Aqueous Shift Reagents for $^{7}Li^{+}$ NMR Transport Studies in Cells

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Abstract

A comparison of the paramagnetic shifts caused on the $^{7}Li^{+}$ NMR resonance by the shift reagents Ln$(PPP)_2^{7-}$, Ln$(TTHA)^{3-}$, Ln$(NTA)_2^{3-}$, Ln$(DPA)_3^{3-}$ and Ln$(HCA)_3^{3-}$ (where $Ln^{3+} = Dy^{3+}$ or Tm^{3+}, and PPP^{5-}, $TTHA^{6-}$, NTA^{3-}, DPA^{2-} and HCA^{2-} represent the ligands triphosphate, triethylenetetraminehexacetate, nitrilotriacetate, dipicolinate, and chelidamate, respectively) was carried out under the same ionic strength conditions. Dy$(PPP)_2^{7-}$, Tm$(PPP)_2^{7-}$ and Dy$(TTHA)^{3-}$ induce, in decreasing order, the largest Li^{+} shifts. However, those produced by Dy$(PPP)_2^{7-}$ and Tm$(PPP)_2^{7-}$ are highly pH dependent. It was found by $^{7}Li^{+}$ (and $^{23}Na^{+}$) NMR spectroscopy that up to seven Li^{+} (or Na^{+}) ions saturate all the binding sites on Dy$(PPP)_2^{7-}$ at pH 7.5. The larger Li^{+} shifts observed at higher pH are due to an increased electrostatic interaction between the shift reagent and Li^{+} ions as a result of deprotonation of the triphosphate ligands. The Li^{+} shifts induced by Dy$(PPP)_2^{7-}$ are smaller in the presence of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+}. The relative order of cation competition in Dy$(PPP)_2^{7-}$ is $Ca^{2+} \geqslant Mg^{2+} > Li^{+} > Na^{+} \geqslant K^{+}$. ^{31}P NMR studies indicate that divalent cations mainly compete with Dy^{3+} for the triphosphate ligand via a scrambling mechanism while monovalent cations compete for the Li^{+} binding sites in the second coordination sphere of the shift reagent. Dy$(TTHA)^{3-}$ is the most promising shift reagent (among those tested) for $^{7}Li^{+}$ NMR studies since it interacts weakly with Li^{+} and yet it produces relatively large shifts which are virtually independent of pH and are less sensitive to the presence of monovalent and divalent cations. Application of Dy$(PPP)_2^{7-}$, Tm$(PPP)_2^{7-}$ and Dy$(TTHA)^{3-}$ to the study of Li^{+} transport in human red blood cells by $^{7}Li^{+}$ NMR spectroscopy is briefly discussed.

Introduction

Lithium is an effective drug in the treatment of bipolar disorders [1]. One of the ways by which lithium may be able to regulate the abnormal intracellular sodium and calcium levels present in nerve cells of bipolar patients is by changing the cell membrane permeability properties. Abnormalities in Li^{+} transport across RBC membranes have been observed in families of hypertensive [2] and bipolar [3] patients, however, it is uncertain whether these abnormalities constitute genetic markers of these diseases.

Several aqueous shift reagents for metal cationic NMR spectroscopy have been reported [4–7] which have found useful applications in membrane transport biochemistry. Recent examples of these applications include the study of transport of alkali metal ions across vesicles [8, 9] and red blood cell (RBC) membranes [7, 10–13]. We have previously shown that Gupta's shift reagent [7], dysprosium(III) triphosphate, could be used to distinguish intra- and extracellular pools of Li^{+} in RBCs [12]. A systematic study was attempted in order to survey the applicability of several shift reagents for $^{7}Li^{+}$ NMR transport studies. Dysprosium(III) and thulium(III) chelates of the ligands shown in Fig. 1 were expected to afford the largest paramagnetic shifts for $^{7}Li^{+}$ NMR [14, 15]. Lithium is present in most biological systems at relatively low concentrations (<1.5 mM) compared to other physiologically relevant cations such as Na^{+} and K^{+} [1]. Hence, shift reagents were tested for their specificity for Li^{+} over other alkali and alkaline-earth metal cations. The pH and metal competition studies described in this paper also provide further insight into the solution structure of the triphosphate shift reagents.

Experimental

Lithium chloride, thulium chloride, dysprosium chloride, sodium triphosphate, glucose, triethylene-

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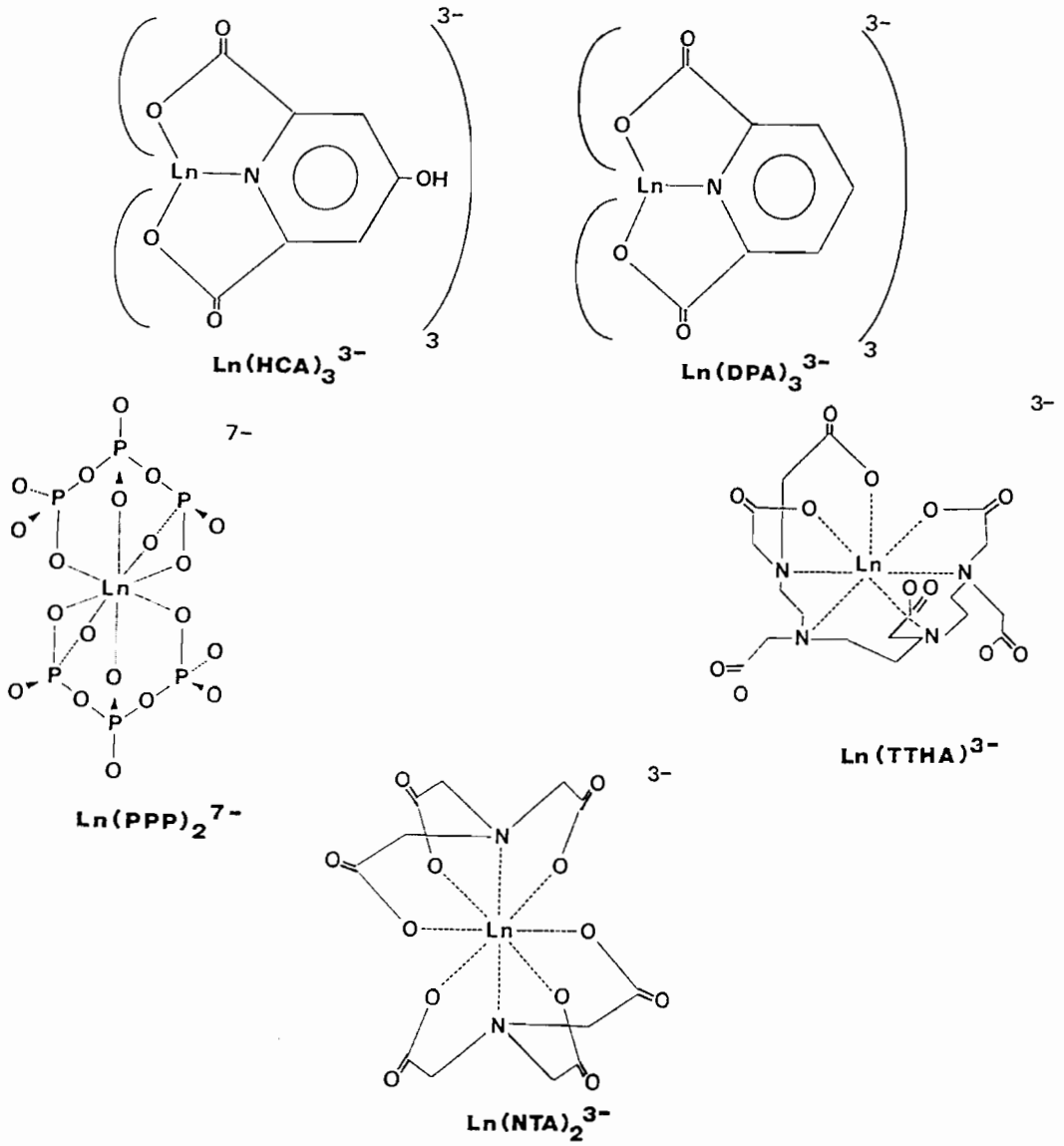

Fig. 1. Structure of shift reagents used in this study. PPP^{5-}, $TTHA^{3-}$, NTA^{3-}, DPA^{2-} and HCA^{2-} represent the ligands triphosphate, triethylenetetraminehexacetate, nitrilotriacetate, dipicolinate (pyridine-2,6-dicarboxylate), and chelidamate (4-oxypiridine-2,6-dicarboxylate), respectively. Water coordination in all of these shift reagents is not represented.

tetraminehexacetic acid (H_6TTHA), chelidamic acid (H_3CA), nitrilotriacetic acid (H_3NTA), dipicolinic acid (H_2DPA), tetramethylammonium chloride and deuterium oxide (99.8%) were supplied by Aldrich. HEPES [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid] was from Sigma. All chemicals were used as received except sodium triphosphate which was recrystallized three times from 40% ethanol.

The shift reagents containing the ligands $TTHA^{6-}$, HCA^{3-}, NTA^{3-} and DPA^{2-} were freshly prepared by *in situ* methods previously described [4–6], with the exception that tetramethylammonium hydroxide was used as a base. With this modified procedure, these shift reagents were in the form of tetramethylammonium salts. This was done so that the bulky counter cation would be less competitive with the alkali and alkaline-earth metal cations being studied.

Tetramethylammonium Dy(III) or Tm(III) triphosphates were prepared by a different method. Recrystallized Na_5PPP was passed down a cation-exchange Dowex-50 column (Dow Chemical Company) loaded with tetramethylammonium chloride. The tetramethylammonium triphosphate thus obtained was recrystallized five times and was checked by ^{23}Na NMR for any residual sodium. Sodium content of tetramethylammonium triphosphate was estimated to be less than 9% of sodium present in Na_5PPP. The tetramethylammonium form of the shift

reagents $Dy(PPP)_2^{7-}$ and $Tm(PPP)_2^{7-}$ was obtained from the respective dysprosium and thulium chlorides by an *in situ* reaction [4] with tetramethylammonium triphosphate.

The HEPES buffer used in all the experiments was adjusted to the physiological pH range with tetramethylammonium hydroxide. The ionic strength of the samples shown in Fig. 2 was adjusted with tetramethylammonium chloride. Ionic contributions from LiCl, shift reagent, HEPES and tetramethylammonium hydroxide used in adjusting pH, were taken into account in estimating the amount of tetramethylammonium chloride required to adjust the solution to the final ionic strength value.

Packed RBCs of healthy donors were supplied by the Chicago Chapter of the American Red Cross. RBC samples were loaded with lithium and processed for NMR measurements according to procedures previously described by us [12b].

^7Li, ^{23}Na and ^{31}P NMR measurements were made at 116.5, 79.4 and 121.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer equipped with a multinuclear probe. For metal NMR studies, 60° pulses were used at a probe temperature of 37 °C. The paramagnetic shifts were measured as the difference of the observed resonance position from the resonance position of the cation in the absence of the shift reagent. Upfield shifts are reported as positive. $^7Li^+$ and $^{23}Na^+$ NMR resonances of sample and reference were measured in separate, spinning 10 mm NMR tubes. No coaxial tube combinations were used. Field-frequency locking on D_2O solvent was used throughout. The above procedures eliminate contributions to the observed $^7Li^+$ and $^{23}Na^+$ NMR shifts from bulk magnetic susceptibility assuming that lanthanide induced shifts on the solvent ^2H resonance are negligible [4]. For ^{31}P NMR studies, a pulse width of 7.2 μs was used with a 0.72 s pulse delay at a probe temperature of 25 °C. The α,γ-phosphate resonance of 0.15 M sodium triphosphate contained in a capillary tube was used as an external standard.

Results

$^7Li^+$ NMR Paramagnetic Shifts Induced by Dysprosium and Thulium Chelates

The efficacy of the dysprosium and thulium chelates (Fig. 1) as shift reagents for $^7Li^+$ NMR studies were tested. For these experiments, the following parameters were held constant: LiCl concentration at 5 mM, pH at 7.5 and the ionic strength at 0.36. Ten different shift reagents were tested, varying their concentrations from 3 to 30 mM. The shift values are plotted against the stoichiometric mole ratio of shift reagent to Li^+, ρ, in Fig. 2.

The shifts induced in the $^7Li^+$ NMR resonance by $Dy(PPP)_2^{7-}$, $Tm(PPP)_2^{7-}$ and $Dy(TTHA)^{3-}$ are

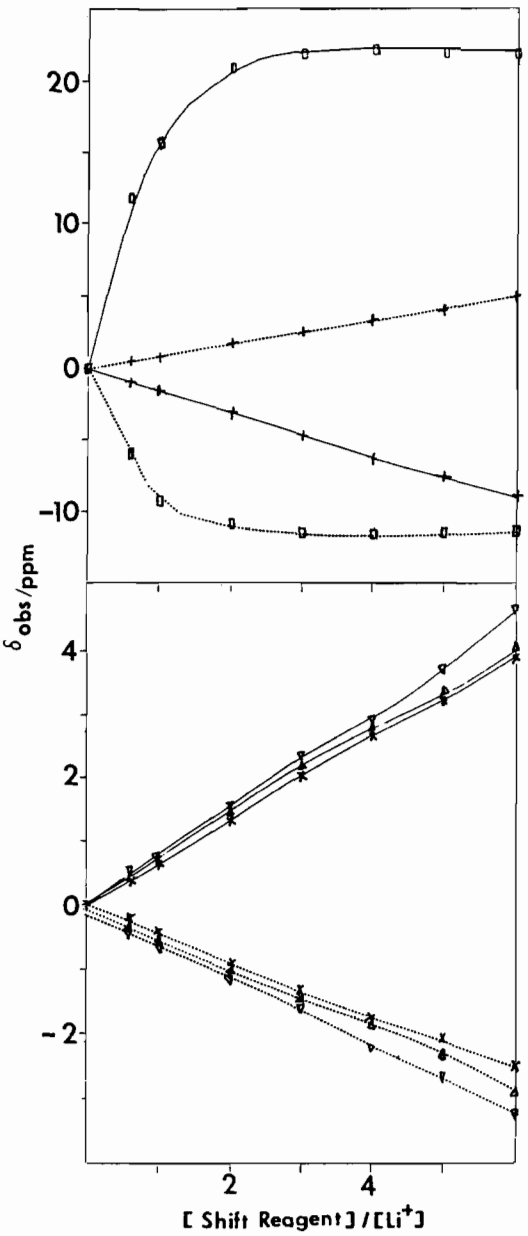

Fig. 2. Dependence of paramagnetic $^7Li^+$ NMR shifts induced by several shift reagents on [shift reagent]/[Li^+] ratio. Full and dotted lines indicate data for Dy^{3+} and Tm^{3+} shift reagents, respectively. The symbols for the ligands PPP^{5-} (□), $TTHA^{6-}$ (+), NTA^{3-} (▽), DPA^{2-} (△), and HCA^{2-} (×) are indicated in parentheses. Each point represents the average of $^7Li^+$ NMR measurements on three separate samples. In all cases, the agreement was within 0.02 ppm. Li^+ concentration was held constant at 5 mM while the shift reagent concentrations varied. All solutions were buffered with 50 mM HEPES and also contained 10 mM glucose and 17% D_2O. The pH was adjusted to 7.5 with tetramethylammonium hydroxide. Ionic strength was adjusted to 0.36 with tetramethylammonium chloride so that all samples had the same ionic strength as that of 30 mM $Dy(PPP)_2^{7-}$. Conditions for NMR measurements are described in 'Experimental'.

significantly larger than those induced by Dy(HCA)$_3^{3-}$, Tm(HCA)$_3^{3-}$, Dy(NTA)$_2^{3-}$, Tm(NTA)$_2^{3-}$, Dy(DPA)$_3^{3-}$, Tm(DPA)$_3^{3-}$ and Tm(TTHA)$^{3-}$, under similar conditions. Slightly larger shifts may have been observed if the solution were free of added salt. However, the shifts are close to their maximum value since a bulky, non-competitive organic counter cation, tetramethylammonium, was used. Much larger $^7Li^+$ NMR shifts can be obtained at alkaline pH with the chelidamate shift reagents (data not shown). The enhanced shifts at alkaline pH are due to the increased electrostatic interaction between Li^+ and the shift reagent, Dy(CA)$_3^{6-}$ or Tm(CA)$_3^{6-}$, resulting from the deprotonation of the chelidamate ligands above pH 8. Similar observations were reported for $^{23}Na^+$ NMR shifts [6]. Based on these studies alone, we conclude that Dy(PPP)$_2^{7-}$, Tm(PPP)$_2^{7-}$ and Dy(TTHA)$^{3-}$ are the preferred shift reagents for $^7Li^+$ NMR studies under physiological conditions.

pH Dependence of $^7Li^+$ and $^{23}Na^+$ NMR Isotropic Shifts

As shown in Fig. 3, the $^7Li^+$ shifts induced by Dy(PPP)$_2^{7-}$ and Tm(PPP)$_2^{7-}$ show a very strong pH dependence while the shift induced by Dy(TTHA)$^{3-}$

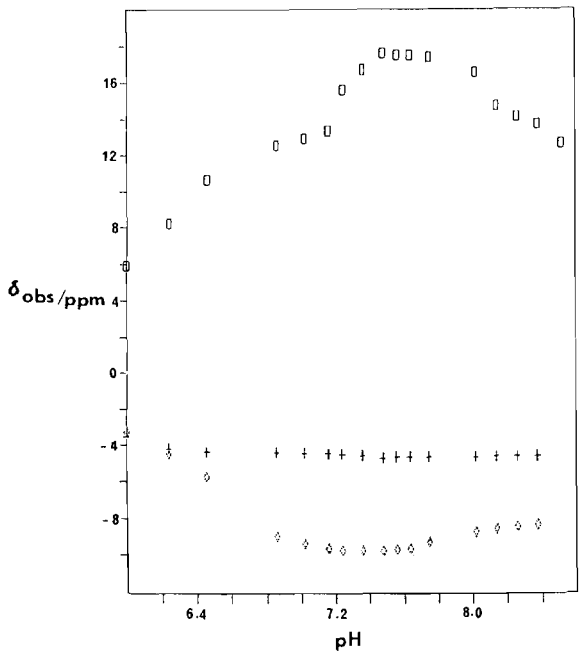

Fig. 3. pH dependence of paramagnetic shifts afforded by Dy(PPP)$_2^{7-}$ (□), Dy(TTHA)$^{3-}$ (+) and Tm(PPP)$_2^{7-}$ (◇). Li^+ concentration was held constant at 5 mM in the three curves. The Dy(PPP)$_2^{7-}$ was kept constant at 5 mM while Dy(TTHA)$^{3-}$ and Tm(PPP)$_2^{7-}$ were held at 15 mM. The reported pH values are not corrected for the deuterium isotope effect. The pH dependence of $^7Li^+$ shifts was found to be reversible by adding tetramethylammonium hydroxide and HCl above and below pH 7.5, respectively. NMR conditions are the same as for Fig. 2.

is quite independent of pH between 6 and 8.5. The decreased $^7Li^+$ NMR shifts observed above pH 8 for the triphosphate shift reagents may be due to increased competition for Li^+ ions from the tetramethylammonium cations, which were increasing in concentration since tetramethylammonium hydroxide was used to adjust the pH. More likely, the decreased shifts are due to OH^- ions replacing the PPP^{5-} ligand at alkaline pH, leading to the formation of $Dy(OH)_3$ [4], as a precipitate of $Dy(OH)_3$ was observed above pH 8.5. The maximum positive shift observed on the Dy(PPP)$_2^{7-}$ occurs at a higher pH than the maximum negative shift on the Tm(PPP)$_2^{7-}$. This behavior may be related to the lower affinity of Dy^{3+} complexes for OH^- relative to the Tm^{3+} analogs [14]. However, the most important region from a physiological standpoint is that below pH 8, where the pH dependence of the paramagnetic shifts afforded by the triphosphate shift reagents is more noticeable.

The titration of 5 mM Dy(PPP)$_2^{7-}$ with excess Li^+ or Na^+ at pH 5.5 and 7.5 was monitored by ^7Li or ^{23}Na NMR spectroscopy, respectively (Fig. 4). Li^+ or Na^+ were found to bind biphasically to the shift reagent at both pH values studied. Extrapolation of the linear portions of the curves results in breaks at $[M^+]/[\text{shift reagent}]$ ratios of approximately 5 and 7 at pH 5.5. and 7.5, respectively. We conclude that up to seven Li^+ (or Na^+) ions are required to saturate all the binding sites on the shift reagent species, Dy(PPP)$_2^{7-}$, DyH(PPP)$_2^{6-}$ and DyH$_2$(PPP)$_2^{5-}$, present at pH 7.5 [16]. At pH 5.5, the major species present in solution are DyH$_2$(PPP)$_2^{5-}$ and Dy(PPP)$^{2-}$ [16]. Thus, the smaller number of cations bound at pH 5.5 (Fig. 4) and the decreased $^7Li^+$ shifts below pH 7.0 (Fig. 3) are presumably due to the presence of species with reduced negative charge at lower pH.

The $^7Li^+$ (or $^{23}Na^+$) paramagnetic shifts are maximized at $[M^+]/[\text{shift reagent}]$ ratios of less than 1. This observation is evidence for a preferred Li^+ binding site in the second coordination sphere of the shift reagent (Fig. 5B) [17]. However, solution structures that involve a preferential binding site for Li^+ in close proximity to the lanthanide ion (Fig. 5A and C) [4, 18] cannot be ruled out. Figure 4 shows that gradual decreases in $^7Li^+$ (and $^{23}Na^+$) shifts continue to occur after a ratio of 1. These results are in agreement with multinuclear NMR studies on dysprosium(III) triphosphate [17] carried out at pH 7.5 that suggest a solution structure where alkali countercations are present in the second coordination sphere of the complex thereby neutralizing its −7 charge (Fig. 5B). Thus, the first equivalent of Li^+ added to Dy(PPP)$_2^{7-}$ at pH 7.5 occupies a preferential binding site with a specific θ value and therefore a specific pseudo-contact shift. Further addition of Li^+ leads to exchange between the Li^+ on the preferred site and those on other locations (Fig. 5B). Each Li^+ location

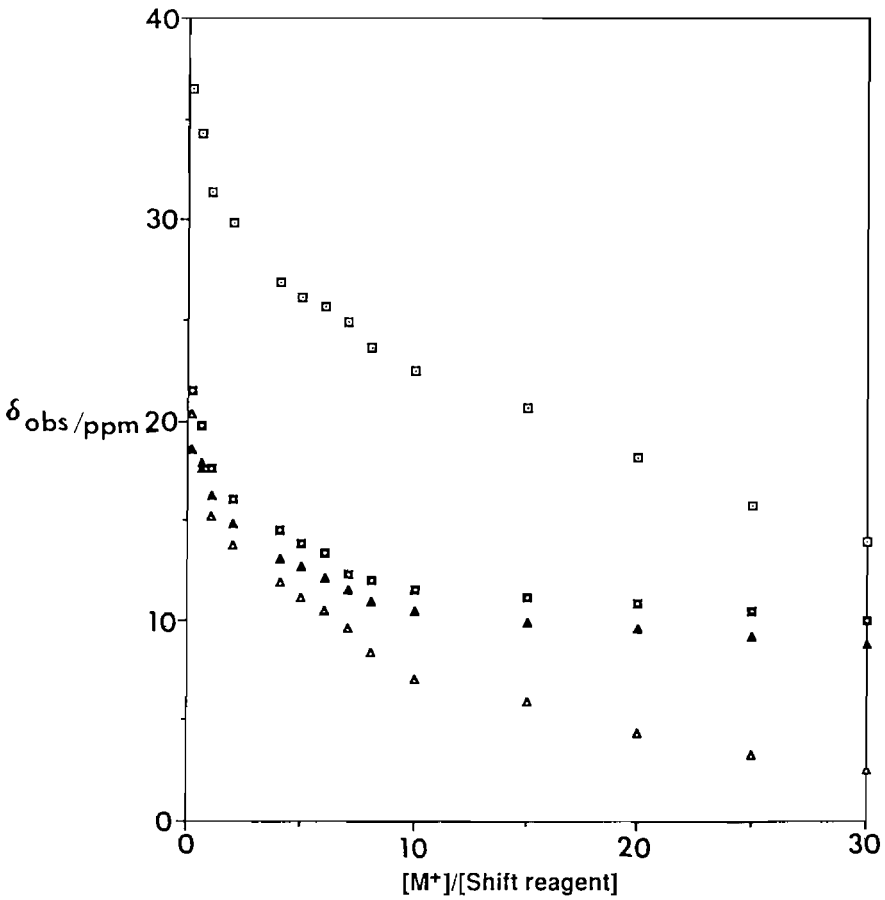

Fig. 4. Titration of $^7Li^+$ (closed symbols) and $^{23}Na^+$ (open symbols) NMR paramagnetic shifts at pH 7.5 (squares) and 5.5 (triangles). $Dy(PPP)_2^{7-}$ concentration was held constant at 5 mM and Li^+ or Na^+ concentration was varied. The shift reagent used was in the tetramethylammonium form. NMR conditions are the same as for Fig. 2.

has its own θ and corresponding pseudo-contact shift. Averaging leads to a decrease of the $(3\cos^2\theta - 1)/r^3$ term and therefore to a decrease of the $^7Li^+$ paramagnetic shift. After the addition of a maximum of seven Li^+ ions depending on the species present at pH 7.5, further addition of Li^+ results in cation exchange between Li^+ in the second coordination sphere and the bulk solution. This results in an asymptotical decrease in $^7Li^+$ paramagnetic shift down to zero.

Based on plots of $\delta_{obs}/[Li^+]$ versus δ_{obs} of the linear portions of the curves at pH 7.5 for $[Li^+]$/[shift reagent] $\leqslant 2$ or $\geqslant 10$, respectively, the first and last Li^+ association constants were calculated to be approximately 740 and 136 M^{-1} ($r = 0.98$). The last Na^+ association constant at pH 7.5 (135 M^{-1}) is of the same order of magnitude as those obtained previously by an independent ^{23}Na NMR study (120–360 M^{-1}) [18]. The changes of $^7Li^+$ NMR shifts were less drastic in similar experiments performed with $Dy(TTHA)^{3-}$ (data not shown), indicating weaker binding ($K < 50$ M^{-1}) of Li^+ to this shift reagent relative to $Dy(PPP)_2^{7-}$.

We further investigated the pH dependence of the shifts induced by the triphosphate shift reagents by ^{31}P NMR titrations.

^{31}P NMR Titration Studies of $Dy(PPP)_2^{7-}$

^{31}P NMR spectra of $Dy(PPP)_2^{7-}$ at pH 6.5, 7.4 and 8.3 in the absence of LiCl (Fig. 6) exhibit an $A(X_2)$ type spectrum indicating that the outer phosphates of the triphosphate ligands are equivalent at all measured pH values. The chemical shift position of the α,γ-resonances is in general more sensitive to pH than that of the β-resonance (Table 1). This observation is in agreement with preferential protonation of $Dy(PPP)_2^{7-}$ at the terminal phosphate groups [16, 19–21]. While the chemical shift position of the β-resonance of $Dy(PPP)_2^{7-}$ is virtually unaffected by the addition of Li^+, that of the α,γ-resonances is shifted downfield. From these results alone, one cannot conclude that Li^+ (or H^+) preferentially binds to the outer phosphate groups since ^{31}P complexation shifts are rather complex in nature. They depend not only on direct coordination effects (for example, inductive or cation charge effects) but also on

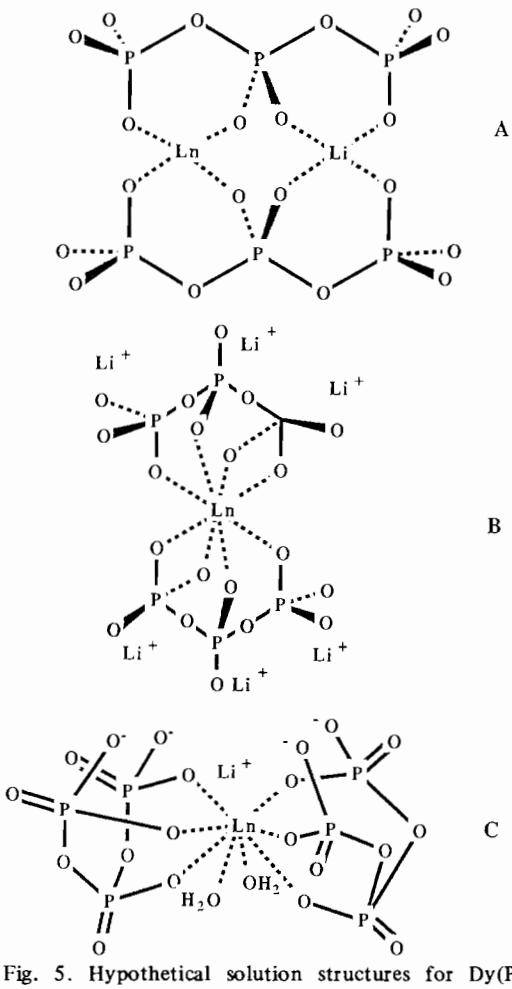

Fig. 5. Hypothetical solution structures for $Dy(PPP)_2^{7-}$ based on studies reported in refs. 4 (A), 17 (B) and 18 (C).

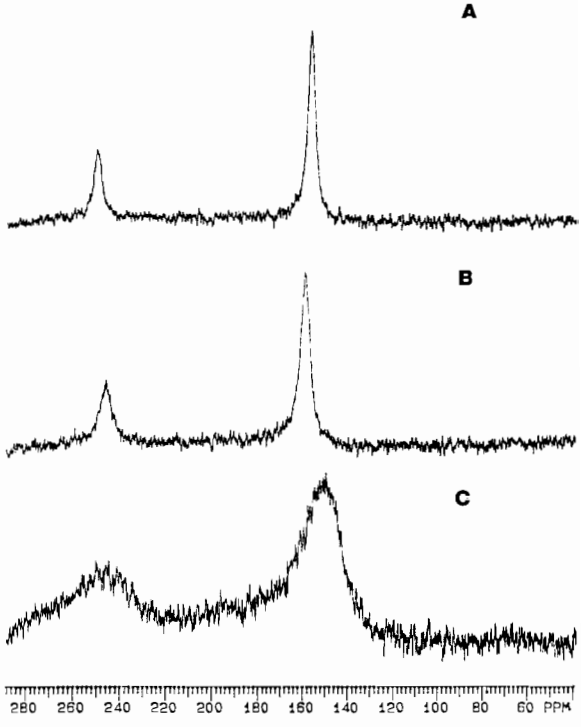

Fig. 6. ^{31}P NMR (121.4 MHz, 25 °C) spectra of $Dy(PPP)_2^{7-}$ at pH 8.3 (A), 7.4 (B) and 6.5 (C) in the absence of LiCl. The shift reagent in the tetramethylammonium form was used at a concentration of 5 mM and the ratio of Dy^{3+} to triphosphate ligand was 1:2. Line broadening of 25 Hz was used.

indirect effects, such as changes in O–P–O torsional angles [22]. Moreover, Li^+ binding to the triphosphate complex could also change the Dy^{3+} dipolar contribution to the ^{31}P shift.

The effect of pH on the linewidths of the ^{31}P NMR resonances is more significant than that observed on the chemical shifts (Table 1). The linewidths of the ^{31}P NMR resonances corresponding to the two outer phosphates increase more drastically at pH 6.5 than

TABLE 1. ^{31}P NMR chemical shifts and linewidths of α,γ- and β-phosphate resonances of $Dy(PPP)_2^{7-}$ in the absence and presence of 100 mM LiCl

	$[Dy^{3+}]/[PPP^{5-}]$ Ratio					
	1:2 at pH			1:4 at pH		
	6.5	7.4	8.3	6.5	7.4	8.3
Without LiCl						
$\delta(\Delta\nu_{1/2})$ [a] α,γ	156(646)	160(473)	157(378)	148(987)	151(633)	152(473)
β	243(898)	248(492)	250(428)	238(1132)	241(696)	242(497)
With 100 mM LiCl						
$\delta(\Delta\nu_{1/2})$ [a] α,γ	139(800)	152(586)	142(575)	[b]	141(803)	147(663)
β	[b]	247(548)	245(632)	[b]	238(897)	240(682)

[a]Chemical shifts and linewidths measured in ppm and Hz, respectively. [b]Resonance too broad to be measured accurately.

does that of the central phosphate. Moreover, the linewidths of all phosphate resonances from the shift reagent are broader in the presence of Li^+ ions. The line broadening observed at pH 6.5 or upon addition of Li^+ ions is due to a shorter τ_{cpx}, the residence time of the ligand in the complexed state. Since the outer phosphate groups of $Dy(PPP)_2^{7-}$ are preferentially protonated or bound to Li^+ [16, 19–21], one would expect the interaction between protonated α,γ-phosphates and the Dy^{3+} ion at low pH and/or in the presence of Li^+ ions to be weaker than the corresponding one with deprotonated β-phosphates. Hence the shorter residence times and wider linewidths for the α,γ-resonances relative to those of the β-resonance.

Below pH 6.0, the chemical shifts of the α,γ-phosphate resonances start changing drastically and eventually coalesce at approximately pH 3.0 [18]. For Dy(III) triphosphate, the chemical shift difference between bound and free phosphate resonances is larger for the central phosphate than for the outer phosphate resonances [18]. Thus, upon lowering the pH below 6.0 there will be a relatively large increase in the linewidth of the β-phosphate resonance while a change in chemical shift will be first observed for the α,γ-phosphate resonance. However, the approach to coalescence, and its effect on linewidths and chemical shift positions, is not likely to be felt at the three pH values reported here.

Two different $[Dy^{3+}]/[ligand]$ ratios, 1:2 and 1:4, respectively, were studied (Table 1). In both cases, significant line broadening occurred at pH 6.5 and/or upon addition of Li^+ ions. The stability constants for $Dy(PPP)_2^{7-}$ and $Dy(HPPP)_2^{5-}$ were previously measured at 35 °C and found to be 9.5 and 7.2 (in log K units), respectively [16]. Thus, for the metal/ligand ratios used in these experiments, the 1:2 Dy^{3+}–PPP complex will be the predominant species present in solution and a ligand exchange mechanism [18] may be operating.

$$Dy(PPP)_2^{7-} \longrightarrow Dy(PPP)^{2-} + PPP^{5-}$$

High $[H^+]$ will favor the dissociation of $Dy(PPP)_2^{7-}$ because of the weaker interaction between the protonated triphosphate ligand and the Dy^{3+} ion. Addition of Li^+ is also expected to facilitate ligand exchange mechanism since it can stabilize the negatively charged species.

Thus, one can conclude that the line broadening observed on the ^{31}P signals of the triphosphate ligand in the presence of Li^+ and at low pH is dominated by intermolecular ligand exchange processes and cannot be used unambiguously to construe any information about intramolecular exchange processes, like those presumably involved in Li^+ (and H^+) binding.

Competition between Li^+ and Other Cations for Shift Reagents

Polyvalent cations present in solution can drastically decrease the $^7Li^+$ NMR paramagnetic shift by competing effectively with Li^+ for the same shift reagent. In physiological solutions, the divalent cations most likely to be encountered are Ca^{2+} and Mg^{2+}. Figure 7 shows the effects of Ca^{2+} and Mg^{2+} on the absolute magnitudes of the $^7Li^+$ NMR shifts induced by $Dy(PPP)_2^{7-}$ and $Dy(TTHA)^{3-}$. For

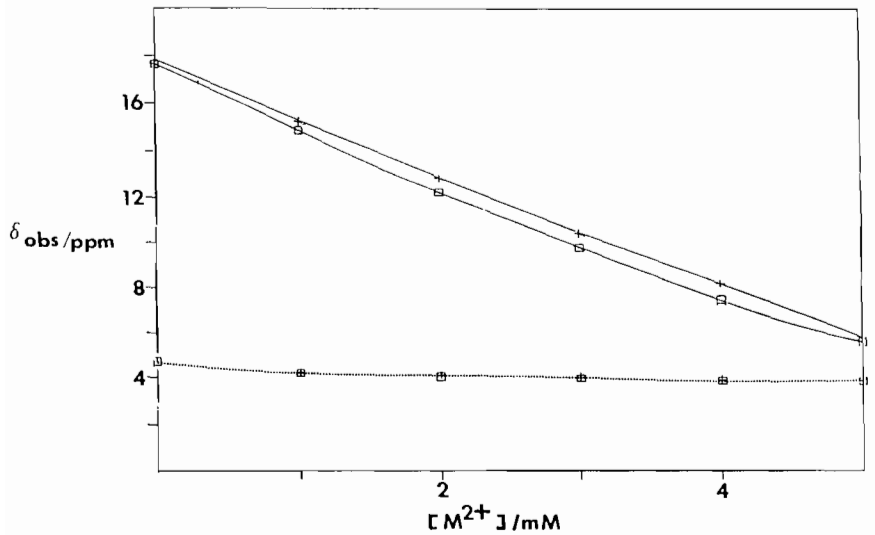

Fig. 7. Competition between Ca^{2+} (□) or Mg^{2+} (+) and Li^+ for $Dy(PPP)_2^{7-}$ (full lines) and $Dy(TTHA)^{3-}$ (dotted lines). The shift reagents' counter cation was tetramethylammonium ion. LiCl concentration was held constant at 5 mM. The concentration of $Dy(PPP)_2^{7-}$ was 5 mM while that of $Dy(TTHA)^{3-}$ was 15 mM, otherwise the experimental conditions were the same as for Fig. 2. In $Dy(PPP)_2^{7-}$ solutions, precipitation was observed when the concentration of Mg^{2+} or Ca^{2+} rose above 3 mM or 2 mM, respectively.

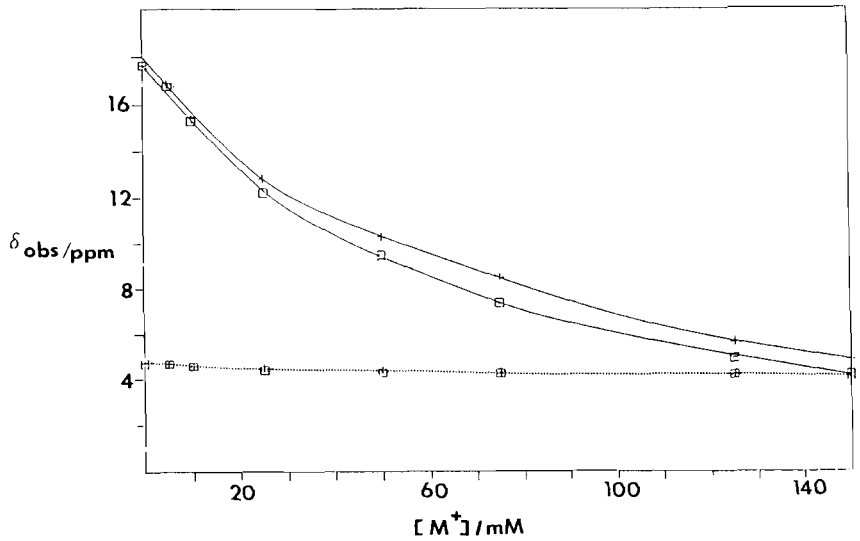

Fig. 8. Competition between Na^+ (□) or K^+ (+) and Li^+ for $Dy(PPP)_2^{7-}$ (full lines) and $Dy(TTHA)^{3-}$ (dotted lines). Same experimental conditions as for Fig. 7.

physiologically relevant concentrations of Ca^{2+} and Mg^{2+} (less than 2 mM, in general) the shifts induced by $Dy(PPP)_2^{7-}$ are significantly larger than those produced by $Dy(TTHA)^{3-}$. Overall, the shifts induced by either shift reagent are sensitive to the presence of Ca^{2+} or Mg^{2+}, with those caused by $Dy(PPP)_2^{7-}$ being extremely sensitive to the presence of these two cations, when compared to $Dy(TTHA)^{3-}$. In physiological solutions, Na^+ and K^+ are encountered in much higher concentrations compared to Li^+. As observed with divalent cations, the shifts induced by either shift reagent are also sensitive to the presence of K^+ or Na^+ (Fig. 8). Likewise, the shifts caused by $Dy(PPP)_2^{7-}$ are more sensitive to the presence of monovalent cations than $Dy(TTHA)^{3-}$. In the competition experiments involving Na^+ and K^+, there is a sluggish decrease in $^7Li^+$ paramagnetic shifts even in the presence of excess Na^+ or K^+. By contrast, there is a steep decrease in $^7Li^+$ shifts even in the presence of low concentrations of Ca^{2+} and Mg^{2+}. Assuming that the Li^+ ion populates the preferential binding site on the triphosphate shift reagent, in the presence of other monovalent cations Li^+ will be forced to exchange with other sites in the second coordination sphere of the shift reagent having different θ values. As a consequence, the term $(3\cos^2\theta - 1)/r^3$ will be averaged out and the induced $^7Li^+$ paramagnetic shift will decrease.

A quantitative interpretation of the competition between Li^+ and other cations for $Dy(PPP)_2^{7-}$ is difficult to obtain as a result of the complex solution chemistry of this shift reagent. However, based on the results depicted on Figs. 7 and 8, the relative order of cation competition for $Dy(PPP)_2^{7-}$ at pH 7.5 is predicted to be $Ca^{2+} \geqslant Mg^{2+} > Li^+ > Na^+ \geqslant K^+$. The decreasing order of association constants for monovalent cations follows the increasing order of ionic radii lending support to an electrostatic model. As expected the competition between divalent cations and Li^+ is much larger than that observed with monovalent cations. Ca^{2+} competition is slightly larger than that provided by Mg^{2+} despite the fact that Ca^{2+} has a larger ionic radius and a smaller affinity for PPP^{5-} relative to Mg^{2+} [19]. This indicates that a different mechanism (*vide infra*) may significantly contribute toward the competition reaction between divalent cations and Li^+.

The addition of Ca^{2+} to $Dy(PPP)_2^{7-}$ was monitored by ^{31}P NMR (Fig. 9). We observed a decrease in the intensity of the resonances of Dy^{3+}-complexed phosphates (Fig. 9A) accompanied by the formation of unshifted but broadened triphosphate resonances (Fig. 9B). The chemical shifts of the latter resonances match those of $CaPPP^{3-}$ (Fig. 9C) and are different from any tetramethylammonium triphosphate present in slow exchange prior to addition of Ca^{2+} (Fig. 9D). Similar ^{31}P NMR observations were found upon addition of Mg^{2+} salts to $Dy(PPP)_2^{7-}$. By contrast, no changes were observed on the ^{31}P NMR spectrum of $Dy(PPP)_2^{7-}$ upon addition of Na^+ and K^+ ions.

These observations indicate two distinct classes of competition mechanisms for divalent and monovalent cations. Divalent cations can compete with the Ln^{3+} ion for the ligand in a scrambling type reaction, or alternatively, they can compete with the monovalent cations for the second coordination sphere of the shift reagents. The ^{31}P NMR studies (Fig. 9) indicate that the scrambling mechanism is operating to a large

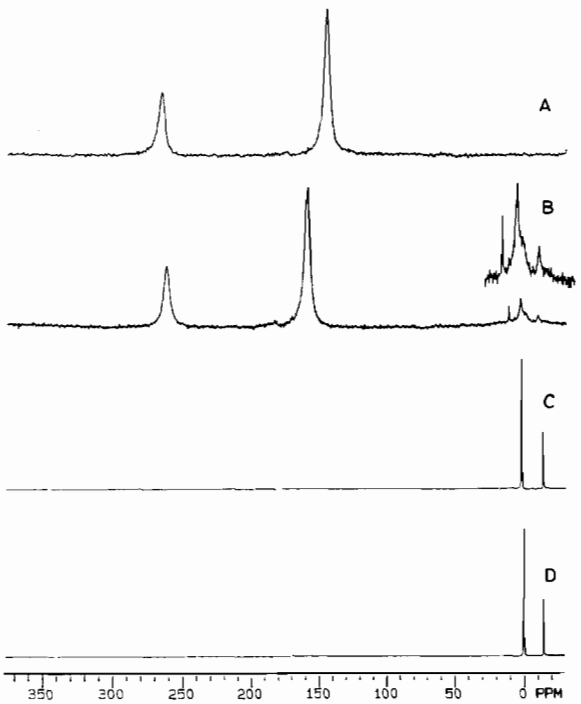

Fig. 9. ^{31}P NMR (121.4 MHz, 25 °C) spectra. (A) 5 mM $Dy(PPP)_2^{7-}$ at pH 7.4. The shift reagent was in the tetramethylammonium form and a Dy^{3+} to ligand ratio of 1:2 was used. Line broadening of 25 Hz was used. (B) Same as in (A) except that 1 mM $CaCl_2$ was also present. The third sharp resonance from the right present in the (B) insert is due to inorganic phosphate resulting from Ca^{2+}-catalyzed triphosphate hydrolysis. (C) 5 mM $(NMe_4)_5PPP$ and 5 mM $CaCl_2$ at pH 7.4. (D) 5 mM $(NMe_4)_5PPP$ at pH 7.4.

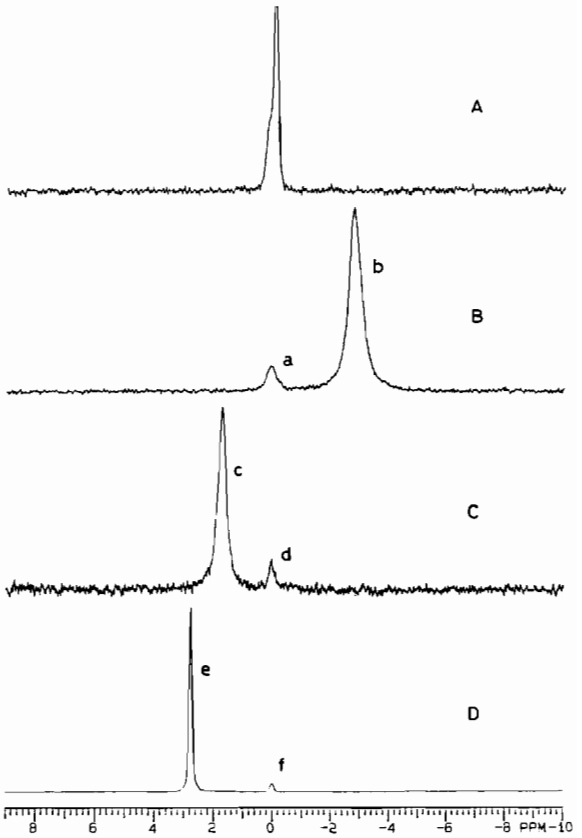

Fig. 10. (A) ^7Li NMR (116.5 MHz, 37 °C) spectrum of gently packed RBCs in a medium containing 3.5 mM LiCl, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 50 mM HEPES at pH 7.5. Packed RBCs were incubated with 3.5 mM LiCl at 37 °C for 12 h prior to NMR measurements. 17% D_2O was present for field frequency lock. Hematocrit was 13%. (B) ^7Li NMR spectrum of the same RBC suspension as in (A) except that 5 mM $Na_7Dy(PPP)_2 \cdot 3NaCl$ was present in the medium instead of 50 mM NaCl. (C) ^7Li NMR spectrum of a similar RBC suspension as in (A) except that 10 mM $Na_7Tm(PPP)_2 \cdot 3NaCl$ was present in the medium instead of 100 mM NaCl. (D) ^7Li NMR spectrum of the same RBC suspension as in (A) except that 10 mM $Na_3Dy(TTHA)$ was present in the medium instead of 30 mM NaCl. a, d, and f represent inner pools of Li^+ while b, c, and e represent outer pools of Li^+. A pulse width of 15 μs was used which corresponds to a flip angle of 45°. The acquisition time was 1 s followed by a delay of 6.5 s, except for (A) where the delay was 50 s. A total of 8 scans were taken for each spectrum with a total accumulation time of approximately 1 min except for (A) which took 7 min.

extent when $Ln(PPP)_2^{7-}$ is present along with Ca^{2+} or Mg^{2+} On the other hand, Na^+ and K^+ ions effectively compete for Li^+ binding sites in the second coordination sphere of the triphosphate complex.

Analogous ^{13}C NMR studies on the $Dy(TTHA)^{3-}$ shift reagent suggested that this complex is not susceptible to the ligand scrambling reaction since no new unshifted ligand resonances were found upon addition of Ca^{2+} (data not shown). Thus, the small decrease observed in the $^7Li^+$ NMR shifts induced by $Dy(TTHA)^{3-}$ (Fig. 7) is probably due to simple competition between Ca^{2+} or Mg^{2+} and Li^+. It is important to note that complexes of the type LnL^{3-n} (where Ln^{3+} and L^{n-} represent the lanthanide ion and ligand, respectively) are more resistant to ligand scrambling reactions than those with a stoichiometry of the type LnL_2^{3-2n} or LnL_3^{3-3n} since the trivalent lanthanide ions have higher affinities than di- and monovalent cations for most ligands. The ligand affinity of the lanthanide ion is enhanced in cases where only one polydentate ligand is coordinated, as in $Dy(TTHA)^{3-}$.

Application of Shift Reagents to the Study of Li^+ Transport in RBC

The application of the three most promising $^7Li^+$ NMR shift reagents discussed in this paper is exemplified in Fig. 10 for a Li^+-loaded RBC suspension. Figure 10A shows that the intra- and extracellular $^7Li^+$ NMR resonances are not resolved in spectra of RBC suspensions to which no shift reagent

has been added. The lack of resolution of the two $^7Li^+$ NMR resonances follows from the fact that Li^+ exists predominantly in the form of an aquo ion in both the intra- and extracellular components of RBC suspensions [12, 13]. In addition, alkali metal chemical shifts are only weakly dependent on ligation and/or solvation [23]. Clear separation of the NMR signals corresponding to the two Li^+ pools can be achieved by addition of 5 mM $Dy(PPP)_2^{7-}$, 10 mM $Tm(PPP)_2^{7-}$ or 10 mM $Dy(TTHA)^{3-}$ to the RBC suspension (Fig. 10B–D). In all samples containing shift reagent, only the $^7Li^+$ NMR signal corresponding to extracellular Li^+ is shifted indicating that the shift reagents used in this study are not cell membrane permeable. Although Li^+ is present in the RBC suspension at a lower concentration than Na^+ or K^+, it effectively competes with these monovalent cations for the three shift reagents tested. Similar reports have appeared for $^{23}Na^+$ and $^{39}K^+$ NMR studies of RBCs [7, 10, 11].

Discussion

Springer and coworkers [4] have hypothesized a solution structure (Fig. 5A) for the interaction of Na^+ with $Ln(PPP)_2^{7-}$ based on X-ray crystallographic studies of Na_5PPP [24]. The paramagnetic effect of Gd^{3+} on the T_1 relaxation rate of the ^{23}Na nucleus in the $Na{\cdot}Gd(PPP)_2^{6-}$ complex was also investigated by Gupta [25] and the Gd^{3+} to Na^+ distance was calculated to be 4 Å. Both the X-ray studies of Na_5PPP and the relaxation studies of $Gd(PPP)_2^{7-}$ suggest a specific Na^+ binding site close to the Ln^{3+} ion (Fig. 5A). More recently, a different structure for $Dy(PPP)_2^{7-}$ has been proposed that also entails a preferential binding site for Na^+ (Fig. 5C) [18]. Although these two structures differ in the mode of Dy^{3+} coordination to PPP^{5-}, the distance between Dy^{3+} and Na^+ in both complexes is similar. Therefore, the dipolar shift, which is inversely proportional to the cube of the distance of the binding site from the paramagnetic center, would be similar for the two models (Fig. 5A and C) assuming that the angular dependence of the dipolar shift is approximately the same.

The PPP^{5-} ligand can also act as a tetradentate ligand in $Ca(PPP)^{3-}$ and in $Nd(PPP)_2^{7-}$ [19, 26]. An alternative structure for $Ln(PPP)_2^{7-}$ based on a tetradentate PPP^{5-} ligand is also given in Fig. 5B, where no specific Na^+ or Li^+ binding site is present. Peters and coworkers [17] have investigated the solution structure of $Dy(PPP)_2^{7-}$ by multinuclear NMR spectroscopy and postulated a model for this shift reagent, similar to that of Fig. 5B, where cations compete for seven sites in the second coordination sphere of the complex. The triphosphate ligand is coordinated to Dy^{3+} via two oxygens of one outer phosphate, one oxygen of the other outer phosphate, and one oxygen of the central phosphate group.

^{31}P NMR spectra of Dy^{3+}-complexed triphosphate are dependent on pH and addition of Li^+ ions (Fig. 6 and Table 1). However, chemical shift changes observed could not be unambiguously interpreted in terms of preferential Li^+ coordination or protonation to specific phosphate groups. Similarly, the linewidths changes caused by H^+ and Li^+ are primarily due to intermolecular ligand exchange processes and are not helpful in establishing which one of the three solution structures (Fig. 5) for the interaction of Li^+ with $Dy(PPP)_2^{7-}$ is correct. ^{17}O NMR spectroscopy constitutes an alternative probe of the triphosphate ligand. Unfortunately, it would not help settle the issue of a preferential binding for Li^+ in $Dy(PPP)_2^{7-}$ since it was found that the triphosphate ligand is in slow exchange and that the ^{17}O NMR signals for the Dy^{3+}-complexed ligand are broadened beyond detection [17].

We found that pH has a dramatic effect on the shift the triphosphate reagent induces in the $^7Li^+$ NMR resonance (Fig. 3). This behavior would be expected if H^+ and Li^+ were competing for the same phosphate binding sites on the dysprosium ligands. Since the $[Li^+]/[\text{shift reagent}]$ ratio in the $Ln(PPP)_2$-Li_n^{n-7} complex is greater than 1:1 (Fig. 4), the additional Li^+ ions could not occupy the same binding site as shown in Fig. 5A and C, except on a time averaged basis.

Potentiometric equilibrium measurements carried out on the association of Dy^{3+} ion with protonated and deprotonated triphosphate anions have shown [16] that, in the pH region where $^7Li^+$ NMR shifts are highly pH dependent, various protonated complexed forms of the 1:2 complex exist at pH 7.5, such as $DyH(PPP)_2^{6-}$ and $DyH_2(PPP)_2^{5-}$. At pH 5.5, extensive dissociation into the 1:1 $Dy(PPP)^{3-}$ complex occurs. If the mechanism of interaction between Li^+ and the shift reagent is predominantly electrostatic, one would expect a weaker interaction between Li^+ (or other cations) and the triphosphate shift reagent at low pH as a result of the lower negative charge of the species, $Dy(HPPP)_2^{5-}$ and $Dy(PPP)^{3-}$, present in solution. We found by $^7Li^+$ NMR spectroscopy that up to seven Li^+ ions may saturate all the binding sites on the second coordination sphere of $Dy(PPP)_2^{7-}$ at pH 7.5 (Fig. 4). Thus, our studies support a model for the solution structure of $Dy(PPP)_2^{7-}$ where up to seven Li^+ ions (at pH 7.5) are bound in the second coordination sphere of the complex (Fig. 5B). The first equivalent of Li^+ added to $Dy(PPP)_2^{7-}$ may occupy a preferential binding site with a specific θ value and therefore a specific pseudo-contact shift. Further addition of Li^+ leads to exchange between the Li^+ on the preferred site and those on other locations.

Although the pH dependence of $^7Li^+$ NMR shifts is a matter of concern in some studies, one could also take advantage of this property to estimate pH changes in biological systems. This is particularly important in the case of $^7Li^+$ NMR transport studies since there are examples of Li^+ transport that generate transmembrane proton gradients. For instance, Li^+-induced Cl^- transport in red blood cells suspended in a bicarbonate medium occurs via the anion-exchange, or band 3, protein and is accompanied by H^+ transport [27].

$Dy(PPP)_2^{7-}$, $Tm(PPP)_2^{7-}$ and $Dy(TTHA)^{3-}$ (Fig. 1) can induce very large shifts in the $^7Li^+$ NMR resonance (Fig. 2). Under equivalent pH and ionic strength conditions, the shift induced by $Dy(PPP)_2^{7-}$ is much greater than that induced by other shift reagents used in this study. The high charge of these three complex ions certainly must serve to increase the fraction of Li^+ ions bound to these shift reagents. However, the nitrilotriacetate, dipicolinate, and chelidamate shift reagents all have an overall charge of -3 at the pH used in these studies and yet they give much smaller paramagnetic shifts than $Dy(TTHA)^{3-}$. These observations suggest a more specific type of interaction, other than electrostatic, at least in the case of $Dy(TTHA)^{3-}$.

Based on a charge effect alone, one might predict lower $^7Li^+$ NMR shifts for $Dy(TTHA)^{3-}$. The large shifts produced by $Dy(TTHA)^{3-}$ may result from some specific binding site for Li^+, possibly involving the carboxylate oxygen atoms [14]. Two proposals for the solution structure of $Ln(TTHA)^{3-}$ complexes have been reported [28, 29]. The solution structure of $Ln(TTHA)^{3-}$ proposed by Yingst and Martell [29] is shown in Fig. 1. Both studies agree that in the $TTHA^{6-}$ ligand one or more carboxylate groups remain uncoordinated to the lanthanide ion. The oxygen atoms in these uncoordinated carboxylate groups could serve as binding sites for Li^+, H^+ or other metal cations. Since the potential Li^+ (or H^+) binding sites would be further from the paramagnetic ions than the coordinated carboxylates, it is not surprising that the $^7Li^+$ NMR shifts induced by $Dy(TTHA)^{3-}$ are relatively smaller than those given by the triphosphate reagents and less pH dependent.

The results in Fig. 2 indicate that the corresponding Dy(III) and Tm(III) complexes always shift the $^7Li^+$ NMR resonance in opposite directions, as predicted by dipolar shift theory [15]. Provided that the paramagnetic shift is purely dipolar in nature and that all other factors are equal, the ratio of the shift induced by Dy(III) over that induced by Tm(III) should be -1.9. The ratio observed for the five ligands studied are in good agreement with this prediction. Similar observations were made for $^{23}Na^+$ NMR shifts [4]. $Dy(TTHA)^{3-}$ causes $^7Li^+$ NMR shifts in the opposite direction from those caused by $Dy(PPP)_2^{7-}$. As reported by Chu *et al.* [4], this behavior has to do with the geometric relationships of the Li^+ or Na^+ binding sites on the two shift reagents to the symmetry elements of their respective asymmetric magnetic susceptibility tensors.

Although $Dy(PPP)_2^{7-}$ and $Tm(PPP)_2^{7-}$ induced the largest $^7Li^+$ NMR shifts, these shift reagents may not be the best for applications to certain biological systems. In addition to their strong pH dependence and extreme sensitivity to the presence of Na^+, K^+, Ca^{2+} or Mg^{2+}, there are problems with their hydrolytic instability [20, 26]. Recently, the possibility of living cells using PPP^{5-} as an energy source has also been demonstrated [30, 31]. Our ^{31}P NMR studies also indicate that addition of Ca^{2+} to $Dy(PPP)_2^{7-}$ induces hydrolysis of the triphosphate ligand (Fig. 9B). However, the triphosphate shift reagents will continue to be useful in systems where inorganic pyrophosphatase activity is low, as in RBCs for example. Therefore, although the $Dy(TTHA)^{3-}$ complex produces smaller shifts compared to $Dy(PPP)_2^{7-}$, it stands as the most promising $^7Li^+$ NMR shift reagent for biological applications since it has a relatively low affinity for Li^+ and is fairly unaffected by pH changes and the presence of physiologically important cations such as Na^+, K^+, Ca^{2+} or Mg^{2+}.

We conclude that the shift reagents $Dy(TTHA)^{3-}$, $Dy(PPP)_2^{7-}$ and $Tm(PPP)_2^{7-}$ (in order of preference) show great promise for probing Li^+ ion distribution in RBCs by ^7Li NMR spectroscopy (Fig. 10). Application of these shift reagents to the investigation of abnormal Li^+ transport in RBCs of bipolar and hypertensive patients is now under way in our laboratory.

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