

large complex caused DNA precipitation. At low DNA concentrations, a large number of Mn complex molecules will be bound to a given molecule of DNA, while, at higher DNA concentrations, the complex molecules are distributed among many DNA molecules. The change in the trend of the shift in E_p of $Mn^{III}P(Dis)_2$ in the presence of different concentrations of DNA, for example, from positive potentials at low DNA concentrations to more negative values at higher DNA concentrations, indicates that the adsorption process dominates the electrochemical response, over the effect of binding to DNA, at the early stages of the titration. This occurs because adsorption and precipitation are present only at low DNA concentrations. Although we were unable to quantify the binding of $Mn^{III}P(Dis)_2$ to DNA, the binding site size was estimated to be about 15 bp. This value agrees with the arithmetic addition of the individual binding site sizes of $Mn^{III}P$ ($s = 3-4$ bp)¹ and Dis ($s = 4-6$ bp).⁸⁻¹¹

The effect of DNA on the Soret band of $Mn^{III}P(Dis)_2$ complex was the same as that for $Mn^{III}P$ alone; however, the magnitude of the increase in the absorbance was greater for $Mn^{III}P$ at low NP concentrations. This may occur because the distamycin portion of the complex competes with the metalloporphyrin ring for sites in the double helix.

Comparison of the electrochemical and spectroscopic behavior of $Mn^{III}P$ and $Mn^{III}P(Dis)_2$ in the presence of DNA suggests that these two species associate with DNA in a similar fashion.

Conclusion

In this paper we reported the binding of two DNA groove-binding molecules, $Mn^{III}P$ and Dis, by voltammetric methods. Two distamycin molecules bind one $Mn^{III}P$, probably by axial coordination of the oxygen atom of the formyl end to the Mn(III) center. Bis(distamycin) compounds are important, as shown by Dervan and others,³⁶⁻³⁹ as potential recognition agents of large sequences of A-T rich DNA.

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Contribution from the Department of Chemistry, Loyola University of Chicago, 6525 North Sheridan Road, Chicago, Illinois 60626, and Department of Chemistry and Center of Neurosciences of Coimbra, University of Coimbra, 3000 Coimbra, Portugal

Physical Basis for the Resolution of Intra- and Extracellular ^{133}Cs NMR Resonances in Cs^+ -Loaded Human Erythrocyte Suspensions in the Presence and Absence of Shift Reagents^{8,11}

Lisa Wittenkeller,[†] Duarte Mota de Freitas,^{*†} Carlos F. G. C. Geraldes,[‡] and Angelo J. R. Tomé[‡]

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For human red blood cells (RBCs) loaded with Cs^+ and suspended in a shift reagent (SR) free medium, the extracellular $^{133}Cs^+$ NMR resonance was shifted upfield from the intracellular resonance. However, in the presence of the SRs $Dy(PPP)_2^{7-}$, $Dy(TTHA)^{3-}$, and $Tm(DOTP)^{5-}$ [where Dy^{3+} and Tm^{3+} denote dysprosium and thulium ions and PPP^{5-} , $TTHA^{6-}$, and $DOTP^{8-}$ represent the triphosphate, triethylenetetraminehexaacetate, and 1,4,7,10-tetraazacyclododecane- N,N',N'',N''' -tetrakis(methylenephosphonate) ligands, respectively], the extracellular $^{133}Cs^+$ NMR resonance was shifted downfield from the intracellular resonance. The magnitudes of the $^{133}Cs^+$ shifts observed with $Tm(DOTP)^{5-}$ were much larger than those for $Dy(TTHA)^{3-}$ and $Dy(PPP)_2^{7-}$ at the same concentration. The direction of the $^{133}Cs^+$ shift induced by $Dy(PPP)_2^{7-}$ was the opposite of that previously reported for $^7Li^+$, $^{23}Na^+$, and $^{39}K^+$ NMR resonances. The negative sign of the pseudocontact ^{133}Cs shift induced by $Dy(PPP)_2^{7-}$ is related to the large size of the Cs^+ cation and its location in the equatorial region formed by the cone around the effective magnetic axis of the triphosphate SR. At physiologically relevant RBC concentrations, 2,3-diphosphoglycerate (DPG), of all intracellular phosphates tested, caused the largest $^{133}Cs^+$ shift. The $^{133}Cs^+$ resonance in carbonmonoxygenated RBC lysate shifted downfield by approximately 2.0 ppm with increasing hemoglobin concentration, whereas an increase in the diamagnetic susceptibility of the sample induced by hemoglobin is expected to induce an upfield shift of 0.1 ppm. The $^{133}Cs^+$ resonance was shifted downfield with increasing concentrations of two unrelated proteins, carbonmonoxyhemoglobin and lysozyme. We conclude that, in the absence of SRs, the physical basis for the resolution of intra- and extracellular ^{133}Cs NMR resonances in Cs^+ -loaded human RBC suspensions arises from Cs^+ binding to intracellular phosphates, in particular DPG, and from the nonideality of intracellular water induced by hemoglobin.

Introduction

Aqueous shift reagents (SRs) have been introduced in the past decade for the study of biologically important metal cations by nuclear magnetic resonance (NMR) spectroscopy.¹ Because of their high negative charges, SRs are not soluble in the interior

of the lipophilic membrane and are repelled by the negatively charged head groups of phospholipids. Thus, SRs remain in the extracellular compartment during NMR experiments conducted on cell suspensions. With the use of SRs, the extracellular resonance is shifted away from the intracellular resonance, thus allowing the simultaneous observation of the two pools of metal ions. Information on metal cation transport and distribution in cell suspensions, and on enzymatic activity, is then easily obtained by metal NMR spectroscopy in the presence of SRs.^{2,3}

In cell suspensions, the presence of SRs in the suspension medium leads to transmembrane differences in chemical shifts that result in resolution of intra- and extracellular $^7Li^+$, $^{23}Na^+$,

* To whom correspondence should be addressed.

[†] Loyola University of Chicago.

[‡] University of Coimbra.

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¹ Abbreviations used in this paper: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BMS, bulk magnetic susceptibility; COHb, carbonmonoxyhemoglobin; CORBC, carbonmonoxygenated red blood cell; deoxyRBC, deoxygenated red blood cell; $DOTP^{8-}$, 1,4,7,10-tetraazacyclododecane- N,N',N'',N''' -tetrakis(methylenephosphonate); DPG, 2,3-diphosphoglycerate; Ht, hematocrit; NMR, nuclear magnetic resonance; P_i, inorganic phosphate; PPP^{5-} , triphosphate; PVP-100, poly(vinylpyrrolidone)-100; SR, shift reagent; $TTHA^{6-}$, triethylenetetraminehexaacetate.

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$^{39}\text{K}^+$, and $^{87}\text{Rb}^+$ NMR resonances.⁴⁻⁷ In the absence of SRs, transmembrane differences in relaxation times provide an alternative means of distinguishing intra- and extracellular pools of Li^+ and K^+ ions.^{8,9} However, intra- and extracellular $^{133}\text{Cs}^+$ NMR resonances in Cs^+ -loaded cell suspensions exhibit a unique behavior. The chemical shifts of the two ^{133}Cs NMR resonances in Cs^+ -loaded human erythrocyte and perfused rat heart suspensions are distinguishable without the incorporation of a SR in the suspension medium.¹⁰ Similar ^{133}Cs NMR observations were recently made with living plant tissue.¹¹ On the other hand, with vesicular preparations, triphosphate was required for separation of the $^{133}\text{Cs}^+$ NMR resonances.¹²

Because of the large ionic radius of Cs^+ , the NMR chemical shift range of ^{133}Cs is much larger relative to those of other alkali metal nuclei and is known to be extremely sensitive to the chemical environment and is affected by the counterions present, as well as by temperature and solvent composition.¹³ The ^{133}Cs nucleus has a nuclear spin of $7/2$, a small quadrupole moment, and a relaxation rate approximately 200 times smaller than that of the other NMR-detectable alkali metal nuclei. The Cs^+ ion is 100% visible by ^{133}Cs NMR spectroscopy. These factors make ^{133}Cs NMR spectroscopy an ideal technique for investigating the distribution and transport of Cs^+ in biological systems.¹⁰⁻¹²

The discovery of a convenient tool for Cs^+ analysis comes at a time when much interest in the biochemistry and physiology of Cs^+ in biological systems exists.¹⁴ Understanding the general properties of alkali metal ions in transport and enzyme activation typically involves the use of Cs^+ as a tracer of K^+ transport and distribution in vivo because Cs^+ is considered to be physiologically similar to K^+ . Investigation of the toxicologic problems is of concern because these are related in part to uptake of radioactive $^{137}\text{Cs}^+$, which has a long biological half-life and is a product of nuclear explosions. Once generated, $^{137}\text{Cs}^+$ is passed quickly down the food chain and eventually settles in muscle and bone tissues. Cs^+ is also used for treatment of depression. Typically, Li_2CO_3 has been administered for this purpose. Cs^+ was found, however, to be absorbed more rapidly, without some of the side effects associated with Li^+ salts. Since Cs^+ salts were first evaluated for their antidepressant activity by Messiha and co-workers,¹⁴ several studies have appeared on Cs^+ effects on neuromuscular systems, the immune system, motor functions, and tumor growth.¹⁴

Our aim in this study was to investigate the origin of the chemical shift resolution of the two $^{133}\text{Cs}^+$ NMR resonances observed in suspensions of human RBCs loaded with Cs^+ in SR-free and SR-containing media. In addition to investigating the unusual ^{133}Cs -shifting properties of $\text{Dy}(\text{PPP})_2^{7-}$ relative to other SRs, we examined the effects of Cs^+ binding to the RBC membrane, intracellular phosphates, and hemoglobin, magnetic susceptibility, and nonideality of intracellular water on the ^{133}Cs

NMR chemical shift of intracellular Cs^+ .

Materials and Methods

Chemicals. All chemicals were supplied by either Aldrich or Sigma and were used without further purification. The macrocyclic ligand 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetrakis(methylene-phosphonic) acid (H_3DOTP) was synthesized and characterized as described elsewhere.¹⁵ Packed RBCs were supplied by Life Source.

Shift Reagents. $\text{Dy}(\text{PPP})_2^{7-}$ was prepared by the dropwise addition of sodium triphosphate, Na_3PPP , to DyCl_3 (final ratio 2.5:1) during vortexing.⁶ $\text{Dy}(\text{TTHA})^{3-}$ was prepared according to a published procedure.¹⁶ $\text{Tm}(\text{DOTP})^{5-}$ was prepared from TmCl_3 and H_3DOTP as described previously.¹⁷ Solutions for the $^{133}\text{Cs}^+$ shift measurements with the shift reagents $\text{Dy}(\text{TTHA})^{3-}$ and $\text{Tm}(\text{DOTP})^{5-}$ were made in a HEPES buffer in the absence of Na^+ . In the Na^+/Cs^+ competition experiments, $\text{Dy}(\text{PPP})_2^{7-}$ in the Na^+ form was dissolved in HEPES buffer, and the Na^+ concentration was varied as described in the caption of Figure 2.

RBC Preparations. Fresh RBCs were collected and washed at least three times in an isotonic 5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl. They were then centrifuged at 2000g for 6 min at 4 °C in a Savant refrigerated centrifuge. Cs^+ loading of RBCs is a rather slow process.^{10,18} We developed loading conditions that led to higher intracellular RBC Cs^+ concentrations than previously reported.¹⁰ Cs^+ loading was accomplished by incubation of the washed, packed RBCs at 45% hematocrit and 37 °C from 1 to 12 h with 150 mM CsCl, 5 mM glucose, and 5 mM HEPES, pH 7.4. The intracellular Cs^+ concentrations varied from 0.59 to 15.0 mM, depending on the incubation time used. The Cs^+ -loaded RBCs were then washed and resuspended at 27–93% hematocrit in the isotonic buffers as described in the figure legends.

Hematocrit measurements were conducted with an IEC MB micro-hematocrit unit (Needham Heights, MA). We applied a 2% correction to the measured hematocrit to account for trapped suspension medium.¹⁹ The osmolarity of all suspension media was adjusted with sucrose and measured with a Wescor vapor pressure osmometer (Wescor Inc., Logan, UT). Except for the experiments in which the effect of osmolarity on the intracellular Cs^+ shift was investigated, RBCs were suspended in isotonic media (osmolarity approximately 300 mosM). The viscosity of the CsCl buffer solution C in Table I was adjusted with PVP-100²⁰ and measured with a Brookfield cone plate viscometer.

Deoxygenated RBCs (deoxyRBC) were prepared by gentle passage of moist nitrogen gas through each sample.^{21,22} To prevent cell drying, we moistened the gas initially by bubbling it through the resuspension medium. Carbonmonoxxygenated RBCs (CORBC) were prepared in a similar fashion by bubbling each sample with CO gas.^{21,22} DeoxyRBC and CORBC were repacked prior to resuspension and their oxygenation states were verified by examination of the ^{31}P NMR spectra of the suspensions (vide infra). Unsealed RBC membranes were prepared by hypotonic cell lysis.²³ RBC lysates were prepared by repeated rapid freezing of washed packed RBCs in an ethanol dry ice mixture and thawing in a water bath at 37 °C.²² Various RBC lysate concentrations were obtained by dilution with doubly distilled water. Homoglobin stripped of DPG and purified according to a published procedure²⁴ was bubbled with CO for 30 min prior to analysis with an IBM 9420 UV/vis spectrophotometer at 550 and 430 nm for determination of the concentration of carbonmonoxyhemoglobin (COHb). Samples containing COHb were placed in septum-sealed NMR tubes that had been purged with nitrogen for removal of any oxygen.

NMR Spectroscopy. ^{133}Cs , ^{13}C , and ^{31}P NMR measurements of cell suspensions were made at 39.4, 75.4, and 121.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer (Loyola University) equipped

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with a variable-temperature unit. Some ³¹P NMR measurements were made on a General Electric GN-500 NMR spectrometer (University of Texas at Dallas). The flip angles of the excitation pulses were 54° (18 μs) for ¹³³Cs, 67° (8.7 μs) for ¹³C, and 45° (45 μs) for ³¹P. The spectral widths were 8000, 16 500, and 10 000 Hz for ¹³³Cs, ¹³C, and ³¹P, respectively. The ¹³³Cs, ¹³C, and ³¹P chemical shifts are reported relative to 0.15 M CsCl, tetramethylsilane, and phosphoric acid, respectively, which were measured separately in spinning 10-mm NMR tubes. The reported line widths represent the widths of the resonances at half-height (in hertz) after subtraction of line broadening used in Fourier transformation. The ¹³³Cs NMR spectra of SR-free cell suspensions were recorded with a 25-s delay to allow for complete relaxation of extracellular Cs⁺ signals (*T*₁ = 13.6 s).¹⁰ With the exception of those of intact RBC suspensions, the spectra of all samples were recorded while the samples were spinning. The ²³Na and ¹³³Cs NMR experiments in SR-containing aqueous solutions were made at 52.9 and 26.4 MHz, respectively, on a Varian XL-200 NMR spectrometer (University of Coimbra) equipped with a variable-temperature unit. The flip angles of the excitation pulses were 90° (22 μs) for ²³Na and 45° (58 μs) for ¹³³Cs. The ¹³³Cs NMR spectra in aqueous solution were recorded with a total relaxation delay of 9 s. For all experiments, the probe temperature was ambient. The reported paramagnetic shifts were measured relative to samples that were free of SR. Downfield and upfield paramagnetic shifts reported in Figure 2 were defined as negative and positive, respectively.

Field-frequency locking on D₂O present in the aqueous media was used throughout. No coaxial tube combinations were used, except for the experiments in which the contribution of the nonideality of intracellular water to the intracellular ¹³³Cs⁺ shift was investigated (vide infra). Locking on D₂O and placing reference and sample solutions in separate NMR tubes with the same geometry eliminate contributions to the observed shifts from volume magnetic susceptibility, assuming that lanthanide-induced shifts of the solvent ²H resonance are negligible.^{16,25} For some experiments (Figures 5 and 6), the ¹³³Cs⁺ chemical shifts were referenced relative to 0.15 M CsCl placed in the bulb of a spherical bulb/cylindrical capillary (Wilmad Glass Co., Inc., Buena, NJ) supported in the outer 10-mm NMR tube with a vortex plug. The spherical bulb/cylindrical capillary reference assembly used eliminated any chemical shift contribution from the diamagnetic susceptibility of the sample resulting from an increase in protein concentration, as was previously reported.^{26,27} The methods used for referencing of ¹³³Cs⁺ chemical shifts are indicated in the figure captions and footnotes to the tables. The concentration of intracellular Cs⁺, [Cs⁺]_{in}, expressed in mmol of Cs⁺/L of RBCs, was calculated from the equation

$$[\text{Cs}^+]_{\text{in}} = \{A_{\text{in}}[\text{Cs}^+]_{\text{s}}\} / \{A_{\text{s}}[\text{hematocrit}]\}$$

where *A*_{in} is the peak area under the intracellular ¹³³Cs⁺ resonance and [Cs⁺]_s and *A*_s are the concentration and peak area of the resonance of the standard 0.15 M CsCl/D₂O solution. The concentration of extracellular Cs⁺ was obtained from a similar expression except that *A*_{in} was replaced by *A*_{out} and [hematocrit] was replaced by (1 - [hematocrit]). Relative peak areas of ¹³³Cs⁺ signals were obtained by means of the integration routines included in the software provided by the manufacturer for the Varian VXR-300 NMR spectrometer.

Results

Resolution of Intra- and Extracellular ¹³³Cs NMR Resonances in the Presence and Absence of SRs. Figure 1 shows the ¹³³Cs NMR spectra of Cs⁺-loaded CORBCs suspended in a Cs⁺ medium without SR (Figure 1A) or with Tm(DOTP)⁵⁻ (Figure 1B), Dy(TTHA)³⁻ (Figure 1C) or Dy(PPP)₂⁷⁻ (Figure 1D). We made the assignments of intra- and extracellular ¹³³Cs resonances in SR-free RBC suspensions (Figure 1A) by observing the changes in peak areas during Cs⁺ loading, as previously reported.¹⁰ As shown in Figure 1A, the intra- and extracellular ¹³³Cs⁺ NMR resonances were resolved without the incorporation of a SR, with the extracellular resonance being upfield. For the samples containing SR, we made peak assignments by centrifuging and separating the extracellular suspension from the Cs⁺ loaded RBCs. The ¹³³Cs chemical shifts were recorded for the SR-containing media alone (no RBCs) and were approximately the same as for the extracellular resonance in the RBC suspension. Upon incorporation of a SR, Tm(DOTP)⁵⁻ (Figure 1B), Dy(TTHA)³⁻

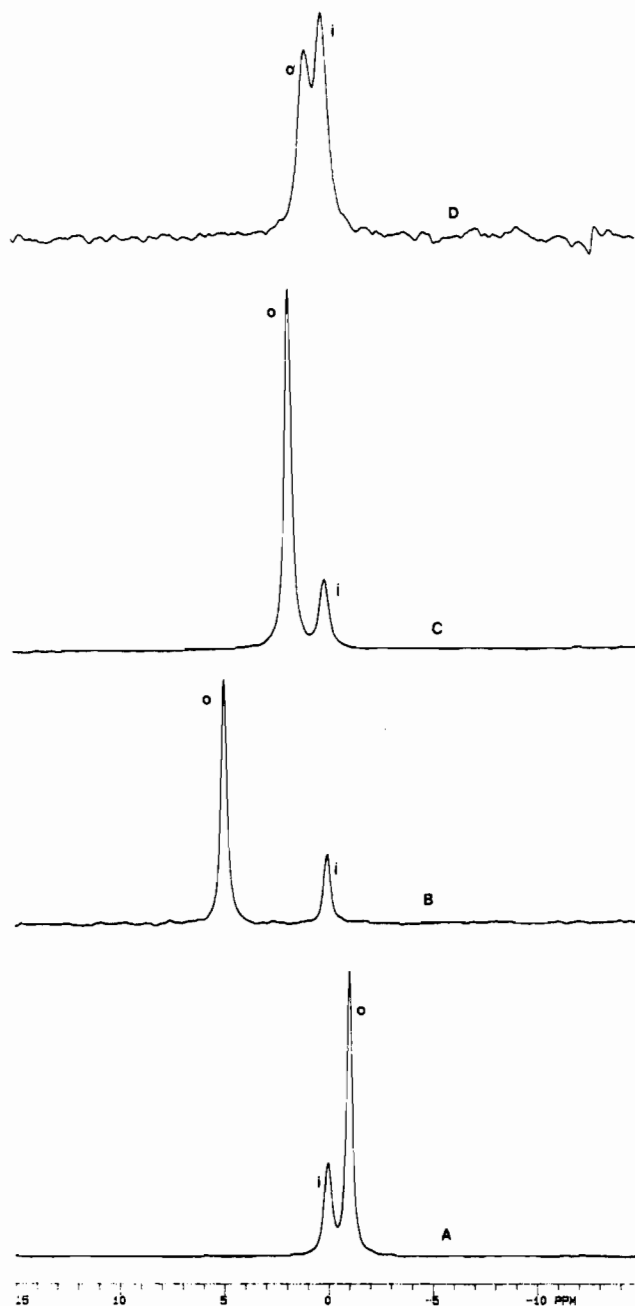


Figure 1. ¹³³Cs NMR spectra: (A) Cs⁺-loaded CORBCs resuspended at 33% hematocrit in an isotonic medium containing 10 mM CsCl, 140 mM NaCl, 5 mM glucose, and 5 mM HEPES, pH 7.4 (initial concentrations [Cs⁺]_{in} = 4.5 mM and [Cs⁺]_{out} = 5.1 mM); (B) RBC suspension similar to that in part A at 40% hematocrit, except that 5.0 mM Tm(DOTP)⁵⁻ replaced 25 mM NaCl in the medium (initial concentrations [Cs⁺]_{in} = 2.5 mM and [Cs⁺]_{out} = 5.3 mM); (C) RBC suspension similar to that in part A at 33% hematocrit, except that 5.0 mM Dy(TTHA)³⁻ replaced 15 mM NaCl in the medium (initial concentrations [Cs⁺]_{in} = 4.1 mM and [Cs⁺]_{out} = 7.9 mM); (D) RBC suspension similar to that in part A at 45% hematocrit, except that 5.0 mM Dy(PPP)₂⁷⁻ replaced 50 mM NaCl in the medium (initial concentrations [Cs⁺]_{in} = 6.1 mM and [Cs⁺]_{out} = 5.8 mM). Each spectrum was recorded in 1000 transients in a septum-sealed NMR tube bubbled with moist CO gas. The symbols i and o denote intra- and extracellular ¹³³Cs⁺ NMR resonances. The spectra are referenced to a sample of 0.15 M CsCl in D₂O which was measured separately.

(Figure 1C), or Dy(PPP)₂⁷⁻ (Figure 1D), in the Cs⁺-containing suspension medium, the extracellular ¹³³Cs⁺ NMR resonance was shifted downfield. The largest downfield shifts of the extracellular ¹³³Cs⁺ resonance were observed in Cs⁺-loaded RBC suspensions containing Tm(DOTP)⁵⁻ in the medium.

Transmembrane Cs⁺ distribution ratios were measured for Cs⁺-loaded RBCs suspended in a Cs⁺-containing media at 37 °C

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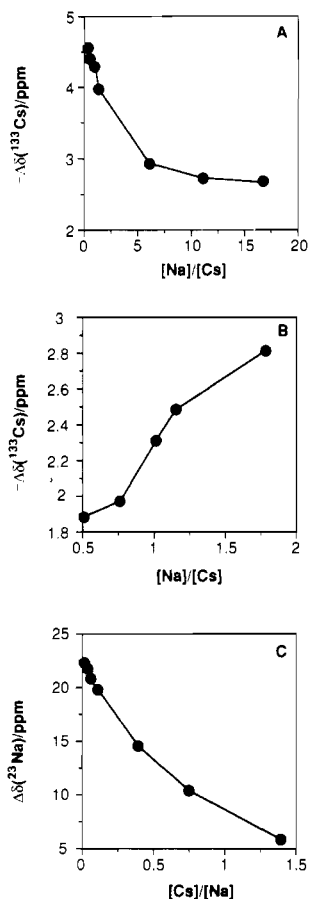


Figure 2. Competition between Na^+ and Cs^+ for shift reagents. (A) Effect of $[Na^+]/[Cs^+]$ ratio on the $^{133}Cs^+$ NMR paramagnetic shifts induced by 4.8 mM $Dy(TTHA)^{3-}$. $[Cs^+]$ was held constant at 10 mM while $[Na^+]$ was varied. (B) Effect of $[Na^+]/[Cs^+]$ ratio on the $^{133}Cs^+$ NMR paramagnetic shifts induced by 4.5 mM $Dy(PPP)_2^{7-}$. $[Cs^+]$ was held constant at 80 mM while $[Na^+]$ was varied. (C) Effect of $[Cs^+]/[Na^+]$ ratio on the $^{23}Na^+$ NMR paramagnetic shift induced by 4.5 mM $Dy(PPP)_2^{7-}$. $[Na^+]$ was held at 60 mM while $[Cs^+]$ was varied. The reported paramagnetic shifts were measured relative to samples that were free of SR. Downfield and upfield paramagnetic shifts were defined as negative and positive, respectively.^{16a}

over a 4-h period. We found that a steady-state transmembrane Cs^+ distribution was reached after 3 h of incubation time. The steady-state Cs^+ distribution ratio ($[Cs^+]_{in}/[Cs^+]_{out}$) across the RBC membrane was approximately the same (0.27 ± 0.01) in the presence and absence of $Tm(DOTP)^{5-}$ or $Dy(TTHA)^{3-}$. However, the Cs^+ distribution ratio observed in $Dy(PPP)_2^{7-}$ -treated RBC suspensions (as is apparent from the relative peak areas shown in Figure 1D) was significantly higher than those observed in Cs^+ -loaded RBCs suspended in $Tm(DOTP)^{5-}$ - or $Dy(TTHA)^{3-}$ -treated media, or in SR-free medium.

$^{133}Cs^+$ NMR Paramagnetic Shifts Induced by Several Dy^{3+} and Tm^{3+} Shift Reagents in Aqueous Solution. The efficacy of the chelates $Dy(PPP)_2^{7-}$, $Dy(TTHA)^{3-}$, and $Tm(DOTP)^{5-}$, which were studied previously for $^7Li^+$ and $^{23}Na^+$ NMR shifts,^{4,16,28} was tested for $^{133}Cs^+$ NMR studies. We obtained plots of the $^{133}Cs^+$ paramagnetic shift of solutions containing 10 mM Cs^+ as a function of ρ (stoichiometric molar ratio of SR to Cs^+) for these three SRs (data not shown). The induced $^{133}Cs^+$ shifts are significantly smaller than those observed for $^7Li^+$ and $^{23}Na^+$ for the same ρ values.^{4,16,28} As the SR concentration increased, the observed $^{133}Cs^+$ shifts increased linearly and did not approach a limiting value except for $Tm(DOTP)^{5-}$. The general shape of the curves for $Dy(TTHA)^{3-}$ and $Dy(PPP)_2^{7-}$ indicates much weaker Cs^+ binding to SRs relative to that for Li^+ and Na^+ ions, whereas the

Table I. $^{133}Cs^+$ NMR Chemical Shifts Caused by Cs^+ Binding to Intracellular RBC Components^a

sample	$\delta(^{133}Cs)/ppm$		
	buffer A ^b	buffer B ^c	buffer C ^d
2.0 mM ATP	0.46	0.43	0.46
0.2 mM ADP	0.23	0.23	0.33
5.4 mM DPG	0.81	0.81	0.83
1.0 mM P_i	0.22	0.27	0.27
2.7 mM COHb ^e	2.02	2.10	2.11

^aShifts are reported relative to a reference sample of 0.15 M $CsCl$ in D_2O placed in a spinning 10-mm NMR tube and measured separately. The reported values represent an average of measurements conducted in three separately prepared samples. Errors are less than 0.05 ppm. ^b $CsCl$ buffer A consisted of 10 mM $CsCl$, 140 mM $NaCl$, 5 mM glucose, and 5 mM HEPES, pH 7.4. The osmolarity was approximately 300 mosM. ^c $CsCl$ buffer B consisted of 10 mM $CsCl$, 5 mM glucose, 5 mM HEPES, pH 7.4, 140 mM KCl , 5 mM $NaCl$, 2.4 mM $MgCl_2$, and 1 μM $CaCl_2$. ^d $CsCl$ buffer C had the same composition as buffer B, except that the viscosity of this solution was adjusted to 5.6 cP with PVP-100. ^eBecause of denaturation problems associated with Hb preparations at high concentration, the concentration of COHb used in this study was below the physiologic value in human RBC (4.8–5.4 mM).³⁰ However, for the COHb experiments we only used 5 mM $CsCl$ in buffers A–C. Thus, the concentration ratio of Cs^+ to COHb is close to the physiologic value in human RBC.

hyperbolic nature of the curve for $Tm(DOTP)^{5-}$ indicates strong Cs^+ binding.²⁹ In contrast to the results for $^7Li^+$ and $^{23}Na^+$, where the shifts induced by $Dy(PPP)_2^{7-}$ and $Tm(DOTP)^{5-}$ are, under the same concentration conditions, larger than those induced by $Dy(TTHA)^{3-}$,^{4,16,28} with $^{133}Cs^+$ the relative shifting ability was $Tm(DOTP)^{5-} \gg Dy(TTHA)^{3-} > Dy(PPP)_2^{7-}$. The $^{133}Cs^+$ shifts induced by $Dy(PPP)_2^{7-}$ were also of opposite sign relative to those reported previously for $^7Li^+$ and $^{23}Na^+$ resonances.^{4,16,28}

Figure 2 shows the results of competition experiments between Cs^+ and Na^+ for the SRs $Dy(TTHA)^{3-}$ and $Dy(PPP)_2^{7-}$. In the case of $Dy(TTHA)^{3-}$, addition of Na^+ decreases the $^{133}Cs^+$ shifts (Figure 2A), indicating competition between Na^+ and Cs^+ for the same binding site on the SR. The common binding site(s) is(are) presumably the carboxylate group(s) in the equatorial region which is(are) not bound to the lanthanide ion.¹⁶ However, in the case of $Dy(PPP)_2^{7-}$, addition of Na^+ to a Cs^+ /SR solution caused a small increase in the $^{133}Cs^+$ shift (Figure 2B), whereas addition of Cs^+ to a Na^+ /SR solution caused a decrease in the $^{23}Na^+$ shift (Figure 2C).

Effect of Cs^+ Binding to Intracellular Phosphates, Hemoglobin, and Cell Membrane on the Intracellular $^{133}Cs^+$ Shift. We used three media to study the contribution of the intracellular RBC components at concentrations close to the physiologic values³⁰ to the intracellular $^{133}Cs^+$ chemical shift (Table I). The compositions of the media are indicated in the footnotes to Table I. Buffer A had the same composition as did the suspension medium used in Figure 1A. Buffer B contained competing intracellular cations, and buffer C, in addition to competing intracellular cations, had its viscosity adjusted with PVP-100²⁰ to approximately 5 cP to mimic that of the intracellular RBC compartment.³¹ Table I shows that the chemical shift of the $^{133}Cs^+$ NMR resonance was at least 0.8 ppm for physiologically relevant concentrations of DPG in buffer A. In contrast, the $^{133}Cs^+$ NMR shifts in the presence of typical RBC concentrations of the intracellular phosphates ATP, ADP, and P_i were approximately 57, 28, and 27%, respectively, of those observed with DPG in buffer A.

To determine whether the large Cs^+ -DPG shift was simply due to the high DPG concentration relative to that of other intracellular phosphates, we also measured the $^{133}Cs^+$ NMR chemical shifts for each intracellular phosphate at the same concentration (2 mM)

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Table II. Effects of Cs⁺ Loading, Oxygenation, and SR on Chemical Shifts and Line Widths of Intracellular RBC ³¹P NMR Phosphate Resonances^{a,b}

	DPG			ATP		
	3	2	P _i	γ	α	β
A. Cs⁺-Loaded DeoxyRBCs						
δ/ppm	6.2	5.5	4.6	-2.9	-7.8	-16.9
Δν _{1/2} /Hz	42	28	19	42	48	137
B. Cs⁺-Free DeoxyRBCs						
δ/ppm	5.9	5.2	4.3	-3.3	-8.1	-17.2
Δν _{1/2} /Hz	26	22	20	46	49	125
C. Cs⁺-Loaded CORBCs						
δ/ppm	5.7	4.9	4.2	-3.4	-8.3	-17.4
Δν _{1/2} /Hz	25	20	21	37	36	104
D. Cs⁺-Free CORBCs						
δ/ppm	5.6	4.8	4.2	-3.3	-8.3	-17.4
Δν _{1/2} /Hz	17	19	15	43	37	125
E. Cs⁺-Loaded DeoxyRBCs in SR Suspension						
δ/ppm	5.8	5.1	4.0	-3.6	-8.3	-17.7
Δν _{1/2} /Hz	36	36	35	51	55	126
F. Cs⁺-Loaded CORBCs in SR Suspension						
δ/ppm	5.5	4.7	4.2	-3.5	-8.3	-18
Δν _{1/2} /Hz	56	120	37	56	55	150

^aSamples A and C were suspended in the same medium as for Figure 1A. Samples B and D were suspended in 150 mM NaCl, 5 mM glucose, and 5 mM HEPES, pH 7.4. Samples E and F were suspended in the same medium as for Figure 1C. All suspension media contained 15% D₂O. RBCs were loaded with 150 mM CsCl for 3 h. ^bShifts are reported relative to a reference sample of phosphoric acid in D₂O, placed in a spinning 10-mm NMR tube and measured separately. The reported ³¹P line widths represent the widths of the resonances at half-height after subtraction of 20 Hz used in line broadening. The reported NMR parameters represent the average of measurements conducted on at least two separately prepared samples. Errors in chemical shift and line width are less than 0.1 ppm and 1.0 Hz, respectively.

in buffer A. The observed shifts were 0.46, 0.25, 0.75, and 0.36 ppm for ATP, ADP, DPG, and P_i, respectively. These values were comparable to those measured in buffer A for each of the intracellular phosphates at known physiological RBC concentrations (see Table I). The downfield shift of the ¹³³Cs⁺ NMR resonance observed in the presence of COHb at concentrations close to its normal range in human RBC³⁰ was larger than the downfield shift induced by DPG. However, the downfield shift induced by COHb is not due to specific Cs⁺ binding to COHb (vide infra). In the presence of competing intracellular cations (buffer B) and with the solution viscosity adjusted to 5 cP (buffer C), the ¹³³Cs⁺ NMR chemical shifts in the presence of each intracellular RBC component were virtually unchanged relative to that observed in buffer A (Table I). Of all intracellular phosphates, DPG continued to induce the largest ¹³³Cs⁺ shift in the presence of competing cations within a physiologic viscosity range. When all of the intracellular components were present in buffer C, the shift observed was 0.99 ppm (*n* = 3). This is less than the calculated value, 4.00 ppm, obtained by addition of the shifts produced separately by each RBC component. The shifts were not additive, presumably because there is competition between intracellular components for the Cs⁺ ion.

Negatively charged phospholipids or amino acid side chains in RBC membrane proteins are also potential sites for Cs⁺ interaction. The ¹³³Cs⁺ NMR parameters of 10 mM CsCl in the presence of unsealed RBC membranes suspended in buffer A (at 3 mg/ml protein concentration) were δ = 0.07 ppm and Δν_{1/2} = 8.3 Hz (*n* = 3). Upon addition of DPG to the same membrane samples, the ¹³³Cs⁺ NMR parameters were δ = 1.01 ppm and Δν_{1/2} = 15.3 Hz (*n* = 3).

Contribution of Magnetic Susceptibility to the Intracellular ¹³³Cs⁺ Shift. ³¹P NMR spectra were recorded for Cs⁺-free RBCs suspended in a Cs⁺-free medium or for Cs⁺-loaded RBCs in a Cs⁺-containing medium (Table II). Both deoxyRBC and

Table III. ¹³³Cs NMR Chemical Shifts and Line Widths for Cs⁺-Loaded RBC Suspensions in the Presence and Absence of 5 mM Dy(TTHA)³⁻^{a,b}

	Cs ⁺ _{in}		Cs ⁺ _{out}	
	with SR	without SR	with SR	without SR
A. Cs⁺-Loaded DeoxyRBCs				
δ/ppm	2.1	2.3	3.8	1.1
Δν _{1/2} /Hz	10	10	4.9	3.9
B. Cs⁺-Loaded CORBCs				
δ/ppm	2.1	1.9	3.7	1.3
Δν _{1/2} /Hz	13	9.5	10	7.1

^aRBCs were loaded with 150 mM CsCl for 3 h. [Cs⁺]_{in} = 2.5 mM. The SR-free samples were suspended in medium A (see Table I). The SR-treated samples were suspended in a medium similar to A, except that 5.0 mM Dy(TTHA)³⁻ replaced 15 mM NaCl. DeoxyRBC and CORBC samples were prepared as described under Materials and Methods. ^bChemical shifts are reported relative to a reference sample of 0.15 M CsCl in D₂O, placed in a spinning 10-mm NMR tube and measured separately. The reported ¹³³Cs⁺ line widths represent the widths of the resonances at half-height after subtraction of 3.0 Hz used in line broadening. The reported NMR parameters represent the average of measurements conducted on at least two separately prepared samples. Errors in chemical shift and line width are less than 0.1 ppm and 1.0 Hz, respectively.

CORBC suspensions were analyzed. As a further test of the effect of a magnetic field gradient across the RBC membrane, we also examined Cs⁺-loaded RBCs suspended in a medium containing 5.0 mM DyTTHA³⁻. Deoxygenation of Cs⁺-free or Cs⁺-loaded RBC suspensions caused broadening and downfield shifts of the ³¹P resonances of intracellular phosphates. In contrast, Cs⁺ loading of CORBC suspensions led to virtually no changes in the line widths or chemical shifts of the ³¹P NMR resonances. Incorporation of 5.0 mM Dy(TTHA)³⁻ in the medium of Cs⁺-loaded RBC suspensions led to upfield shifts (relative to SR-free samples) and broadening of the ³¹P NMR spectra of both deoxyRBC and CORBC suspensions. However, in suspensions containing SR, the spectral changes were larger with CORBC than with deoxyRBC.

The effect of the degree of oxygenation on the ¹³³Cs NMR spectra of the SR-free and SR-containing Cs⁺-loaded RBC suspensions was also examined (Table III). As reported above for ³¹P NMR spectra of RBC suspensions in SR-free media, deoxygenation also caused small downfield shifts of the intracellular ¹³³Cs⁺ NMR resonance of Cs⁺-loaded RBC suspensions in SR-free media. The presence of Dy(TTHA)³⁻ in the suspension medium of deoxyRBCs induced an upfield shift of the intracellular ¹³³Cs⁺ resonance. In contrast, CORBCs gave rise to a broad and downshifted intracellular ¹³³Cs⁺ resonance in the presence of SR. Incorporation of Dy(TTHA)³⁻ into the suspension media of either deoxy or CO Cs⁺-loaded RBCs caused broadening and downshifting of the extracellular ¹³³Cs⁺ resonance, as reported above (Figure 1). In SR-free media, the chemical shift separation between intra- and extracellular ¹³³Cs⁺ NMR resonances from Cs⁺-loaded RBCs was 1.2 and 0.6 ppm, respectively, in deoxyRBC and CORBC suspensions. In SR-treated media, the chemical shift separation was 1.7 and 1.6 ppm, respectively, in deoxyRBC and CORBC suspensions.

For further characterization of the volume and diamagnetic susceptibility contributions of hemoglobin toward the intracellular ¹³³Cs⁺ shift, we examined the effect of hemoglobin concentration on the ¹³³Cs NMR spectra by varying the hematocrit concentration (Figure 3) or osmolarity (Figure 4) in CORBC suspensions or by diluting the CORBC lysate (Figure 5).

We varied the hematocrit concentration, [Ht], by changing the volume ratio of packed RBCs to suspension medium (buffer A). The chemical shifts of intra- and extracellular ¹³³Cs⁺ resonances were measured at different hematocrit values (Figure 3). The ¹³³Cs⁺ chemical shift extrapolated for the suspension medium alone ([Ht] = 0) was 1.7 ± 0.1 ppm upfield from that extrapolated for packed cells ([Ht] = 100%). In CORBC suspensions, as the

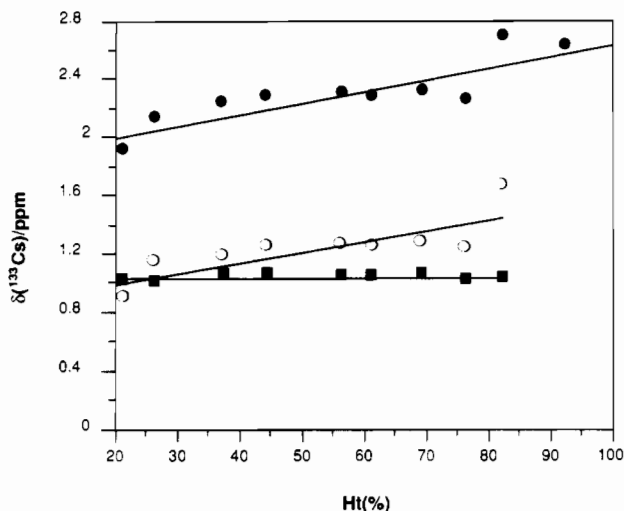


Figure 3. Dependence of intra- and extracellular $^{133}\text{Cs}^+$ NMR chemical shifts on hematocrit concentration for Cs^+ -loaded CORBCs ($n = 2$) suspended in buffer A (see Table I). The symbols \bullet and \circ represent the intra- and extracellular $^{133}\text{Cs}^+$ shifts, respectively, and the symbol \blacksquare denotes the transmembrane chemical shift difference. The shifts were determined relative to a sample of 0.15 M CsCl in D_2O which was measured separately. A 2% correction was applied to the measured hematocrit concentration to account for trapped suspension medium.¹⁹ Errors in chemical shift are less than 0.06 ppm.

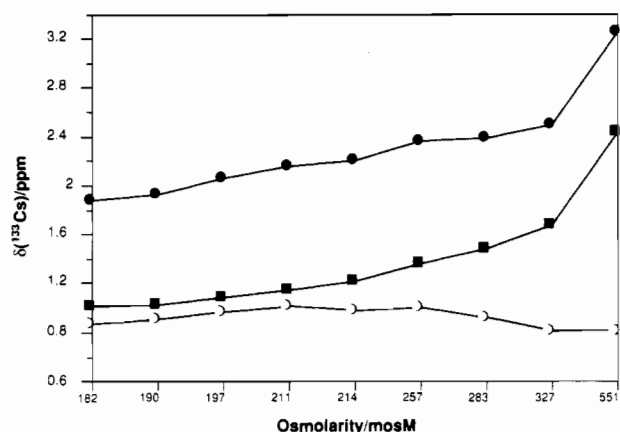


Figure 4. Dependence of intra- and extracellular $^{133}\text{Cs}^+$ NMR chemical shifts on osmolarity for Cs^+ -loaded CORBC suspensions ($n = 2$). The osmotic pressure of the suspension medium was adjusted by addition of 0.3 mL of sucrose (ranging in concentration from 0 to 1000 mM) to 2.0 mL of Cs^+ -loaded CORBCs suspended in 0.6 mL of buffer A. The symbols \bullet and \circ represent the intra- and extracellular $^{133}\text{Cs}^+$ shifts, respectively, and the symbol \blacksquare denotes the transmembrane chemical shift difference. The shifts were determined relative to a sample of 0.15 M CsCl in D_2O which was measured separately. Errors in chemical shift are less than 0.1 ppm.

hematocrit increased, the intracellular $^{133}\text{Cs}^+$ resonance and, to a less extent, the extracellular $^{133}\text{Cs}^+$ resonance were shifted downfield. The chemical shift separation between intra- and extracellular $^{133}\text{Cs}^+$ resonances in Cs^+ -loaded CORBC suspensions increased slightly (by approximately 0.1 ppm) when the hematocrit increased from 25 to 95%. The intracellular Cs^+ concentration varied between 1.2 and 4.8 mM, whereas the extracellular Cs^+ concentration varied between 4.6 and 14 mM, when the Ht increased from 25 to 95%.

The chemical shifts of intra- and extracellular $^{133}\text{Cs}^+$ resonances were measured at different osmolarity values (Figure 4). The osmolarity was increased by addition of increasing amounts of the nonelectrolyte sucrose to the suspension medium. In CORBC suspensions, as the osmolarity increased, the intracellular $^{133}\text{Cs}^+$ resonance was shifted downfield, whereas the extracellular $^{133}\text{Cs}^+$ resonance was shifted slightly upfield. The chemical shift separation between intra- and extracellular $^{133}\text{Cs}^+$ resonances in

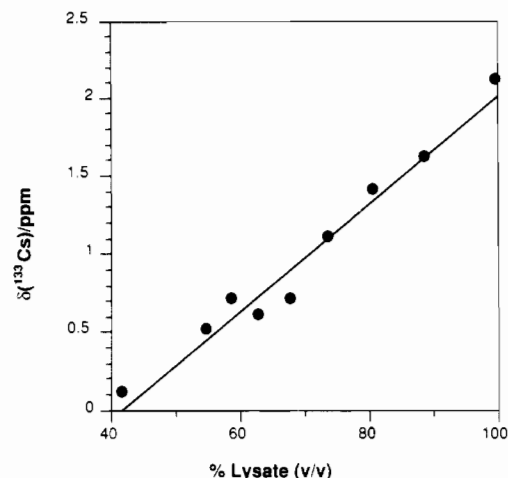


Figure 5. Dependence of the $^{133}\text{Cs}^+$ shift on CORBC lysate concentration ($n = 2$). Concentrated CORBC lysates were prepared from Cs^+ -loaded RBCs, as described under Materials and Methods. The various lysate concentrations were obtained by addition of varying amounts of distilled water to concentrated RBC lysate. The CsCl concentrations ranged from 2.4 mM at a lysate concentration of 100% to 1.0 mM at a lysate concentration of 42%. The lysate concentration is given in units of % (v/v), the percentage ratio of the initial volume of concentrated lysate and the volume of each sample after dilution. The reported $^{133}\text{Cs}^+$ shifts were measured relative to 0.15 M CsCl placed in the bulb of a spherical bulb/cylindrical capillary reference assembly. Errors in chemical shift are less than 0.05 ppm.

Cs^+ -loaded CORBC suspensions underwent an approximately 2-fold increase as the osmolarity increased from 182 to 551 mosM. The intracellular Cs^+ concentration varied between 1.7 and 2.3 mM, whereas the extracellular Cs^+ concentration varied between 4.6 and 7.5 mM, when the osmolarity increased from 182 to 551 mosM.

Contribution of Nonideality of Water to the Intracellular $^{133}\text{Cs}^+$ Shift. Concentrated RBC lysate bubbled with CO gas was diluted with distilled water to yield different COHb concentrations. Under these experimental conditions, the Cs^+/Hb concentration ratio was constant. Figure 5 shows the $^{133}\text{Cs}^+$ chemical shifts in RBC lysate referenced to 0.15 M CsCl placed in the bulb of a spherical bulb/cylindrical capillary reference assembly. Because of the spherical geometry of this reference assembly, the reference and sample solutions experience the same changes in magnetic field strength.^{26,27} An increase in CORBC lysate concentration was accompanied by a 2.1 ppm downfield shift of the $^{133}\text{Cs}^+$ NMR resonance. The smaller downfield shift of the $^{133}\text{Cs}^+$ NMR resonance observed with increasing CORBC lysate concentration did not include the contribution from an increase in the diamagnetic susceptibility of COHb. An increase in CORBC lysate concentration from 42 to 100% resulted in an increase of 7.7 Hz in the line width of the $^{133}\text{Cs}^+$ NMR resonance.

Figure 6 shows the effect of increasing concentrations of COHb and lysozyme on the chemical shift and line width of the $^{133}\text{Cs}^+$ NMR resonance. Both lysozyme and COHb caused downfield $^{133}\text{Cs}^+$ shifts, with the shifts induced by COHb being slightly larger. Whereas an increase in COHb concentration caused an increase from approximately 3.0 to 28 Hz in the line width of the $^{133}\text{Cs}^+$ NMR resonance, a similar increase in lysozyme concentration had a very small effect on the line width. An increase in protein concentration resulted in an increase in the diamagnetic susceptibility of the sample. However, because of the reference method employed, the observed downfield $^{133}\text{Cs}^+$ shifts measured in the presence of increasing COHb or lysozyme concentrations were due to a protein property independent of magnetic susceptibility. We also measured the $^{133}\text{Cs}^+$ NMR parameters in PVP-100 samples which had the same viscosity as did the protein samples (data not shown). In contrast to the case of COHb solutions, an increase in solution viscosity had no significant effect on the line width of the $^{133}\text{Cs}^+$ NMR resonance measured in PVP-100 solutions of varying concentrations. Unlike the case of

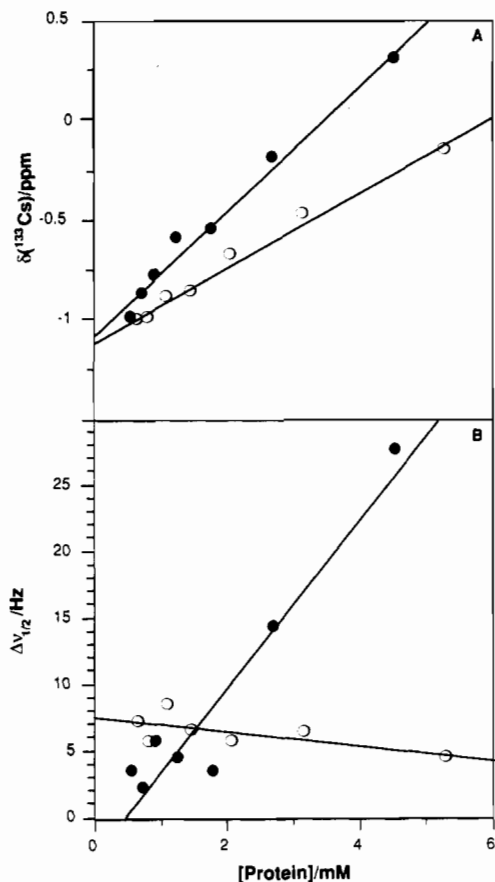


Figure 6. Effect of varying concentrations of (●) COHb and (○) lysozyme on (A) chemical shifts and (B) line widths of ¹³³Cs⁺ NMR resonances (*n* = 2). The initial Cs⁺ concentration was 10 mM. The COHb concentration is expressed in terms of tetramer concentration whereas the concentration of lysozyme is expressed in terms of monomer concentration. The protein samples were diluted by addition of varying amounts of distilled water. The reported chemical shifts were measured relative to 0.15 M CsCl placed in the bulb of a spherical bulb/cylindrical capillary reference tube assembly. Errors in chemical shift and line width are less than 0.05 ppm and 0.5 Hz, respectively.

COHb or lysozyme, an increase in PVP-100 concentration did not lead to a downfield shift of the ¹³³Cs⁺ resonance.

Cs⁺ Binding to DPG. To characterize the interaction of Cs⁺ with DPG, we obtained ³¹P NMR and ¹³C NMR spectra for DPG solutions in the presence and absence of Cs⁺. The chemical shifts and line widths of ³¹P and ¹³C NMR spectra of DPG, in the presence and absence of competing ions at physiologically relevant concentrations, are given in Table IV. ³¹P peak assignments were made as previously reported.^{32,33} ¹³C peak assignments were based on standard chemical shifts for carboxylic, methylene, and methyne carbons. The chemical shifts of the ³¹P and ¹³C NMR resonances of DPG did not change upon addition of Cs⁺ or competing ions. However, the line widths increased upon addition of Cs⁺, but did so to a lesser extent in the presence of competing ions. Using ¹³³Cs NMR spectroscopy (data not shown), we determined the Cs⁺-DPG association constant from plots of δ_{obs}/[Cs⁺] vs [Cs⁺] (in mM) at pH 7.4. The good linear fit (*r*² = 0.90) of the ¹³³Cs⁺ chemical shift data in the presence of DPG is consistent with a stoichiometry of 1:1 for the Cs⁺-DPG complex. From the slope of the linear plot the dissociation constant was found to be 3.8 ± 0.2 mM at 23 °C.

Discussion

It is known that the resonances of monovalent alkali metal ions are shifted in the presence of lanthanide SRs.^{1-3,34} The direction

Table IV. ³¹P and ¹³C NMR Chemical Shifts and Line Widths of DPG in the Presence and Absence of 10 mM Cs⁺^a

	³¹ P NMR ^b		¹³ C NMR ^b		
	3	2	1	2	3
A. Medium A					
without Cs ⁺	5.5 (22)	4.6 (13)	179 (6)	75 (11)	67 (9)
with Cs ⁺	5.5 (38)	4.6 (43)	179 (12)	75 (20)	67 (18)
B. Medium B					
without Cs ⁺	5.6 (18)	4.6 (16)	179 (18)	75 (16)	67 (14)
with Cs ⁺	5.6 (30)	4.6 (29)	179 (24)	75 (29)	67 (15)

^aMedium A consisted of 10 mM CsCl and 5.4 mM DPG. Medium B consisted of 10 mM CsCl, 5.4 mM DPG, 140 mM KCl, 5 mM NaCl, 2.4 mM MgCl₂, and 1 μM CaCl₂. We did not add HEPES and glucose to the solutions in order to simplify the ¹³C NMR spectra. ^bChemical shifts in ppm are followed by line widths in Hz in parentheses. ³¹P chemical shifts are reported relative to a reference sample of phosphoric acid in D₂O, placed in a spinning 10-mm NMR tube and measured separately. ¹³C chemical shifts are reported relative to tetramethylsilane present in the sample. The reported ³¹P and ¹³C line widths represent the widths of the resonances at half-height after subtraction of 20 and 2.0 Hz, respectively, used in line broadening. The reported NMR parameters are an average for three separately prepared samples. Errors in chemical shift and line width are less than 0.5 ppm and 1.0 Hz, respectively.

of the observed shifts for extracellular ¹³³Cs⁺ resonances in the presence of SRs is dependent on the angle of orientation of the Cs⁺ relative to the principal axis of the lanthanide ion. It is clear from Figure 1 that SRs are not needed for separation of the chemical shifts of intra- and extracellular ¹³³Cs⁺ NMR resonances in Cs⁺-loaded RBC suspensions. The positions of the intra- and extracellular ¹³³Cs signals in the presence of SRs (Figure 1B-D) were inverted relative to the positions when no shift reagent was used (Figure 1A). The Cs⁺-shifting properties of Dy(PPP)₂⁷⁻ are unique in that, for all other alkali metal ions, Dy(PPP)₂⁷⁻ shifts the extracellular resonance in the upfield direction.^{4,16} However, Dy(TTHA)³⁻ and Tm(DOTP)⁵⁻ shift the extracellular Cs⁺ resonance downfield, as for the other alkali metal cations.^{4,16,28} Moreover, the magnitude of the ¹³³Cs⁺ shift induced by Dy(PPP)₂⁷⁻ was considerably smaller than those caused by Dy(TTHA)³⁻ and Tm(DOTP)⁵⁻, at the same SR concentrations. In contrast, for other alkali and alkaline earth metal ions, the magnitude of the shifts induced by Dy(PPP)₂⁷⁻ is considerably larger than those caused by Dy(TTHA)³⁻, but only slightly larger than those induced by Tm(DOTP)⁵⁻, at the same SR concentrations.^{4,16,28} The very large ¹³³Cs⁺ shifts induced by Tm(DOTP)⁵⁻ are presumably associated with the large size of the Cs⁺ cation and its binding via four phosphonate oxygens close to the Tm³⁺ ion and near the cone axis.

The downfield ¹³³Cs⁺ shift induced by Dy(PPP)₂⁷⁻ is probably related to the large ionic radius of the Cs⁺ cation and its location in the equatorial region formed by the dipolar cone angle around the effective magnetic axis of the SR. Addition of Na⁺ to a Cs⁺/SR solution removed the Cs⁺ ions from the Na⁺ preferred sites and caused a small increase in the downfield ¹³³Cs⁺ shift (Figure 2B). However, addition of Cs⁺ to a Na⁺/SR solution caused a relatively large upfield ²³Na⁺ shift (Figure 2C), presumably because Cs⁺ binds to both sides of the cone, with only some weak preference for the equatorial side. Thus, the Na⁺/Cs⁺ competition experiments indicate that Na⁺ and Cs⁺ have distinct binding sites on Dy(PPP)₂⁷⁻, with Na⁺ clearly preferring the axial portion outside the dipolar cone and binding strongly to the SR and with Cs⁺ binding weakly to the equatorial portion inside the cone. The specific effect of Dy(PPP)₂⁷⁻ on the Cs⁺ distribution ratio is presumably due to the higher charge of this SR, because it was not so noticeable in the Tm(DOTP)⁵⁻ or Dy(TTHA)³⁻-containing RBC suspensions. This SR-specific effect on the Cs⁺ distribution across the RBC membrane is in agreement with the higher and lower transmembrane Li⁺ and Na⁺ ratios, respectively,

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previously reported for Li⁺-loaded RBC suspensions containing Dy(PPP)₂.⁷⁻⁸

We investigated the contribution of each intracellular component (Table I) and of the RBC membrane to the observed chemical shift of the intracellular ¹³³Cs NMR resonance in Cs⁺-loaded CORBC suspensions. At concentrations close to physiologic values for ATP, ADP, DPG, and P_i, DPG gave the largest downfield ¹³³Cs⁺ shift of all the intracellular phosphates tested. We also measured the ¹³³Cs⁺ shifts for samples containing the same concentration (2.0 mM) of each intracellular phosphate and found that the interaction of Cs⁺ with DPG was not due to an elevated DPG concentration relative to that of other intracellular phosphates in RBC. We also found evidence for a Cs⁺-DPG interaction in the presence of competing cations and in samples whose viscosity was adjusted to that of the intracellular RBC compartment (Table I). In the absence of DPG, preparations of unsealed RBC membranes gave no significant ¹³³Cs⁺ shifts or line broadening. We conclude that the RBC membrane is not responsible for the downfield shift of the intracellular ¹³³Cs⁺ resonance observed in Cs⁺-loaded RBC suspensions. The chemical shift of the ¹³³Cs⁺ resonance is known to be sensitive to the nature of the counteranion present in solution.¹³ The anion present at the highest concentration in RBCs is Cl⁻. However, the Cl⁻ concentrations inside and outside RBCs are approximately the same and unlikely to be responsible for the observed resolution of ¹³³Cs NMR resonances in Cs⁺-loaded RBC suspensions (Figure 1A). In contrast, biological phosphates are present only in the intracellular compartment. We therefore conclude that Cs⁺ binding to intracellular phosphates, and to DPG in particular, contributes strongly to the appearance of two ¹³³Cs resonances in Cs⁺-loaded RBC suspensions.

Magnetic susceptibility is known to be an important, albeit small, contributor to the observed chemical shifts of NMR resonances in biological samples. The effects of magnetic susceptibility on chemical shifts have sometimes been the cause of misinterpretation of data.^{21,22,25,27,35-40} Cell suspensions consist of two compartments, intra- and extracellular. If the intracellular compartment is spherical, the nuclei inside and outside the cells experience the same magnetic field, and thus there is no chemical shift difference between intra- and extracellular NMR resonances. For any nonsymmetric geometry, a chemical shift difference may be observed. The bulk magnetic susceptibility (BMS) contribution to the chemical shift depends on the volume susceptibility of the medium inside and outside the cells, the cell morphology, and the relative orientation of the sample with respect to the externally applied magnetic field.^{3,25} When NMR experiments are carried out with superconducting magnets, as in the case of this study, paramagnetic substances have a positive susceptibility, which generates a downfield shift, and diamagnetic substances have a negative susceptibility, which generates an upfield shift.^{21,22,25} Because the direction and magnitude of the BMS shifts are dependent on the alignment of the magnetic moments of the individual nuclei with the applied magnetic field, the BMS shifts are of opposite sign in superconducting magnets and in electromagnets.^{21,22,25} The effects of BMS can be changed in a sample by incorporation of a paramagnetic substance, such as a shift reagent, into the suspension medium.^{21,22,25}

In its deoxygenated form, Hb is paramagnetic,³⁵⁻³⁷ inducing a magnetic susceptibility difference between the intra- and extracellular compartments in deoxyRBC suspensions. The magnetic field gradient across the RBC membrane effects both broadening and downfield shifts of the ³¹P resonances.^{21,22} Upon incorporation of a SR into the suspension medium, the magnetic field gradient is decreased, and consequently the line widths sharpen and the

chemical shifts move upfield.^{21,22} In contrast to deoxyHb, COHb is diamagnetic.³⁵⁻³⁷ When a SR is incorporated into a CORBC suspension, a significant magnetic field gradient is generated across the cell membrane, causing broadening and downshifting of the ³¹P NMR resonances. We examined the magnetic susceptibility contribution of hemoglobin to the intracellular ³¹P and ¹³³Cs⁺ shifts by comparing ³¹P and ¹³³Cs NMR spectra of deoxyRBC and CORBC suspensions. Whereas deoxyRBC suspensions gave broad and downfield-shifted ³¹P resonances for DPG and ATP, the corresponding resonances were sharp and were shifted upfield in CORBC suspensions (Table II). The presence of 5.0 mM Dy(TTHA)³⁻ in the medium partially inverts the magnetic field gradient across the RBC membrane in suspensions of Cs⁺-loaded deoxyRBCs because the two compartments now feel a similar magnetic susceptibility. In contrast, incorporation of 5.0 mM Dy(TTHA)³⁻ into the medium of Cs⁺-loaded CORBCs leads to a small enhancement of the magnetic field gradient across the RBC membrane. Thus, magnetic susceptibility differences between the intra- and extracellular compartments are responsible for the chemical shift and line width changes observed in the ³¹P NMR resonances of Cs⁺-free and Cs⁺-loaded RBC samples, as previously reported.^{21,22}

Just as for ³¹P NMR resonances of intracellular phosphates, chemical shift and line width changes occurred for the intra- and extracellular ¹³³Cs⁺ NMR resonances upon deoxygenation or upon incorporation of 5.0 mM Dy(TTHA)³⁻ into the medium (Table III). However, the changes in the ¹³³Cs⁺ NMR parameters were smaller than those observed for ³¹P NMR resonances of intracellular phosphates. The large effects of the SR on the chemical shift and line width of the extracellular ¹³³Cs⁺ resonance are associated with the paramagnetism of the SR and are not due to magnetic susceptibility properties of the SR-treated samples. Unless a significant inhomogeneity component is present, magnetic susceptibility affects the resonance frequency of all nuclei equally. Thus, for a RBC suspension placed in a superconducting magnet, the magnetic susceptibility contributions to the ¹³³Cs⁺ and ³¹P shifts should be approximately the same. However, because inhomogeneity effects are present in blood samples,⁴¹ there may also be a contribution of the gyromagnetic ratios of ¹³³Cs and ³¹P to the observed BMS shift.²⁵ At a constant magnetic field, nuclei with a lower resonance frequency show weaker line-broadening effects.²¹ It is therefore not surprising that shifting and broadening effects were less pronounced in ¹³³Cs than in ³¹P resonances. The chemical shift separation between intra- and extracellular ¹³³Cs⁺ NMR resonances changed by not more than 0.6 ppm upon deoxygenation of Cs⁺-loaded RBCs in the presence or absence of a SR. Even when diamagnetic or paramagnetic susceptibility differences are present across the RBC membrane, as confirmed by ³¹P NMR spectroscopy,^{21,22} a clear separation between intra- and extracellular ¹³³Cs⁺ NMR resonances is observed in Cs⁺-loaded RBC suspensions. If magnetic susceptibility effects alone were responsible for the resolution of ¹³³Cs⁺ NMR resonances in Cs⁺-loaded CORBC suspensions, one would predict that intracellular ¹³³Cs⁺ NMR resonances would be shifted upfield from extracellular ¹³³Cs⁺ NMR resonances when recording the spectrum using a superconducting magnet.^{21,22} Because the relative positions of the ¹³³Cs⁺ NMR resonances observed in Cs⁺-loaded RBC suspensions are the opposite of those predicted, we conclude that magnetic susceptibility effects are not responsible for the resolution of ¹³³Cs⁺ NMR resonances in human RBC suspensions.

In suspension medium alone or in packed cells, only one ¹³³Cs⁺ resonance was observed. However, the chemical shifts of the ¹³³Cs⁺ resonances observed either in suspension medium alone ($\delta_{\text{out}} = 1.0$ ppm; [Ht] = 0) or in packed cells ($\delta_{\text{in}} = 2.6$ ppm; [Ht] = 95%) differed significantly from those observed in Cs⁺-loaded RBC suspensions of intermediate hematocrit concentrations (Figure 3). Dependence of ³¹P chemical shifts on hematocrit concentration was also observed for triethyl phosphate, dimethyl methylene-

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phosphonate, and hypophosphite in oxygenated RBC suspensions.³⁸ As the [Ht] increases, the diamagnetic susceptibility of the sample increases as a result of an increase in COHb concentration. An increase in diamagnetic susceptibility with increasing [Ht] is associated with a decrease in BMS and, with a superconducting magnet, is expected to generate upfield shifts. However, downfield (as opposed to upfield) $^{133}\text{Cs}^+$ shifts of intra- and extracellular resonances were observed with increasing [Ht] (Figure 3). Clearly, an effect independent of magnetic susceptibility is responsible for the downfield intracellular $^{133}\text{Cs}^+$ shifts observed in Cs^+ -loaded RBC suspensions. As the [Ht] varied, the intra- and extracellular Cs^+ concentrations also varied, presumably because Cs^+ transport occurred for Cs^+ -loaded RBCs suspended at different hematocrit concentrations.

Theoretical calculations based on concentric cylindrical compartments with independent magnetic susceptibilities have predicted that the diamagnetic susceptibility of COHb should result in intracellular resonances that are 0.12 ppm upfield from extracellular resonances.^{21,22} However, a recent calculation³⁸ that took into account the interdependence of the magnetic susceptibilities of the two cellular compartments has shown that the contribution of magnetic susceptibility effects to the chemical shift separation between intra- and extracellular resonances in RBC suspensions is dependent on [Ht] and is less (0.06 ppm, [Ht] = 100%; 0.08 ppm, [Ht] = 0) than that previously predicted (0.12 ppm). The observed chemical shift separation between intra- and extracellular $^{133}\text{Cs}^+$ resonances in Cs^+ -loaded RBC suspensions was weakly dependent on Ht, as expected (0.80 ppm, [Ht] = 100%; 1.20 ppm, [Ht] = 0). However, the observed transmembrane chemical shift differences observed with $^{133}\text{Cs}^+$ resonances were much larger than those predicted. We conclude that an effect independent of magnetic susceptibility is responsible for the clear resolution and the relative positions of the $^{133}\text{Cs}^+$ NMR resonances in Cs^+ -loaded CORBC suspensions of intermediate hematocrit concentrations.

An increase in osmolarity of the suspension medium leads to an efflux of intracellular water and a decrease in mean cell volume.³⁹ The resulting increase in COHb concentration gives rise to an increase in the diamagnetic susceptibility of the sample with increasing osmolarity, which is associated with a decrease in BMS and, with a superconducting magnet, is expected to lead to upfield shifts. However, a downfield (as opposed to upfield) $^{133}\text{Cs}^+$ shift, due to a magnetic susceptibility independent effect, of the intracellular resonances was observed with increasing osmolarity (Figure 4). At low osmolarity values, partial cell lysis and a decrease in ionic strength may account in part for the magnitude of the $^{133}\text{Cs}^+$ shifts. The small upfield $^{133}\text{Cs}^+$ shift of the extracellular resonance with increasing osmolarity is presumably due to an increase in the BMS of the extracellular compartment, as previously reported for ^{31}P resonances of phosphoryl compounds in RBC suspensions.³⁹ The chemical shift separation between intra- and extracellular $^{133}\text{Cs}^+$ resonances increased with osmolarity in Cs^+ -loaded RBC suspensions. Similar increases in ^{31}P and ^{19}F NMR resonances were reported for phosphoryl compounds and difluorophosphate in RBC suspensions.^{27,39} As the mean cell volume decreases with increasing osmolarity of the suspension medium, the intracellular concentrations of both biological phosphates and Cs^+ are increased. We conclude that volume susceptibility is not responsible for the resolution and relative positions of the intra- and extracellular $^{133}\text{Cs}^+$ resonances originating from Cs^+ -loaded CORBCs that are suspended in media of varying osmolarity.

The high concentration of COHb present at high CORBC lysate concentration should lead to an increase in the diamagnetic susceptibility of the sample. An increase in diamagnetic susceptibility with increasing RBC lysate concentration is associated with a decrease in BMS and, with a superconducting magnet, is expected to lead to small upfield shifts (0.12 ppm).^{22,38} Such small upfield ^{31}P shifts were, in fact, observed for triethyl phosphate.³⁸ Downfield $^{133}\text{Cs}^+$ shifts were observed with increasing CORBC lysate concentration (Figure 5). The $^{133}\text{Cs}^+$ shifts measured in RBC lysate samples relative to 0.15 M CsCl placed in a spherical

bulb are independent of changes in magnetic susceptibility of the RBC lysate sample.^{26,27,39} We conclude that a magnetic susceptibility independent effect is responsible for the downfield $^{133}\text{Cs}^+$ shifts observed in CORBC lysate samples. Precedents for magnetic susceptibility independent contributions to ^{31}P and ^{19}F chemical shifts in CORBC lysate samples have also been reported for phosphoryl compounds and difluorophosphate.^{27,38-40}

To determine the contribution of the nonideality of water to the intracellular $^{133}\text{Cs}^+$ shift, we measured $^{133}\text{Cs}^+$ chemical shifts and line widths in the presence of increasing concentrations of COHb and lysozyme (Figure 6). To eliminate the diamagnetic susceptibility contribution in the protein samples, we measured the chemical shifts relative to CsCl placed in a spherical bulb/cylindrical capillary reference assembly. The finding that increasing concentrations of two unrelated proteins (COHb and lysozyme) caused downfield $^{133}\text{Cs}^+$ shifts suggests that the observed intracellular $^{133}\text{Cs}^+$ shifts in Cs^+ -loaded RBCs are controlled by a general property of proteins and not by a specific property of Hb. Similar observations were reported for ^{31}P chemical shifts of phosphoryl compounds in the presence of either COHb or lysozyme.⁴⁰ The general property of proteins responsible for the $^{133}\text{Cs}^+$ shifts is unlikely to be viscosity because the $^{133}\text{Cs}^+$ chemical shift changes measured in PVP-100 samples were insignificant relative to those measured in the presence of samples of COHb or lysozyme with the same viscosity. The line widths of the $^{133}\text{Cs}^+$ resonances in the presence of lysozyme or PVP-100 were essentially independent of concentration or viscosity. In contrast, the line widths of the $^{133}\text{Cs}^+$ NMR resonances increased with increasing concentrations of COHb or CORBC lysate, indicating that weak Cs^+ binding to COHb is present. The slightly larger downfield $^{133}\text{Cs}^+$ shifts induced by COHb relative to lysozyme are also consistent with weak Cs^+ binding to COHb. The isoelectric point of hemoglobin is higher than that of lysozyme. At the neutral pH used in our experiments, COHb has a higher overall anionic charge than lysozyme. Nonspecific weak Cs^+ binding is therefore expected to be more important for COHb than for lysozyme. Because both lysozyme and COHb gave downfield shifts, but different line width changes, of the $^{133}\text{Cs}^+$ NMR resonances, we conclude that the weak interaction between Cs^+ and COHb is not solely responsible for the observed downfield shifts of intracellular $^{133}\text{Cs}^+$ resonances in Cs^+ -loaded CORBC suspensions.

In the extracellular compartment, the Cs^+ ion is coordinated to water ligands. In the intracellular compartment, however, hemoglobin is surrounded by ordered water of hydration. Thus, hemoglobin disrupts the hydrogen-bonding network inside RBCs, leading to nonideality of intracellular water. The number of water ligands available for coordination to intracellular Cs^+ is smaller than that available for coordination to extracellular Cs^+ . A decrease in electron shielding of intracellular Cs^+ should result in a downfield shift of the intracellular $^{133}\text{Cs}^+$ resonance, as observed. The downfield shifts of intracellular $^{133}\text{Cs}^+$ NMR resonances resemble the downfield shifts of ^{19}F NMR resonances of intracellular trifluoroacetate and difluorophosphate in RBC suspensions.^{27,42} In contrast, the downfield shifts of intracellular $^{133}\text{Cs}^+$ are in the opposite direction of the upfield shifts of ^{31}P NMR resonances observed for intracellular phosphoryl compounds and difluorophosphate.^{27,39,40} Water coordinates to Cs^+ via oxygen atoms whereas it coordinates to F via hydrogen atoms. In both cases hydrogen bonding results, however, in deshielding of the $^{133}\text{Cs}^+$ and ^{19}F nuclei. The dependence of the chemical shift of intracellular $^{133}\text{Cs}^+$ on the nonideality of water is consistent with the strong dependence of ^{133}Cs chemical shifts on the nature of the solvent.¹³

Therefore, we conclude that the chemical shift separation of $^{133}\text{Cs}^+$ NMR resonances in Cs^+ -loaded RBC suspensions stems from binding of Cs^+ to intracellular phosphates (in particular DPG) and from the nonideality of intracellular water induced by hemoglobin.

The increase in the line widths of all ^{13}C and ^{31}P NMR resonances of DPG upon addition of Cs^+ is consistent with Cs^+ en-

hancement of the spin-spin relaxation times of the three ^{13}C and two ^{31}P resonances of DPG (Table IV). Therefore, the interaction of the Cs^+ ion with DPG most likely involves the carboxylic group on carbon 1 and each of the phosphate groups on carbons 2 and 3 of DPG. The chemical shifts of the ^{13}C and ^{31}P NMR resonances of DPG did not change upon addition of Cs^+ , presumably because the interaction with the carboxylate and phosphate groups of DPG occurs via the oxygen atoms and not directly with the reporter nuclei. From our ^{31}P and ^{133}Cs NMR results (Tables I and IV), we conclude that Cs^+ interacts more strongly with DPG than with ADP or ATP. Cs^+ forms a 1:1 complex with DPG with a K_D of 3.8 ± 0.2 mM. This Cs^+ interaction is unique because DPG is reported to bind Mg^{2+} , Zn^{2+} , and Al^{3+} more weakly than does either ADP or ATP, despite the presence of two basic phosphates.^{32,43,44} The stronger interaction of Cs^+ , relative to Na^+ ,⁴⁵ with DPG may be related to the larger ionic size of Cs^+ and to the capacity of DPG to act as a tridentate ligand. We conclude from our ^{31}P and ^{13}C NMR data (a) that Cs^+ interacts with the phosphate and carboxylate groups of DPG and (b) that the competing ions decrease the observed Cs^+ interaction with DPG.

Comparison of ^{31}P NMR spectra of Cs^+ -loaded and Cs^+ -free RBC suspensions (Table II) indicated that Cs^+ loading broadened and moved the ^{31}P resonances of DPG (and to a smaller extent the resonances of ATP and P_i) downfield in deoxyRBC but not in CORBC suspensions. Deoxygenation did not cause any sig-

nificant changes in the peak areas of the ^{31}P resonances of DPG in Cs^+ -loaded or Cs^+ -free RBC suspensions (data not shown). This lack of change in DPG levels upon deoxygenation is in agreement with previous reports.^{30a,46} DPG binds more strongly to deoxyhemoglobin than to oxyhemoglobin (or its analogue, COHb).^{30a,33} Moreover, more Mg^{2+} is bound to DPG in CORBC than in deoxyRBC suspensions.^{30a} Although less free DPG is available in deoxygenated RBC suspensions to bind Cs^+ , DPG is not complexed as fully to Mg^{2+} .^{30a} Because the affinity of DPG for Mg^{2+} is higher than that for Cs^+ ,³² binding of Cs^+ to free intracellular DPG occurs most strongly in deoxyRBC suspensions. This may explain why the effect of Cs^+ loading on ^{31}P resonances of DPG is more noticeable in deoxyRBC than in CORBC suspensions (Table II). Competition between Cs^+ and hemoglobin for DPG will have the result that smaller amounts of DPG are available to bind to deoxyHb, which, in turn, may result in an increase in the oxygen affinity of hemoglobin. The enhanced oxygen affinity of hemoglobin in the presence of Cs^+ may hinder the release of oxygen to tissues and may provide a mechanism for Cs^+ toxicity.

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Contribution from the Departments of Chemistry, Texas Christian University, Fort Worth, Texas 76129, and The University of Newcastle-upon-Tyne, NE1 7RU, U.K.

Determination of the Molar Extinction Coefficients of the Deuteroferriheme Analogues of Peroxidase Enzyme Compounds I and II

Fanny S. Woo,^{1a,b} Ricardo E. Rodriguez,^{1a,c} Dale A. Huckaby,^{1a} Peter Jones,^{1d} and Henry C. Kelly*,^{1a}

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Absorbance data obtained in the stopped-flow spectrophotometric study of the "in situ" biphasic regeneration of deuteroferriheme (dfh) following its oxidation by NaOCl are utilized to calculate extinction coefficients of species presumed to be involved in series pseudo-first-order redox regeneration processes. Such data are interpreted in terms of a previously proposed mechanism of the type $\text{A} \xrightarrow{k_1} \text{B} \xrightarrow{k_2} \text{C}$ where A denotes the dfh- OCl^- oxidation product; B, a reaction intermediate; and C, the regenerated dfh. Results are consistent with a scheme involving consecutive one-electron reductions of the heme analogues of peroxidase enzyme compounds I and II to regenerate free dfh in a state of monomer-dimer equilibrium. A value $\epsilon \approx 4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 384 \text{ nm}$ is computed at pH 6.85 and 25 °C for the molar extinction coefficient of the compound II analogue (B), which has been depicted as dinuclear $\text{Fe}^{\text{III}}\text{OFe}^{\text{IV}}$. This is intermediate in magnitude between the value $\epsilon \approx 2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ calculated for the dfh-derived compound I analogue (A), depicted as $\text{Fe}^{\text{IV}}_2\text{O}$, and previously calculated values of extinction coefficients for monomeric and dimeric dfh. Such ϵ values are consistent with observed changes in optical density accompanying heme regeneration and with kinetic studies that show $k_1 > k_2$ by about an order of magnitude.

Introduction

A number of studies of the chemistry of iron(III)-porphyrin (heme) models of peroxidase enzyme systems have been focused on the stoichiometry and rates of formation and the subsequent reactivity of oxidized heme species which are functional analogues of enzyme-derived reaction intermediates.²⁻¹⁶ Although such

investigations are complicated by the tendency of the protein-free hemes to undergo dimerization in aqueous solution with consequent

- (1) (a) Texas Christian University. (b) Current address: Rhone Poulenc Co., Houston, TX. (c) Current address: Department of Chemistry, Texas Wesleyan University, Fort Worth, TX 76105. (d) The University of Newcastle-upon-Tyne.
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