Ion-Pairing Interactions between $Co(en)_3^{3+}$ and the ^{23}Na NMR Frequency Shift Reagent $TmDOTP^{5-}$ †

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Received February 6, 1997⊗

Three new formulations of $TmDOTP^{5-}$ ($DOTP^{8-}$ = 1,4,7,10-tetraazacyclododecane-1,4,7,11-tetrakis(methylenephosphonate)) have been prepared in an effort to develop a low-osmolality form of the ^{23}Na frequency shift reagent (SR). Equally concentrated (0.32 M) solutions of $(MegH)_4HTmDOTP$ (Meg = *N*-methylglucamine or meglumine), $Na_4HTmDOTP$, and $[Co(en)_3]_{4/3}HTmDOTP$ have solution osmolalities of 1245, 1040, and 707 mOsm/kg, respectively, comparable to the ionic and non-ionic gadolinium-based MRI contrast agent preparations in clinical use. An analysis of ^{23}Na and ^{59}Co frequency shifts induced by $TmDOTP^{5-}$ indicated that $Co(en)_3^{3+}$ can form both 1:1 and 2:1 adducts with $TmDOTP^{5-}$ with (log) binding constants of 3.1 ± 0.4 and 2.5 ± 0.4, respectively. These values were comparable with those obtained by analysis of the ^1H frequency shifts observed for $Co(en)_3^{3+}$ upon binding to $HoDOTP^{5-}$. The ^1H shifts of $Co(en)_3^{3+}$ signals induced by $YbDOTP^{5-}$ at pH 7.4 were fitted best by a 1:1 binding model with a conditional binding constant of 3.1 ± 0.2. The ^{59}Co and ^1H limiting frequency shifts of $Co(en)_3^{3+}$ could be fitted with a dipolar shift model in which the Co atom of the $Co(en)_3^{3+}$ cation is located 5.0 ± 0.3 Å from the Ln atom of the $LnDOTP^{5-}$ chelate, and with an angle of 40 ± 0.2° between the Co−Ln vector and the 4-fold symmetry axis of the $LnDOTP^{5-}$ complex. Ion pairing of $Co(en)_3^{3+}$ and $TmDOTP^{5-}$ was significant enough in both saline and human blood plasma to reduce the effectiveness of the ^{23}Na frequency SR. Comparisons between all formulations suggested that $Na_4HTmDOTP$ represents the best compromise of lower osmolality with minimal reduction of SR shift potency.

Introduction

The maintenance of a cytolemmal Na^+ gradient is a primary function of the ubiquitous enzyme Na^+-K^+-ATPase. As pump dysfunction has been implicated in numerous diseases including hypertension and sepsis,[1] there is considerable interest in developing methods to monitor intra- and extracellular Na^+ concentrations in intact tissue.[2−6] In comparison to other techniques for quantitating $[Na^+]$ in intact tissues, ^{23}Na NMR in combination with a paramagnetic shift reagent (SR) is one of the more promising methods because it can be minimally invasive and it offers measurement of intracellular sodium with optimal temporal resolution. Paramagnetic SRs used for this purpose are typically highly charged, anionic species that ion-pair with extracellular sodium, $[Na^+_e]$, in a labile equilibrium, and thereby induce a frequency shift that separates its signal from the intracellular Na^+ resonance.[7−9] Any candidate SR for *in vivo* use must produce a large hyperfine frequency shift at relatively low dose (preferably <0.1 mmol/kg), have low chemical toxicity, and be eliminated without loss of chemical integrity. Compared with other SRs reported so far, $TmDOTP^{5-}$ requires the lowest dosage to achieve resolution of intra- and excellular ^{23}Na signals.[6,10−12] Recent successful applications of this SR *in vivo* with small animals have demonstrated its effectiveness, but a complete toxicity profile of the SR has not been reported.

A recent ^{23}Na NMR shift and relaxation rate enhancement study[13] showed that two types of Na^+ binding sites exist on the $TmDOTP^{5-}$ chelate. The first type has a unique position near the 4-fold symmetry axis of the complex with a limiting shift of about 420 ppm to higher frequency. Three or four sites of a second type lie further away from the symmetry axis, and

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† A portion of this work was presented at the 221st National Meeting of the American Chemical Society, New Orleans, March 1996.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

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S0020-1669(97)00128-6 CCC: $14.00

consequently this type has a smaller limiting shift of about 162 ppm, also to higher frequency. The binding constants for Na^+ with $TmDOTP^{5-}$ ($\log K_n = 1.6-2.5$; K in M^{-1}) were found to be about 10-fold larger than those reported[14] for Na^+ binding to $DyTTHA^{3-}$, another SR.

The approximate chemical composition of the $TmDOTP^{5-}$ SR preparation used for all *in vivo* ^{23}Na NMR studies to date has been $Na_4HTmDOTP\cdot 3NaOAc$. An 80 mM stock solution of SR with this formulation has a measured osmolality of 640 mOsm/kg at physiological pH, with contributions from Na^+ (52%), OAc^- (38%), and $Na_3H_nTmDOTP^{-(2-n)}$ ($n = 1$ and 2) (10%).[13] It is undesirable for an infusion or injection solution to have an osmolality much greater than that of blood plasma, ~310 mOsm/kg.[15] Obviously, the osmolality of this solution could be lowered either by removal of the excess sodium salt remaining from the synthesis (the 3 equiv of NaOAc), by employing a polyvalent countercation, or perhaps even better, by using a combination of these. Two different approaches have been taken in the present work to achieve a low-osmolality SR preparation. The first was to prepare a salt-free SR stock solution by precipitating the fully protonated $H_5TmDOTP$ complex from an (excess) salt-containing SR synthesis solution. The isolated $H_5TmDOTP$ was then neutralized with the appropriate base to yield the salt-free formulations corresponding to $Na_4HTmDOTP$, $(MegH)_4HTmDOTP$ (Meg = *N*-methylglucamine or meglumine), and $[Co(en)_3]_{4/3}HTmDOTP$. The second approach was a direct synthesis of the salt-free form of the complex by mixing either thulium carbonate ($Tm_2(CO_3)_3$) or freshly prepared thulium hydroxide ($Tm(OH)_3$) with a suspension of H_8DOTP, followed by neutralization with the bases of the first approach. All three salt-free formulations resulted in SR solutions with considerably lower osmolality. The interaction between $Co(en)_3^{3+}$- and the SR anion was further examined by ^{23}Na, ^{59}Co, and ^1H NMR. These NMR data allowed us to characterize the binding constant and geometry of the $Co(en)_3^{3+}$-$LnDOTP^{5-}$ adduct.

Experimental Section

Chemicals. The macrocyclic ligand 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis (methylenephosphonic acid) (H_8DOTP) was prepared as reported previously.[16] $Na_4HLnDOTP\cdot 3NaX$ (X = Cl^- or OAc^-) complexes were prepared from H_8DOTP and the corresponding LnX_3 salts using procedures also described previously.[17] Thulium hydroxide, $Tm(OH)_3$, was freshly prepared by hydrolysis of a $TmCl_3$ solution with NaOH, followed by filtration and washing. Thulium carbonate, $Tm_2(CO_3)_3$, was prepared in a similar way using $NaHCO_3$ as base in place of NaOH. The (+)- and (−)-$Co(en)_3I_3\cdot H_2O$ complexes were prepared using literature methods[18] and converted to the hydroxide forms by anion exchange chromatography. The chirality of each $Co(en)_3^{3+}$ stereoisomer was checked by circular dichroism spectroscopy. The purity of each $LnDOTP^{5-}$ complex was established by ^{31}P NMR spectroscopy. The pH values of the $LnDOTP^{5-}$ solutions for NMR titrations were adjusted with tetraethylammonium or sodium hydroxide as indicated in the text. All other chemicals were obtained from either Aldrich or Sigma and used without purification.

Preparation of Low-Osmolality SR Formulations. Upon dropwise addition of 4 equivalents of dilute HCl (0.5 M) to a solution of $Na_4HTmDOTP\cdot 3NaCl$, a white precipitate formed (when the solution pH was between 3 and 5)[19] that could be isolated by centrifugation. Excess NaCl was removed by repeated washings and centrifugation of the precipitate (after 2−3 washings, Cl^- could no longer be detected in the filtrate, upon addition of $AgNO_3$). The precipitate was then washed with EtOH and dried at 55 °C for 24 h. Elemental analysis indicated a composition of $H_5TmDOTP\cdot 3H_2O$ [this sample also contained a 5% excess of unchelated H_8DOTP that was present in the original SR preparation) [FW: 795.6. Found (calcd): C, 19.12 (19.00); H, 4.93 (4.97); N, 7.47 (7.39)], and the yield was 80%. The fully protonated $H_5TmDOTP$ was converted to various salt-free forms at physiological pH by neutralization with either NaOH, *N*-methyl glucamine (meglumine), or $Co(en)_3(OH)_3$. The amount of base required to neutralize an 80 mM $H_5TmDOTP$ solution was approximately 4 equiv for NaOH and meglumine and 1.3 equiv for $Co(en)_3(OH)_3$.

$Na_4HTmDOTP$ was also prepared directly using two other thulium salts.

$$Tm(OH)_3 + H_8DOTP + 4NaOH \rightarrow Na_4HTmDOTP \quad (1)$$

$$^1/_2Tm_2(CO_3)_3 + H_8DOTP + 4NaOH \rightarrow Na_4HTmDOTP + CO_2\uparrow \quad (2)$$

In each case, a suspension of thulium hydroxide or carbonate was added to a solution of H_8DOTP in small portions so that the solution would turn clear after each addition (in only a few seconds). Freshly-prepared suspensions of thulium hydroxide or carbonate were much more reactive for this complexation process than the corresponding dried solids. The complexation reactions could be further accelerated by raising the temperature to 70 °C. The pH of the resulting solutions containing equal amounts of Tm^{3+} and ligand was adjusted to 7.4 by slow addition of 0.2 M NaOH. This procedure gave essentially quantitative yields of $Na_4HTmDOTP$.

Osmometric Measurements. Solution osmolalities were measured on a WESCOR 5500 vapor pressure osmometer at room temperature. Each measurement was repeated 3−5 times and the average value reported. The instrument was calibrated with WESCOR 290 and 1000 mmol/kg standards before each sample measurement.

NMR Spectroscopy. ^1H (500 MHz), ^{31}P (202.4 MHz), ^{23}Na (132.33 MHz), and ^{59}Co (118.9 MHz) NMR spectra were recorded at 25 °C on a General Electric GN-500 NMR spectrometer using either a 5 mm (for ^1H and ^{59}Co) or a 10 mm (for ^{31}P and ^{23}Na) probe, both equipped for temperature control. ^1H T_1 measurements were performed using an inversion−recovery pulse sequence. DSS was used as internal reference for ^1H chemical shifts. H_3PO_4 (85%) was used as an external chemical shift reference for ^{31}P resonance. A 50 mM $Co(en)_3I_3$ solution in D_2O and 140 mM NaCl solution in D_2O were used as external references for ^{59}Co and ^{23}Na, respectively. BMS effects on ^{59}Co and ^{23}Na frequency shifts were corrected by using the internal D_2O lock signals. It has been shown by ^{17}O NMR that $DyDOTP^{5-}$ has no inner-sphere coordinated water, so that any ^2H frequency shift due to hyperfine interactions is negligible. The frequency shifting abilities of $[Co(en)_3]_{4/3}HTmDOTP$, $Na_4HTmDOTP\cdot 3NaCl$, $Na_4HTmDOTP$, and $(Et_4N)_4TmDOTP$ were also compared in plasma, isolated by centrifugation of whole blood donated by one of the authors (A.D.S.). ^{23}Na NMR spectra of plasma samples were recorded at 37 °C using a 5 mm probe. D_2O (10%) (v/v) was added for locking purposes. Small volumes of 80 mM SR stock solutions were added to an initial 0.5 mL of plasma to minimize dilution of the plasma during each SR titration.

Calculations. All NMR data were fitted with various binding models by using the Solver feature of Microsoft Excel as described previously.[13]

Results and Discussion

1. Osmolalities of Salt-Free SRs. As anticipated, all salt-free preparations of SR, $(MegH)_4HTmDOTP$, $Na_4HTmDOTP$, and $[Co(en)_3]_{4/3}HTmDOTP$ had significantly lower osmolalities than the currently used SR formulation, $Na_4HTmDOTP\cdot 3NaX$

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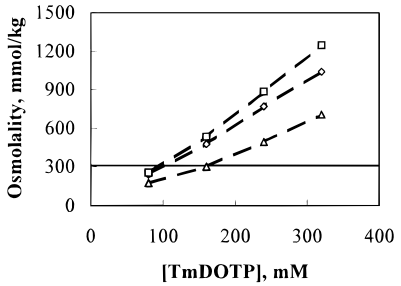

Figure 1. Osmolalities of salt-free SR preparations: $Na_4HTmDOTP$ (◇), $(MegH)_4HTmDOTP$ (□), and $[Co(en)_3]_{4/3}HTmDOTP$ (△). The horizontal line represents the osmolality of human blood plasma.

($X = Cl^-$ or OAc^-). Preparations of equally concentrated (80 mM) solutions of the SR anion had osmolalities of 254, 245, and 174 mOsm/kg, respectively, compared with 640 mOsm/kg for the analogous SR solution containing excess salt. Interestingly, there were even larger differences in the osmolalities of the three salt-free forms at higher concentrations (Figure 1). A comparison of the two monovalent cation formulations shows that Na^+ binds more strongly to the SR anion than does $MegH^+$. At higher SR concentrations, this binding difference was magnified, resulting in significantly lower osmolalities for $Na_4HTmDOTP$ than for $(MegH)_4HTmDOTP$. $[Co(en)_3]_{4/3}$-HTmDOTP had the lowest osmolality at all SR concentrations, as expected for a formulation with fewer countercations. Like the other preparations, however, the osmolality of $[Co(en)_3]_{4/3}HTmDOTP$ was even lower than would be anticipated for an ideal solution, based solely upon the number of ions present. This indicates that the trivalent $Co(en)_3^{3+}$ cation interacts strongly with $TmDOTP^{5-}$, even at the lowest concentrations examined here. From curves such as those shown in Figure 1, one can estimate that the concentration of SR anion achievable before exceeding an isotonic solution (310 mOsm/kg) (solid horizontal line in Figure 1) would be ~95 mM for $Meg_4HTmDOTP$, ~105 mM for $Na_4HTmDOTP$, and ~162 mM for $[Co(en)_3]_{4/3}HTmDOTP$. One can also compare the osmolalities of these three excess-salt-free preparations with those of commercial gadolinium-based MRI contrast reagents (CRs). The osmolalities of *Magnevist* ($(MegH)_2GdDTPA$), *Prohance* (GdHP-DO3A), and *Ominiscan* (GdDTPA-BMA) at 0.5 M are 1960, 630, and 650 mOsm/kg,[20] respectively, while the extrapolated osmolalities of our three excess-salt-free SRs, $Meg_4HTmDOTP$, $Na_4HTmDOTP$, and $[Co(en)_3]_{4/3}HTmDOTP$, at an identical concentration would be 2000, 1700, and 1200 mOsm/kg, respectively. Thus, one would predict that Meg_4-HTmDOTP at 0.5 M would have a solution osmolality comparable to the commercial CR, *Magnevist*, while $Na_4HTmDOTP$ at this same concentration is expected to have a somewhat lower osmolality than this CR. This is encouraging because it is not realistic to consider the discovery of a nonionic SR. The negative charge of the SR species appears to be quite important for its potency.[2,13,14]

2. Estimation of Binding Constants for the $Co(en)_3^{3+}$−SR Adducts. The low osmolality of solutions containing $[Co(en)_3]_{4/3}HTmDOTP$ prompted us to further explore the interaction between $Co(en)_3^{3+}$ and the $TmDOTP^{5-}$ chelate. A decrease of the $TmDOTP^{5-}$-induced ^{23}Na frequency shift was seen upon addition of (+)-$Co(en)_3^{3+}$ (Figure 2, top). This experiment was conducted by titrating a solution of $(Et_4N^+)_5TmDOTP^{5-}$ (15 mM) containing a small amount of Na^+ ($Na^+/TmDOTP^{5-} = 0.4$) with (+)-$Co(en)_3^{3+}$ (as the I^- salt) at pH 10.7 (where the fully deprotonated complex

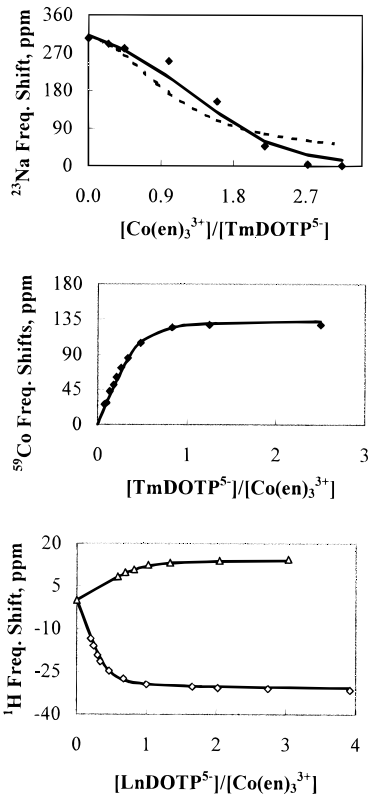

Figure 2. (Top panel: Dependence of TmDOTP-induced ^{23}Na frequency shifts on the $Co(en)_3^{3+}$:$TmDOTP^{5-}$ molar ratio at pH 10.7 and at a Na^+:$TmDOTP^{5-}$ molar ratio of 0.4. The initial sample contained 15 mM $H_5TmDOTP$, 80 mM Et_4NOH, and 6 mM NaCl. Increasing amounts of $Co(en)_3I_3$ were added sequentially. The dotted line represents the fit to a 1:1 binding model while the solid curve represents the fit to a model that includes both a 1:1 and a 2:1 species. Center panel: Dependence of TmDOTP-induced ^{59}Co frequency shifts on the $[TmDOTP^{5-}]$:$[Co(en)_3^{3+}]$ ratio at pH 10. Increasing amounts of $Co(en)_3I_3$ were added sequentially (from right to left). The solid curve represents the fit to a model that includes both a 1:1 and a 2:1 species. Bottom panel: Shifts of $Co(en)_3^{3+}$ protons induced by 15 mM $HoDOTP^{5-}$ at pH 10 (◇) and 10 mM $YbDOTP^{5-}$ at pH 7.4 (△) as a function of $LnDOTP^{5-}$:$Co(en)_3^{3+}$ molar ratio. Increasing amounts of $Co(en)_3I_3$ were added sequentially (from right to left).

$TmDOTP^{5-}$ is the only species). The hyperbolic binding curve was similar in shape to that reported previously for Mg^{2+} binding to $TmDOTP^{5-}$.[21] The first additions of $Co(en)_3^{3+}$ result in very small changes in the observed ^{23}Na shift while further additions of $Co(en)_3^{3+}$ resulted in more dramatic changes as more bound Na^+ ions were displaced from their sites by the more highly charged $Co(en)_3^{3+}$. These data were fitted with a binding model involving formation of 1:1 and 2:1 $Co(en)_3^{3+}$−$TmDOTP^{5-}$ adducts:

$$M + L \rightleftharpoons ML \quad (3)$$

$$M + ML \rightleftharpoons M_2L \quad (4)$$

$$Na + L \rightleftharpoons Na^AL \quad (5)$$

$$Na + L \rightleftharpoons Na^BL \quad (6)$$

where M is $Co(en)_3^{3+}$, L is $TmDOTP^{5-}$, and Na^AL and Na^BL are species with the Na^+ ion bound to $TmDOTP^{5-}$ at site A (limiting shift, 420 ppm) and at site B (limiting shift, 162 ppm), respectively.[13] Previous work has shown that, at low Na^+/$TmDOTP^{5-}$ molar ratios, the 1:1 Na^+−$TmDOTP^{5-}$ adduct (Na^AL) is the main species contributing to the observed ^{23}Na shift.[13] An analysis of the data of Figure 2, top panel, using

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eqs 3−6 yielded stepwise binding constants (log K_1 and log K_2) of 2.9 ± 0.5 and 2.5 ± 0.4 for the 1:1 $Co(en)_3$−$TmDOTP^{2-}$ and 2:1 $[Co(en)_3]_2$−$TmDOTP^{+}$ adducts, respectively. These values should be regarded only as apparent constants, however, since the ionic strength of the solution was not controlled or corrected for during the titration. The ionic strength was more than doubled throughout the $Co(en)_3I_3$ titration (from 231 to 501 mM). Although this effect may also contribute to the observed decrease in ^{23}Na frequency shift from left to right in Figure 2, top, such a contribution would be very small (we have shown previously[13] that Et_4NCl and Me_4NCl have only a small effect on the observed ^{23}Na frequency shift induced by $TmDOTP^{5-}$). The solid curve of Figure 2, top, shows the calculated fitting to the experimental data using these binding constants. This fitting was not nearly as good as that of similar data for Ca^{2+} or Mg^{2+} binding to $TmDOTP^{5-}$,[21] suggesting that our binding model (which admits no nonideality) may indeed be oversimplfied. The binding constants for trivalent $Co(en)_3^{3+}$ derived from the above model were smaller than those determined for divalent Ca^{2+} (log K_1 = 5.7) and Mg^{2+} (log K_1 = 3.9, log K_2 = 2.2) and only slightly larger than those determined for alkali metal ions (Li^+, Na^+ and Cs^+, $\log K_n = 1.3-2.6$, n = 1, 2, and 3).[13,21] This result is not unexpected because the larger size and subsequent poorer fit of $Co(en)_3^{3+}$ on the $TmDOTP^{5-}$ surface may tend to offset any increase in binding interaction due to the greater charge of $Co(en)_3^{3+}$. In fact, this was an important reason for choosing $Co(en)_3^{3+}$, along with its kinetic inertness toward dissociation.

Given that, in the *in vivo* situation, the SR anion and its countercation will be significantly more diluted in the blood plasma and interstitial spaces, and the concentration of Na^+ will be ∼25 times greater, the results from Figures 1 and 2 (top) could be considered encouraging for the use of $Co(en)_3^{3+}$ as a countercation. It significantly lowers the osmolality of the injection solution (Figure 1), without ion pairing with $TmDOTP^{5-}$ to the extent of Ca^{2+} and Mg^{2+} (compare data of Figure 2, top, with Figure 3 of ref 21). Thus, we titrated $[Co(en)_3]_{4/3}HTmDOTP$ into a sample of human blood plasma and measured the resulting ^{23}Na frequency shifts. Those results are compared with titrations into saline in Figure 3 (upper). Here, we also depict curves for the titration of $Na_4HTmDOTP$ into saline solution, human blood plasma, and whole rat blood ($Na_5TmDOTP$, data from ref 22). These results demonstrate that ion pairing of $Co(en)_3^{3+}$ with $TmDOTP^{5-}$ is apparently significant enough to noticeably reduce its shift potency. The curve for the $Co(en)_3^{3+}$ countercation was always below the analogous one for the Na^+ countercation in Figure 3 (upper).

It is also important to note that, for a given SR preparation, the plasma curve was always below the saline curve, and the blood curve was always below the plasma curve in Figure 3 (upper). Plasma contains Mg^{2+} and Ca^{2+} ions, not present in saline solution, which can compete with Na^+ for $TmDOTP^{5-}$. In addition, blood also contains erythrocytes, and their polyanionic external surface may compete with $TmDOTP^{5-}$ for Na^+. (In blood, the appropriate value for [SR] is the extracellular concentration.[22])

Figure 3 (bottom) shows the ^{23}Na frequency shift titration curves for the other two low-osmolality preparations in human plasma and compares them with that for $Na_4HTmDOTP{\cdot}3NaCl$. It is clear that while Et_4N^+ (like $MegH^+$ in Figure 1) and the excess 3 equiv of NaCl (*vide supra*) worsen the osmolality (3NaCl, significantly), they do not seriously reduce the shift

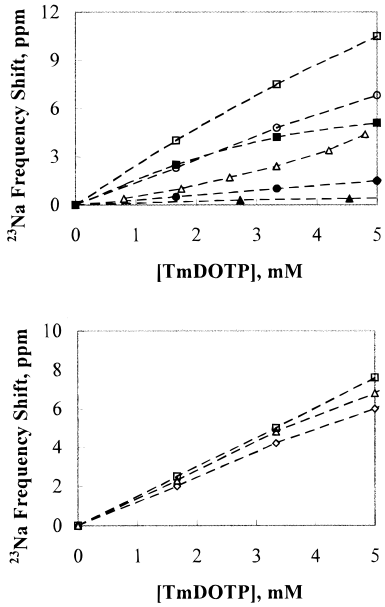

Figure 3. (Upper) ^{23}Na frequency shift induced by $Na_4HTmDOTP$ (□, saline; ○, plasma; △, whole blood) and $[Co(en)_3]_{4/3}HTmDOTP$ (■, saline; ●, plasma; ▲, whole blood) at 37 °C. The data given by (Δ) were taken from ref 22. Lower: Plasma ^{23}Na frequency shifts induced by $(Et_4N)_4HTmDOTP$ (□), $Na_4HTmDOTP$ (△), and $Na_4HTmDOTP{\cdot}3NaCl$ (◇) at 37 °C.

potency of $TmDOTP^{5-}$. Thus, the $Na_4HTmDOTP$ preparation is the best compromise for shift potency and low osmolality.

3. Multinuclear NMR Studies of the $Co(en)_3^{3+}$:$TmDOTP^{5-}$ Ion Pair. Another reason for choosing $Co(en)_3^{3+}$ was the strength of the natural abundance ^{59}Co resonance,[23] the frequency of which is extremely sensitive to many factors, such as temperature[24] and isotope effects.[23] Indeed, the strong ^{59}Co NMR signal of $Co(en)_3^{3+}$ enabled a direct study of the interaction between $Co(en)_3^{3+}$ and the SR anion by monitoring the ^{59}Co frequency shifts induced by $TmDOTP^{5-}$. The interaction between $Co(en)_3^{3+}$ and $TmDOTP^{5-}$ was fast on the ^{59}Co NMR time scale since only a single, concentration-dependent, ^{59}Co resonance was observed. Figure 2 (center panel) shows the ^{59}Co frequency shift as a function of the $[TmDOTP^{5-}]/[Co(en)_3^{3+}]$ molar ratio for a 10 mM $TmDOTP^{5-}$ solution (0.5 mL, pH 10, tetraethylammonium as countercation) titrated with a 100 mM $Co(en)_3^{3+}$ (I^- as the counteranion) solution. The ^{59}Co resonance was shifted to higher frequency by $TmDOTP^{5-}$, similar to what is observed for ^{23}Na. This indicates that the binding loci of $Co(en)_3^{3+}$ and Na^+ are similar.[13,21] With increasing $[TmDOTP^{5-}]:[Co(en)_3^{3+}]$ ratio, the magnitude of the ^{59}Co shift increased significantly at low ratios (<0.5) and then gradually approached a maximum value of 130 ppm in the range $0.5 < [TmDOTP^{5-}]:[Co(en)_3^{3+}] < 1.0$. Such a saturation titration curve is also consistent with formation of both 1:1 and 2:1 adducts between $Co(en)_3^{3+}$ and $TmDOTP^{5-}$.

The ^{59}Co shifts were evaluated using a simple (but not too realistic) model that assumed that the limiting frequency shifts of the 1:1 and 2:1 species were identical

$$\Delta_{obs} = \{[ML] + 2[M_2L]\}\Delta/[M]_{tot} \qquad (7)$$

where L is $TmDOTP^{5-}$, $[M]_{tot}$ is the total concentration of $Co(en)_3^{3+}$ including both free and bound species, and Δ is the limiting shift of the bound $Co(en)_3^{3+}$ cation (135 ppm, for both

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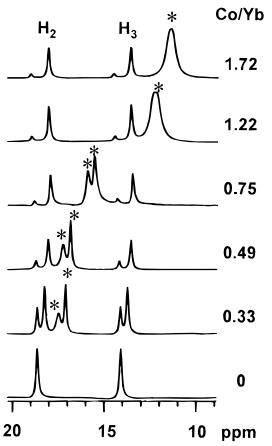

Figure 4. ^1H NMR spectra of various $Co(en)_3^{3+}/YbDOTP^{5-}$ mixtures at pH 7.4 and 10 mM $YbDOTP^{5-}$. The resonances labeled H_2 and H_3 correspond to $YbDOTP^{5-}$ resonances while those labeled with an asterisk (*) correspond to $Co(en)_3^{3+}$ resonances.

the 1:1 and 2:1 species). This yielded stepwise binding constants of $\log K_1 = 3.4 \pm 0.4$ for the 1:1 adduct and $\log K_2 = 2.5 \pm 0.4$ for the 2:1 adduct. The solid curve in Figure 2 (center panel) represents the actual fitting to the experimental data points.

In addition to the large shift, $TmDOTP^{5-}$ also induced severe line broadening in the ^{59}Co resonance. The line width at $[TmDOTP^{5-}]:[Co(en)_3^{3+}]$ ratios > 1 was too large to allow an accurate determination of the ^{59}Co apparent transverse relaxation time. At a $[TmDOTP^{5-}]:[Co(en)_3^{3+}]$ molar ratio of 0.2 (where the 2:1 adduct is the predominant species), the ^{59}Co line width was 5700 Hz ($T_2 \cong 56\ \mu s$), as compared with 170 Hz in SR-free solution, and its T_1 was $200 \pm 60\ \mu s$ (as estimated by null-point measurements after a 180° pulse). Clearly, the quadrupolar[25] and dipolar mechanisms[26] enabled in the adduct are quite efficient, and the apparent differences between T_1 and T_2 suggest that the ^{59}Co resonance of $Co(en)_3^{3+}$ may have an exchange contribution in the presence of $TmDOTP^{5-}$.

To facilitate considerations for future SR preparation improvements, the interaction between $LnDOTP^{5-}$ and $Co(en)_3^{3+}$ was further characterized by monitoring the ^1H NMR spectrum of either $HoDOTP^{5-}$ or $YbDOTP^{5-}$ upon addition of $Co(en)_3^{3+}$. Figure 4 shows a stacked plot of a selected region of the ^1H spectrum of the $YbDOTP^{5-}$ solution after the addition of increasing amounts of (+)-$Co(en)_3^{3+}$. The two resonances, labeled H_2 and H_3, correspond to two $DOTP^{8-}$ ethylenediamine ring protons, the frequencies of which are hyperfine shifted by the Yb^{3+} ion.[17] With the first addition of (+)-$Co(en)_3^{3+}$, the degeneracies of these two $YbDOTP^{5-}$ resonances were lifted; the lower frequency $YbDOTP^{5-}$ peak in each pair corresponds to a single enantiomer of $YbDOTP^{5-}$ that binds more favorably with (+)-$Co(en)_3^{3+}$ (J. Ren, unpublished results). Two new resonances also appeared at 17−17.5 ppm (labeled with asterisks) corresponding to ethylenendiamine protons of (+)-$Co(en)_3^{3+}$, frequency shifted by the $YbDOTP^{5-}$. The detection of two ethylenediamine proton resonances indicates that (+)-$Co(en)_3^{3+}$ binds with a slightly different geometry with the two enantiomers of $YbDOTP^{5-}$. As more (+)-$Co(en)_3^{3+}$ is added, the proton signals of (+)-$Co(en)_3^{3+}$ shift to lower frequencies, toward their normal diamagnetic positions, and broaden somewhat. Figure 2 (bottom panel) summarizes the ^1H shifts of $Co(en)_3^{3+}$ induced by $HoDOTP^{5-}$ at pH 10 and $YbDOTP^{5-}$ at pH 7.4 as a function of the $LnDOTP^{5-}:Co(en)_3^{3+}$ molar ratio (constant $[LnDOTP^{5-}]$). The observed resonances showed a concentration dependent shift, reflecting rapid exchange between free and $LnDOTP^{5-}$-bound $Co(en)_3^{3+}$ cations. The $HoDOTP^{5-}$ titration curve leveled off when the $HoDOTP^{5-}:Co(en)_3^{3+}$ molar ratio reached a value of 0.5 while the $YbDOTP^{5-}$ titration curve leveled off when the $YbDOTP^{5-}:Co(en)_3^{3+}$ molar ratio was nearer 1. The log K values evaluated from the ^1H shifts of $Co(en)_3^{3+}$ induced by $HoDOTP^{5-}$ were 3.0 ± 0.3 for the 1:1 $Co(en)_3^{3+}-HoDOTP^{5-}$ adduct and 1.5 ± 0.2 for the 2:1 $[Co(en)_3^{3+}]_2-HoDOTP^{5-}$ adduct, using a limiting shift of −32.5 ppm. The agreement between the calculated and observed ^1H shifts was excellent (solid lines through the data of Figure 2, bottom panel). The shifts induced by $YbDOTP^{5-}$ were fitted best with a 1:1 binding model, yielding a conditional log K value of 3.1 ± 0.2 and a limiting shift of 14.5 ppm. Inclusion of 2:1 species did not improve this fitting.

4. Geometry of the $Co(en)_3^{3+}-LnDOTP^{5-}$ Adduct. The limiting shifts of the ^1H resonance frequencies of $Co(en)_3^{3+}$ induced by $HoDOTP^{5-}$ and $YbDOTP^{5-}$ contain information about the geometry of the $Co(en)_3^{3+}-LnDOTP^{5-}$ adduct. The low- and high-frequency hyperfine shifts observed for the protons of $Co(en)_3^{3+}$ in the presence of $HoDOTP^{5-}$ and $YbDOTP^{5-}$, respectively, are in the same directions as those observed for the frequencies of $DOTP^{8-}$ ligand protons located within the cone defined by an angle (θ) of 54.7° with the 4-fold symmetry axis of the complex in the corresponding $LnDOTP^{5-}$ complexes.[17] This indicates that the $Co(en)_3^{3+}$ binding site must also be located within this cone. Given that $Co(en)_3^{3+}$ ions must be interacting with the $LnDOTP^{5-}$ complexes *via* ion pairing, the proton shifts of $Co(en)_3^{3+}$ can be described by the dipolar equation

$$\Delta = D_{Ln} A_2^0 \langle r^2 \rangle_{Ln} G \qquad (8)$$

where D_{Ln} is Bleaney's magnetic constant for a given Ln^{3+};[27] $A_2^0\langle r^2\rangle$ is the crystal field parameter of $LnDOTP^{5-}$; and G is the geometric factor ($=(3\cos^2\theta - 1)/r^3$) of the nucleus observed. Here, r is the distance between the Ln^{3+} ion and the $Co(en)_3^{3+}$ proton while θ is the angle between the r vector and the 4-fold symmetry axis of $LnDOTP^{5-}$. The crystal field parameters, $A_2^0\langle r^2\rangle$, have been determined for $HoDOTP^{5-}$ (182 Å^3 ppm), $YbDOTP^{5-}$ (146 Å^3 ppm), and other $DOTP^{8-}$ complexes of the heavy lanthanide ions.[28] According to eq 8, if the geometric factor G is independent of Ln for all $LnDOTP^{5-}$ complexes along the series, the observed frequency shifts for a given nucleus will be proportional to the product $D_{Ln}A_2^0\langle r^2\rangle_{Ln}$. For the $Co(en)_3^{3+}$ cation, the ratio ($\Delta_{Yb}:\Delta_{Ho}$) of the limiting ^1H frequency shift induced by $YbDOTP^{5-}$ and $HoDOTP^{5-}$ was −0.45, in reasonable agreement with the calculated ratio (−0.41) of the $D_{Ln}A_2^0\langle r^2\rangle_{Ln}$ values for these two complexes. This confirmed that the ^1H frequency shifts of $Co(en)_3^{3+}$ induced by $YbDOTP^{5-}$ were indeed dominated by the dipolar hyperfine shift term. Thus, the the average angular geometric parameter, θ, for the $Co(en)_3^{3+}$ protons could be estimated from the observed limiting shifts, if the average distance r is known or can be approximated.

The average proton$-Ln^{3+}$ distance was evaluated from the ^1H longitudinal relaxation rate constant enhancement obtained at $LnDOTP^{5-}:Co(en)_3^{3+} > 1$ (where $Co(en)_3^{3+}$ is greater than 95% bound) using the reduced Solomon−Bloembergen equation:[26]

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$$T_1^{-1} = (4/3)(\mu_0/4\pi)^2\mu_{\text{eff}}^2\gamma^2\beta^2\tau_s/\langle r^6\rangle \quad (9)$$

where $\mu_0/4\pi$ is the magnetic susceptibility parameter for a vacuum, μ_{eff} is the effective magnetic moment of the Ln^{3+} ion, γ is the magnetogyric ratio of ^1H, β is the Bohr magneton, and τ_s is the electron-spin correlation time of $LnDOTP^{5-}$. Using τ_s values of 0.54 and 0.28 ps determined previously[28] for $HoDOTP^{5-}$ and $YbDOTP^{5-}$ and measured ^1H T_1's of 3.0 ± 0.3 and 12.2 ± 0.7 ms for $Co(en)_3^{3+}$, respectively ($[HoDOTP^{5-}]$:$[Co(en)_3^{3+}] = 1.6$ and $[YbDOTP^{5-}]$:$[Co(en)_3^{3+}] = 1.4$), an average Ln−H distance of 5.0 ± 0.3 Å can be estimated for these two ion-paired complexes. Substituting this $\langle r\rangle$ value into eq 8 gives an average angle ($\langle\theta\rangle$) of $40 \pm 2°$ for the $Co(en)_3^{3+}$ protons in the ion-paired complex. The validity of these $\langle r\rangle$ and $\langle\theta\rangle$ values can be further checked by the ^{59}Co frequency shift induced by $TmDOTP^{5-}$. Using a $D_{Ln}A_2^0\langle r^2\rangle_{Tm}$ value of 18 970 Å^3 ppm for $TmDOTP^{5-}$,[28] together with a Tm-Co distance of 5.0 Å and a $\langle\theta\rangle$ value of 40°, the calculated ^{59}Co limiting shift was 131 ppm, in good agreement with the experimental result (135 ppm). The $\langle r\rangle$ and $\langle\theta\rangle$ values found for $Co(en)_3^{3+}$ are similar to those previously estimated for Cs^+ (ionic radius: 1.67 Å) bound to $TmDOTP^{5-}$ where the binding occurs at the outer oxygen axial oxygens of the phosphonate groups.[21] The success of the assumption that the cobalt and the hydrogen nuclei of the cation are at the same averaged location may reflect rapid inter-ion pair exchange and/or rapid intra-ion pair rotation of the countercation.

Summary

Three new, excess-salt-free formulations of the ^{23}Na shift reagent $TmDOTP^{5-}$ have been prepared and characterized. The osmolalities of these depended upon the identity and charge of the countercation, with the osmolalities decreasing in the order $MegH^+ > Na^+ > Co(en)_3^{3+}$. The tripositive cation $Co(en)_3^{3+}$ forms an ion-pair complex(log $K = 2.5-3.1$) with $TmDOTP^{5-}$ at pH 7.4, which is somewhat stronger than that with the smaller monovalent ion, Na^+ (log $K = 1.6-2.5$).[13] Although the sugar cation $meglumineH^+$ reportedly binds to $GdDOTP^{5-}$ with a higher affinity (log $K = 2.96$)[29] than Na^+, our osmolality measurements indicate that $MegH^+$ more fully dissociates from $TmDOTP^{5-}$ than does Na^+ at equivalent concentrations. Measured ^{59}Co and ^1H frequency shifts indicate that $Co(en)_3^{3+}$ forms an ion pair with $TmDOTP^{5-}$ in a geometrical position similar to that of Na^+ on the charged surface of the SR, but at a considerably longer distance from the paramagnetic center. Finally, the SR formulation $Na_4HTmDOTP$ appears to represent the best compromise of lower osmolality with minimal reduction of SR potency.

Acknowledgment. Support of this research by grants from the Robert A. Welch Foundation (AT-584) and the NIH Biotechnology Research Program (RR02584) is gratefully acknowledged. We also thank Dr. Z. Kovacs for synthesis of H_8DOTP. C.S.S. acknowledges an NIH grant (R01-GM32125) and Contract DE-AC02-76CH00016 with the DOE and its office of Health and Environmental Research to BNL, as well as early discussions with Mr. Christian Labadie and Professor Albert Haim.

IC970128Q

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