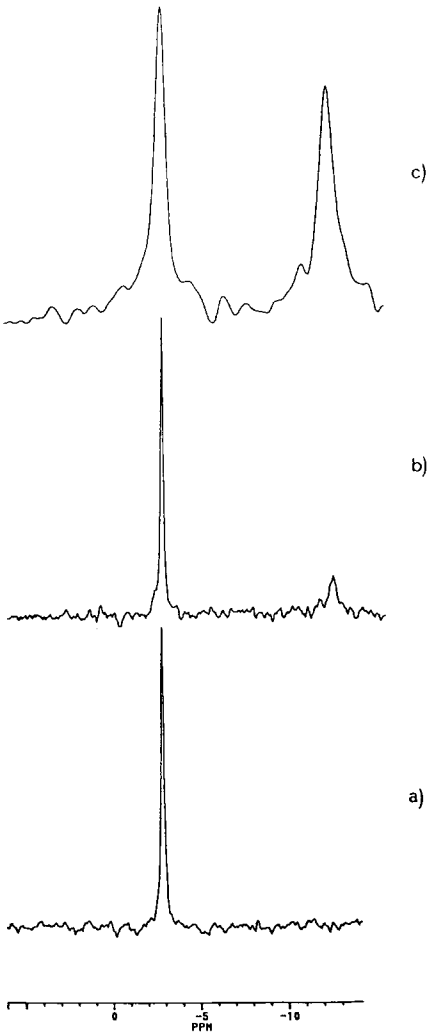


Figure 5. ^{31}P $\{^{1}H\}$-NMR spectra in chloroform-*d* at 24 °C of: (**a**) DPPC (10^{-3} M); (**b**) DPPC (10^{-3} M) + $Al(acac)_3$(10^{-2} M); (**c**) DPPC (10^{-3} M) + $Al(malt)_3$ (10^{-2} M).

The observed reactivity of DPPC in $CDCl_3$ or water compares well with the data referring to the ^{31}P-NMR experiments performed in aqueous suspensions of human ghosts. In fact, in 1% Triton this material is disrupted to give a micellar system. Although the lower phospholipid concentration makes it difficult to show evidence of any reactivity of this system with $Al(acac)_3$ and $Al(malt)_3$, clear evidence of reactivity is observed upon employing 0.1 M solutions of Al $(lact)_3$ at neutral pH values.

The results depicted in this paper make possible the first molecular interpretation of the established membrane toxicity of Al(III). The ability of this metal center to destabilize plasma and model membranes (see the introduction), to influence membranes biophysics (Khachaturian, 1986) and ultimately to alter the physical properties of neuronal membranes (Pettegrew, 1986) can now be interpreted in the light of at least one 'simple' event of coordination chemistry. Of course, the interactions of Al(III) species with the head groups of plasmatic membranes *in vivo* or *in vitro*, under pathological conditions,

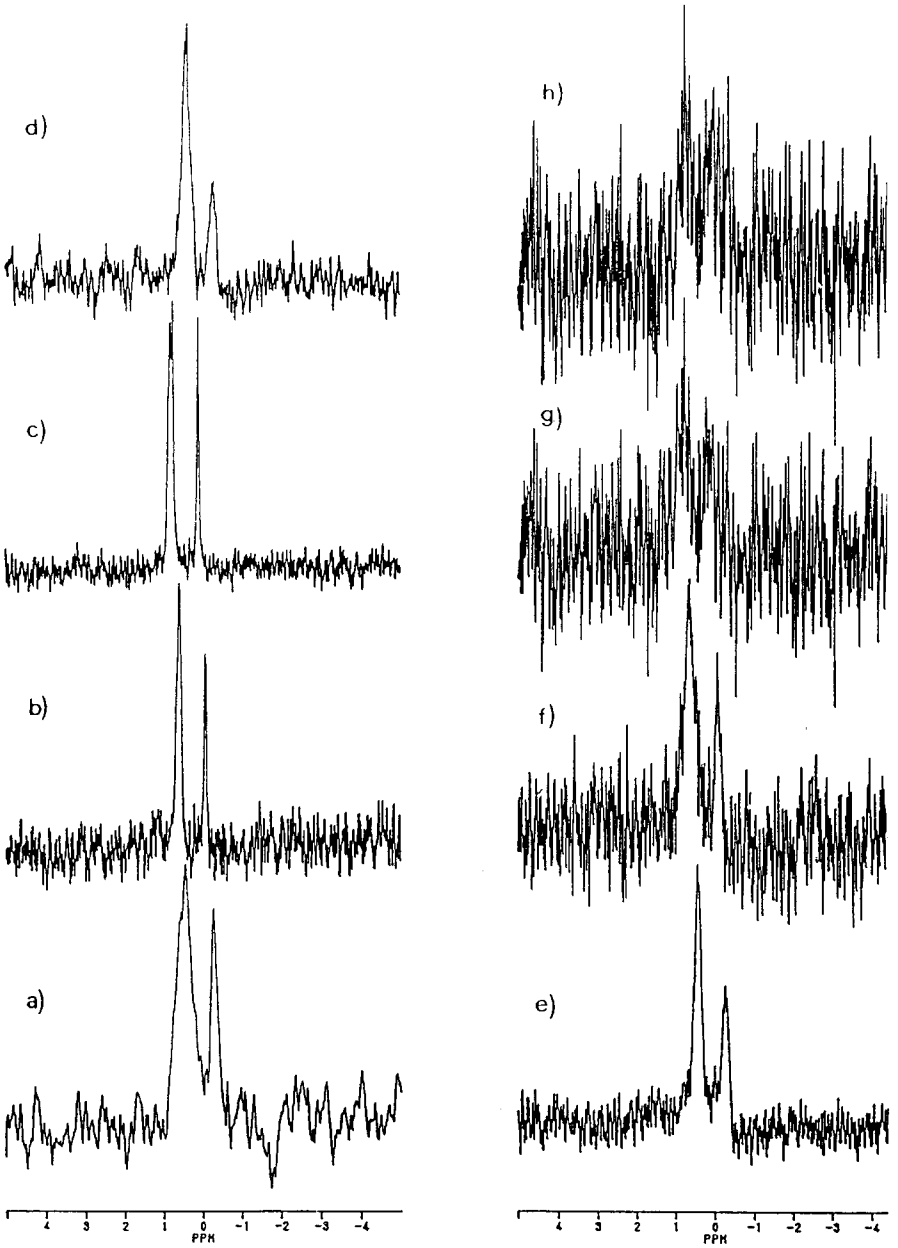


Figure 6. ^{31}P $\{^1H\}$-NMR spectra in chloroform-*d* of 1% Triton X-100 Tris buffered (pH 7.4) aqueous standard ghosts solutions at 24 °C (**a**), 37 °C (**b**), 50 °C (**c**) and back to 24 °C (**d**) compared with those of the ghosts solutions reacted with an equal volume of 10^{-1} M $Al(lact)_3$ in 1% Triton X-100 Tris-buffered solutions at 24 °C (**e**), 37 °C (**f**), 50 °C (**g**) and back to 24 °C (**h**).

are not expected to be massive, thus involving a large percentage of membrane phospholipids. In fact, the formation of relatively few reasonably stable Al(III)–phospholipid bonds, per aggressed cell, could well be able to significantly affect the supramolecular order of the membrane bilayer, thus seriously affecting its integrity. It is well known that a dismetabolism of aluminum is currently linked by various authors to the pathogenesis of some encephalopathies, including Alzheimer's Disease (Perl & Good, 1993; Kruck 1993), and the specific membrane toxicity of Al(III) has been considered relevant, in this connection, to the pathogenesis of this most elusive neurodegenerative disease (Pettegrew, 1986; Khachaturian, 1986).

Acknowledgments

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