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NOTPME: a ^{31}P NMR probe for measurement of divalent cations in biological systems

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1,4,7-Triazacyclononane-*N*,*N*',*N*''-tris(methylenephosphonate monoethylester) (NOTPME) has been synthesized, characterized and analyzed for use as a ^{31}P NMR indicator of intracellular Mg^{2+} and Zn^{2+} ions. The ^{31}P NMR spectrum of this chelate in the presence of metal ions shows characteristic resonances for the free chelate, $Mg(NOTPME)^-$, $Zn(NOTPME)^-$, and $Ca(NOTPME)^-$. The K_d values indicate that this chelate has a 10-fold higher affinity for Mg^{2+} than for Ca^{2+} at physiological pH values. In the presence of Mg^{2+}, NOTPME is readily loaded into red blood cells. A ^{31}P NMR spectrum of red cells taken after several washings shows resonances characteristic of entrapped NOTPME and the $Mg(NOTPME)^-$ complex, the relative areas of which report an intracellular free Mg^{2+} concentration of 0.32 mM. The ^{31}P chemical shifts of the free chelate and its metal complexes are far downfield from the typical phosphorus-containing metabolites observed in biological systems, thus making it possible to monitor intracellular cation concentrations and cell energetics simultaneously.

Magnesium chelator; ^{31}P NMR indicator

1. INTRODUCTION

Many biological systems require the diamagnetic cations, Ca^{2+}, Mg^{2+}, and Zn^{2+} to regulate or catalyze various reactions [1–3]. An evaluation of the role of divalent cations in cell function has been limited by the availability of direct methods for measuring free cation concentrations in cells and tissues. Presently available methods for measurement of divalent cations include indirect calculations based on equilibrium reactions [4], ion-selective micro-electrodes [5,6], and null point measurements using metallochromic dyes that are either microinjected into cells [7] or placed into the extracellular space with subsequent lysis of cells [8,9]. Virtually all of these methods are invasive in nature and require sample destruction prior to analysis. In contrast, NMR has made considerable advances as a non-invasive tool in measuring intracellular free divalent cation concentrations in perfused organs and intact cells.

Recently, fluorinated chelators have been effectively used to measure intracellular free Ca^{2+} [10,11] and Mg^{2+} [12,13] in cells and perfused organs by ^{19}F NMR. These chelators work quite well but do suffer some sensitivity loss due to linewidth exchange broadening [13,14]. The synthetic routes to these compounds are often quite complex and this limits the possible chelate structures and hence metal-ion selectivity that may be designed into the chelate. In this study, we present our initial observations on a new macrocyclic chelate, 1,4,7-triazacyclononane-*N*,*N*',*N*''-tris(methylenephosphonate monoethylester) (NOTPME, see structure) as a ^{31}P NMR indicator of intracellular free Mg^{2+} in biological systems.

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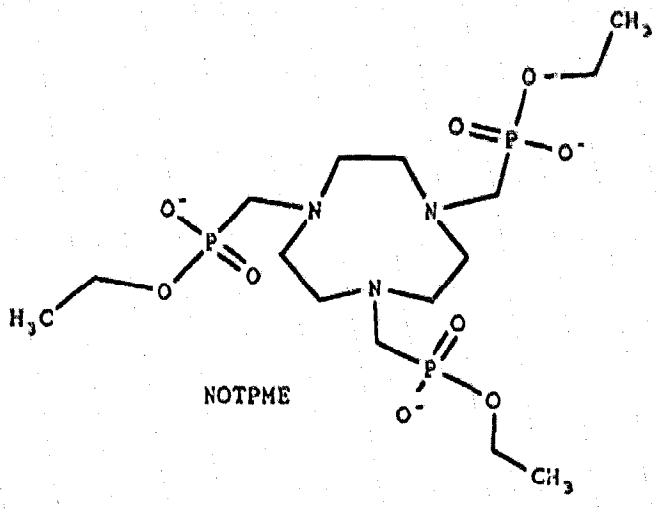
2. MATERIALS AND METHODS

The title chelate was obtained as white crystals of Na_3NOTPME upon hydrolysis of the corresponding diester in NaOH (40.8% yield). The product was characterized by elemental analysis and ^{1}H NMR. ^{1}H NMR (D_2O, HDO peak set as reference at 4.90 ppm), δ (ppm): 1.27 (t, 9H, $-CH_3$), 2.93 (d, 6H, $-P-CH_2$), 2.95 (s, 12H, $N-CH_2$), 3.95 (p, 6H, $O-CH_2$). The diester (NOTPDE) was obtained as a yellow viscous oil in 96% yield by reacting 1,4,7-triazacyclononane, diethylphosphite, and paraformaldehyde in refluxing benzene with azeotropic removal of the water by continuous distillation.

NMR spectra were obtained on a General Electric GN-500 NMR spectrometer using a 10 mm broad band probe tuned to 202.4 MHz for ^{31}P detection. ^{31}P chemical shifts are reported relative to 85% H_3PO_4 as an external standard. Probe temperatures were accurate to ± 0.5°C. Samples of NOTPME used for K_d determinations by NMR consisted of 115 mM KCl, 20 mM NaCl, 10 mM glucose and 10 mM Hepes buffered with Tris base at pH 7.4. Varying concentrations (typically 0.5–100 mM) of Mg^{2+}, Ca^{2+} or Zn^{2+} were added to the sample and the resulting ^{31}P NMR spectrum obtained. Resonance areas were determined by integration of peaks using the GE software.

Whole blood was obtained from the authors (ADS, RR) in heparinized tubes and centrifuged at 3000 × *g* for 6 min to remove the buffy coat. RBCs were then washed 3 times in 5 mM phosphate-buffered saline at pH 7.4. RBCs at 50% hematocrit were suspended in the loading medium containing 100 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.4, 1 mM adenine, 20 mM glucose, and 10 mM NOTPME and incubated at 37°C for 12 h. No lysis of RBCs were observed during the loading procedure. The RBCs were centrifuged



and the supernatant discarded. The RBCs were washed 3 times with phosphate-buffered saline at pH 7.4 before resuspending in isotonic medium. The intracellular pH in RBCs before and after loading was 7.12 and 6.98, respectively.

3. RESULTS

3.1. *Complexation studies*

Fig. 1 displays a ^{31}P NMR spectrum of a solution containing 20 mM NOTPME, 30 mM Mg^{2+}, 2.5 mM Zn^{2+}, and 60 mM Ca^{2+} at pH 7.4. All three metal ion-bound species are in slow exchange with 'free' NOTPME. The linewidths of all four resonances are about 9 Hz with proton decoupling and 22 Hz without decoupling (J_{PH} = 18 Hz). The T_1s and nuclear Overhauser enhancements were equal (1.25 s and 1.2, respectively) within experimental error for all four species. Separate titrations for each metal ion were performed and the area of the resonances corresponding to free and metal bound NOTPME were obtained by integration and their concentrations estimated. The K_d values obtained at 25°C and 37°C by NMR for the Mg^{2+} and Ca^{2+} complexes are reported in Table I. It was not possible to obtain K_d values for Zn(NOTPME)$^-$ from the NMR data because Zn^{2+} was completely bound to NOTPME at any ligand concentration that may be conveniently

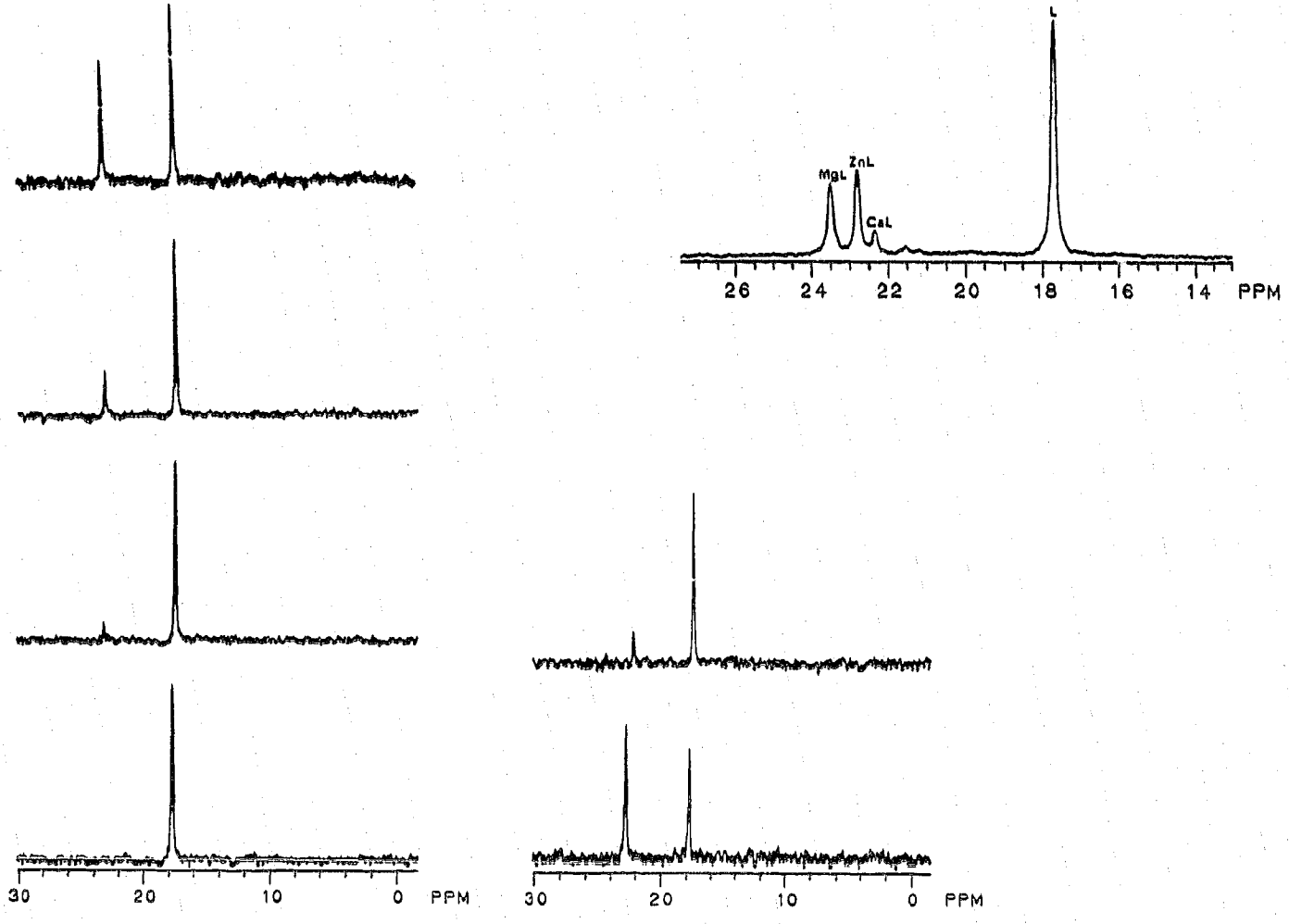


Fig. 1. ^1H-Decoupled ^{31}P NMR spectra of a solution containing 20 mM NOTPME, 2.5 mM $ZnCl_2$, 30 mM $MgCl_2$, 60 mM $CaCl_2$, and 50 mM Hepes, pH 7.4.

Table I

Stability constants of the NOTPME complexes with Ca^{2+}, Mg^{2+}, and Zn^{2+}

Metal ion	K_d (at 25°C)	K_d (at 37°C)
Ca^{2+}	50 mM	47.62 mM
Mg^{2+}	5.77 mM	4.66 mM
Zn^{2+}	(10^{-11} M)*	-

*K_d estimated from the thermodynamic value (log K_d = 15.4) by considering the ligand protonation constants (pK_1 = 11.8, pK_2 = 3.65, and pK_3 = 1.4) and the proton concentration at pH 7.4

detected by NMR. The K_d value reported for Zn(NOTPME)$^-$ in Table I was estimated from the thermodynamic stability constant value measured by potentiometry. The data shows that NOTPME has a binding constant for Mg^{2+} that is in an acceptable range for measurement of free Mg^{2+} concentrations under typical cellular conditions. Furthermore, this chelate shows a significant binding selectivity for Mg^{2+} over Ca^{2+} that is not available in any known non-macrocyclic chelate. In comparison, the fluorinated chelator, MF-APTRA, reported by London and coworkers [12] has a K_d of 1 mM for Mg^{2+} and 12 μM for Ca^{2+}.

Fig. 2 shows the ^{31}P NMR chemical shifts of the NOTPME complexes of Ca^{2+}, Mg^{2+}, and Zn^{2+} as a function of pH between 6 and 10 for solutions containing 1:1 metal ion and ligand (both 5 mM). Under these conditions the Zn(NOTPME)$^-$ resonance is visible over this entire pH range, the Mg(NOTPME)$^-$ resonance is detectable only above pH 6.4, and the Ca(NOTPME)$^-$ resonance is visible only above pH 7.8. The resonances remain in slow exchange over the entire pH range, but the amount of each metal ion bound species is too low to detect below the indicated pH value due to the normal pH dependence of the K_d values. These differences clearly demonstrate the greater selectivity of NOTPME for Mg^{2+} over Ca^{2+} at physiological pH (ca7.4). As shown, the chemical shifts of the metal ion bound species are virtually independent of pH over this range.

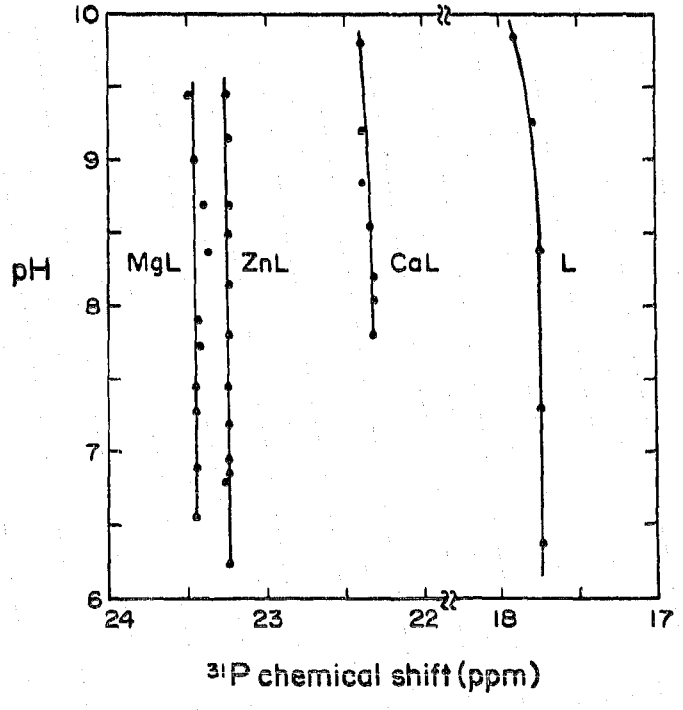


Fig. 2. pH dependence of the ^{31}P NMR chemical shifts of 5 mM Mg(NOTPME)$^-$, Zn(NOTPME)$^-$, Ca(NOTPME)$^-$, and NOTPME (from left to right).

3.2. *Determination of intracellular free Mg^{2+} concentration in human erythrocytes*

Fig. 3 illustrates one application of NOTPME to detect intracellular free Mg^{2+} in RBCs. NOTPME loaded RBCs were prepared by incubating RBCs at 37°C in a loading medium containing NOTPME in the presence of 5 mM $MgCl_2$ for 12 h. NOTPME loading into RBCs was not observed in the absence of $MgCl_2$ in the loading medium. $MgCl_2$ could also be replaced by $ZnCl_2$ in the NOTPME loading medium. NOTPME loaded RBCs were washed 3 times in phosphate buffer to remove all extracellular ligand before suspending them in isotonic saline containing 2 mM $MgCl_2$. After recording the NMR spectra of NOTPME loaded RBCs, the samples were centrifuged and the supernatant analyzed for any leakage of NOTPME from the cells during the time course of the NMR experiment. No NOTPME was visible in the supernatant and therefore the entire signal must have arisen from the intracellular NOTPME. About 1 hour after the cells were washed, the spectrum shown in Fig. 3 was collected. Two

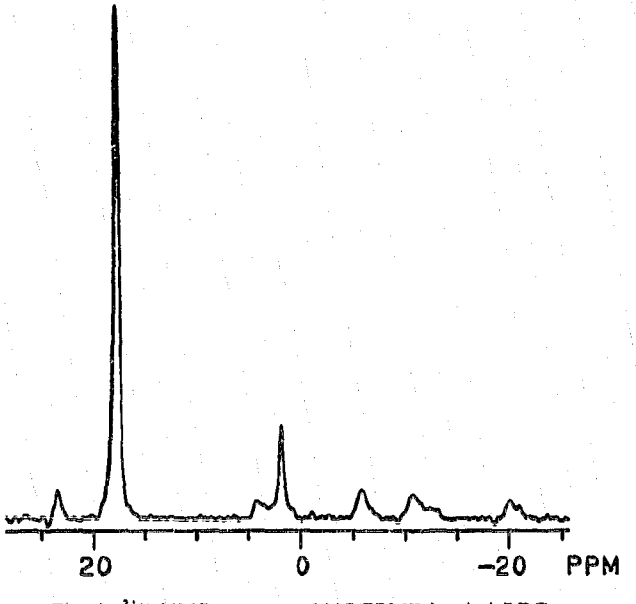


Fig. 3. ^{31}P NMR spectra of NOTPME loaded RBCs.

resonances were observed corresponding to free and Mg^{2+} bound NOTPME and the areas of those resonances reported an intracellular free Mg^{2+} concentration of 0.32 ± 0.04 (n = 3) mM using peak integration and the K_d reported in Table I. The normal range for intracellular free Mg^{2+} levels in RBCs normally range between 0.2 and 0.3 mM [12,15,16]. Upon addition of the divalent cation ionophore A23187 to the suspension medium, the intracellular free Mg^{2+} concentration increased to 1.90 mM (data not shown). This result indicates that NOTPME can easily detect small changes in free Mg^{2+} levels in intact cells.

Separate atomic absorption measurements verified that intracellular Mg^{2+} did indeed increase during loading of NOTPME, suggesting that the chelate is transported as a complex with Mg^{2+}. Upon washing and resuspending the NOTPME loaded RBCs in an isotonic saline, Mg^{2+} efflux occurs during the 1 h period before the NMR spectrum was recorded (perhaps via both Na^+-dependent and Na^+-independent Mg^{2+} efflux mechanisms [17]) bringing the intracellular free Mg^{2+} back to the normal values.

4. DISCUSSION

NOTPME has several advantages over the fluorinated chelators such as BAPTA and APTRA for measurement of intracellular Mg^{2+} in biological systems. These include (i) ease of synthesis, (ii) higher affinity for Mg^{2+} than for Ca^{2+}, (iii) no significant exchange contribution to the bound and free ligand resonances, and (iv) the three magnetically equivalent ^{31}P nuclei in this chelate provides an attractive NMR nucleus for biological tissue since cell energetics may be measured simultaneously. The significant selectivity for Mg^{2+} over Ca^{2+} exhibited by NOTPME at physiological pH is of great advantage for monitoring Mg^{2+} in biological systems without concern about interference from Ca^{2+}. It is important to emphasize that the resonances of NOTPME and its magnesium complex do not overlap with the phosphorus containing metabolites in tissue. Thus NOTPME provides a versatile approach for measuring changes in intracellular free magnesium as well as changes in metabolite concentrations by ^{31}P NMR during various physiological interventions. Although it is fortuitous that the K_d for Mg(NOTPME)$^-$ at physiological pH is in the range required for measurement of intracellular Mg^{2+} in many biological systems, it may prove possible to fine tune the affinity of NOTPME for Mg^{2+} by changing the alkyl substituents on the phosphonate oxygens or by adding an alkyl chain to the carbon which links the phosphonate groups to the triaza ring. We are currently exploring these possibilities as well as the synthesis of hydrolyzable ester derivatives of NOTPME which may be loaded without cotransport of Mg^{2+} into RBCs.

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