Li^+ T_1 relaxation times presumably can be used as probes of Li^+ binding to the RBC membrane and for determining whether the extent and site of Li^+ binding are different in RBCs from bipolar, hypertensive, and normotensive controls. Thus, the MIR approach (because of the its total noninvasiveness, easy visualization of Li^+ pools, and ability to probe interactions between the Li^+ ion and RBC components) will be the method of choice to investigate whether Li^+ transport and distribution parameters in RBCs can be used with confidence as genetic markers of bipolar disorders[36] and hypertension.[37]

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Registry No. $Dy(PPP)_2^{7-}$, 81868-53-3; $Dy(TTHA)^{3-}$, 91264-39-0; Li, 7439-93-2; Na, 7440-23-5.

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Effects of Negatively Charged Shift Reagents on Red Blood Cell Morphology, Li^+ Transport, and Membrane Potential

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Lanthanide shift reagents have been used extensively in multinuclear magnetic resonance (NMR) applications in order to obtain information regarding ion distribution and transport in cellular systems. The aqueous reagents used in this study were $Dy(PPP)_2^{7-}$, $Tm(PPP)_2^{7-}$, $Dy(TTHA)^{3-}$, $Dy(PcPcP)_2^{7-}$, and $Dy(DOTP)^{5-}$, where Dy^{3+} and Tm^{3+} represent dysprosium and thulium ions and PPP^{5-}, $TTHA^{6-}$, $PcPcP^{5-}$, and $DOTP^{8-}$ denote the triphosphate, triethylenetetraminehexaacetate, bis(dihydroxyphosphinylmethyl)phosphinate, and 1,4,7,10-tetrazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetrakis(methanephosphonate) ligands, respectively. The apparent size and shape of Li^+-free RBCs (red blood cells), studied by both scanning electron microscopy and Coulter counter methods, were unchanged by the presence of the above shift reagents at concentrations lower than 10 mM. However, Li^+ incubation changed both the shape and size of RBCs. The rates of Na^+-Li^+ exchange in Li^+-loaded RBCs measured by ^7Li NMR spectroscopy in the presence of $Dy(PPP)_2^{7-}$, $Tm(PPP)_2^{7-}$, or $Dy(PcPcP)_2^{7-}$ were significantly higher than the rates measured in the absence of shift reagents by atomic absorption or in the presence of $Dy(TTHA)^{3-}$ or $Dy(DOTP)^{5-}$ by ^7Li NMR spectroscopy. ^{31}P and ^{19}F NMR measurements of the membrane potential of Li^+-free RBCs revealed that the shift reagents studied (except for $Dy(TTHA)^{3-}$) do change the membrane potential, with the most negatively charged reagents having the largest effect. Thus, shift reagents must be used with caution in physiological NMR studies and in particular RBC applications.

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Introduction

Cells undergo a variety of shape changes at different stages in a cell cycle or in the process of cell maturation and differentiation. In the absence of hydrodynamic forces, the red blood cell (RBC) shape normally observed is that of a biconcave disc (hence the name discocyte).[1,2] Several references in the literature suggest that the energy-dependent spectrin–actin network may play a role in maintaining the shape of RBCs.[3-6] Alterations in spectrin phosphorylation by ATP depletion have been shown to be associated with crenation (shrinkage) in RBCs.[1,2,7] Other studies have shown that factors such as pH, ionic strength, and several drugs cause alterations of the discocyte shape.[8-12] Thus, it is apparent that RBC shape may be controlled by both energy-requiring processes and physicochemical interactions.

Several paramagnetic lanthanide complexes have been applied as shift reagents for NMR-detectable alkali-metal cations.[13-17] These reagents have become popular in recent years for distinguishing intra- and extracellular ions, in particular Li^+, Na^+, and K^+. In order to test the suitability of shift reagents for clinical and biological research, we have examined the effects of the negative charge on some of the most widely used shift reagents on RBC morphology, membrane potential, and Li^+ transport rates. The structure of the ligands used in this study are shown in Figure 1. The ligands were selected such that the shift reagents used in this study had overall charges ranging from −3 to −7. The application of the chosen shift reagents to $^7Li^+$ and $^{23}Na^+$ NMR

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Table I. Composition of Suspension Media Used in This Study[a]

medium ref	composition
A	150 mM NaCl, 5 mM Na_2HPO_4, pH 7.4
B	150 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 10 mM TrisCl, pH 7.5
C	112.5 mM choline chloride, 10 mM glucose, 85 mM sucrose, 0.1 mM ouabain, 10 mM TrisCl, pH 7.5
D	129 mM (or 120 mM) NaCl, 10 mM glucose, 0.1 mM ouabain, 7 mM (or 10 mM) $Na_3Dy(TTHA)$, 10 mM TrisCl, pH 7.5
E	91.5 mM choline chloride, 10 mM glucose, 0.1 mM ouabain, 7 mM $(Me_4N)_3Dy(TTHA)$, 10 mM TrisCl, pH 7.5
F	125 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 5 mM $Na_5Dy(DOTP)$, 10 mM TrisCl, pH 7.5
G	87.5 mM choline chloride, 10 mmM glucose, 0.1 mM ouabain, 5 mM $(Me_4N)_5Dy(DOTP)$, 10 mM TrisCl, pH 7.5
H	120 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 3 mM $Na_7Dy(PPP)_2$·3NaCl, 10 mM TrisCl, pH 7.5
I	82.5 mM choline chloride, 10 mM glucose, 0.1 mM ouabain, 3 mM $(Me_4N)_7Dy(PPP)_2$·3NaCl, 10 mM TrisCl, pH 7.5
J	120 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 3 mM $Na_7Dy(PcPcP)_2$·3NaCl, 10 mM TrisCl, pH 7.5
K	82.5 mM choline chloride, 10 mM glucose, 0.1 mM ouabain, 3 mM $(Me_4N)_7Dy(PcPcP)_2$·3NaCl, 10 mM TrisCl, pH 7.5
L	100 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 5 mM $Na_7Tm(PPP)_2$·3NaCl, 10 mM TrisCl, pH 7.5

[a] The osmolarity of the media was adjusted with sucrose to 300 ± 10 mosM whenever necessary.

transport studies in cellular systems had been previously reported,[13-17] except for $Dy(PcPcP)_2^{7-}$. To the best of our knowledge, only the Gd^{3+} analogue of the phosphonate shift reagent has been reported as a contrast agent for imaging studies.[18] The effect of shift reagents on the RBC membrane potential was investigated by using published ^{31}P and ^{19}F NMR methods.[19,20] The effect of shift reagent on membrane potential is of paramount importance to establish the clinical applicability of shift reagents. The effect of shift reagents on the measurement of Li^+ transport rates in RBCs is particularly important because of the reported abnormalities in Li^+ absorption in RBCs from hypertensive[21] and bipolar patients.[22] Lithium is of no therapeutic value in the treatment of hypertension. However, lithium salts are the preferred drugs in the treatment and maintenance of both manic and depressive episodes of bipolar disorders.[23] On the basis of studies of Li^+ transport in RBCs of hypertensive and bipolar patients, a membrane abnormality hypothesis has evolved.[22,24] Before application of ^7Li NMR spectroscopy to the investigation of abnormal Li^+ binding and transport in RBCs, the specific effects of shift reagents on Li^+-loaded RBCs must be evaluated. This study also shows that the charge effects reported in the accompanying paper are not specific for $Dy(PPP)_2^{7-}$, but rather they are general effects inherent to highly negatively charged shift reagents.

Materials and Methods

Chemicals. Lithium chloride, dysprosium(III) chloride, thulium(III) chloride, glucose, sodium triphosphate, sodium cacodylate, HEPES, paraformaldehyde, triethylenetetraminehexaacetic acid (H_6TTHA), sodium trifluoroacetate, trifluoroacetamide, hypophosphorous acid, and D_2O (99.8%) were supplied by Aldrich Chemical Co. Ouabain, a plant steroid that inhibits the sodium pump, was from Sigma. Glutaraldehyde and osmium tetroxide were from Ladd Research Industries. 1,4,7,10-Tetrazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetrakis(methanephosphonic acid) (H_8DOTP) was prepared according to published procedures.[17] Bis(dihydroxyphosphinylmethyl)phosphinic acid (H_5PcPcP) was synthesized by Medichem Research Inc., Chicago, IL. $Dy(PPP)_2^{7-}$, $Tm(PPP)_2^{7-}$, $Dy(TTHA)^{3-}$, and $Dy(PcPcP)_2^{7-}$ were prepared according to the procedure of Chu et al.,[13] while $Dy(DOTP)^{5-}$ was made according to Sherry et al.[17]

RBC Suspensions and Li^+ Incubation. Packed RBCs of healthy donors were supplied by the Chicago Chapter of Life Source Blood Bank. The compositions of all suspension media used in this study are described in Table I. RBCs were washed three times by centrifugation at 2000*g* for 10 min with medium A at 4 °C. Washed RBCs were separated from the plasma and buffy coat by aspiration and then stored at 4 °C before use. RBCs were incubated with different levels of Li^+ according to a modification of the procedure of Zimmermann and Soumpasis.[25] RBCs at 5% hematocrit were suspended in an isotonic medium containing varying concentrations of LiCl (1.5–140 mM), 10 mM glucose, and 20 mM TrisCl at pH 7.4 and incubated at 37 °C for 1 h. The Li^+-loaded cells were then washed twice in medium A and resuspended in an isotonic solution containing varying concentrations of LiCl as indicated above. NaCl concentrations in the suspension media were adjusted so that the osmolarity was maintained at 300 ± 5 mosM. The osmolarity of all cell suspensions was measured with a Wescor vapor-pressure osmometer. For experiments involving lanthanide shift reagent (LSR) treated cells, RBCs were suspended at 13% hematocrit in a solution containing 5 mM KCl, 10 mM glucose, 10 mM HEPES buffer at pH 7.5, varying concentrations of LiCl and NaCl, and the desired LSR. The concentrations of the LSRs used in this study were 3, 5, or 15 mM $Dy(PPP)_2^{7-}$, 5 or 7 mM $Tm(PPP)_2^{7-}$, 10 or 20 mM $Dy(TTHA)^{3-}$, 3 mM $Dy(PcPcP)_2^{7-}$, and 5 mM

Table II. Comparison of Average RBC Size Obtained for Li^+-Free RBCs Treated with Various Lanthanide Shift Reagents with the Diameters and Volumes Obtained by Using the SEM and CC Instruments, Respectively[a,b]

sample	*n*	av diam/μm SEM	av vol/fL CC
RBC controls	636	7.56 ± 0.14	82 ± 4
RBCs treated with 3 mM $Dy(PPP)_2^{7-}$	892	7.64 ± 0.21	88 ± 2
RBCs treated with 5 mM $Tm(PPP)_2^{7-}$	720	7.61 ± 0.18	86 ± 6
RBCs treated with 3 mM $Dy(PcPcP)_2^{7-}$	667	7.68 ± 0.12	88 ± 6
RBCs treated with 10 mM $Dy(TTHA)^{3-}$	718	7.55 ± 0.16	82 ± 6
RBCs treated with 5 mM $Dy(DOTP)^{5-}$	694	7.59 ± 0.12	85 ± 4

[a] Values are expressed as mean ± standard error of the mean. $p > 0.001$. Thus, no significant changes in the diameters of the RBCs were observed on shift reagent treatment. [b] The suspension medium for the RBC control sample was medium B (Table I). The letter indicated as follows in parentheses corresponds to the suspension media that was used for each shift reagent: $Dy(PPP)_2^{7-}$ (H), $Tm(PPP)_2^{7-}$ (L), $Dy(PcPcP)_2^{7-}$ (J), $Dy(TTHA)^{3-}$ (D), and $Dy(DOTP)^{5-}$ (F).

Table III. Comparison of Average Diameters and Volumes of Li^+- and Dy^{3+}-Treated RBCs Obtained by the SEM and CC Instruments, Respectively[a]

sample	*n*	av diam/μm SEM	av vol/fL CC
RBC control	636	7.56 ± 0.14	82 ± 4
RBCs treated and suspended in 1.5 mM LiCl	720	7.96 ± 0.22	92 ± 3
RBCs treated and suspended in 5 mM LiCl	692	8.12 ± 0.12	112 ± 6
RBCs treated and suspended in 140 mM LiCl	768	8.48 ± 0.10	134 ± 8
RBCs treated and suspended in 0.06 mM $DyCl_3$	732	7.62 ± 0.10	86 ± 4

[a] Values are expressed as mean ± standard error of the mean. $p < 0.001$ for RBCs treated with 5 mM or 140 mM LiCl. $p > 0.001$ for RBCs treated with 1.5 mM LiCl or 0.06 mM $DyCl_3$. Thus, significant changes in RBC size were observed only upon treatment with 5 mM LiCl or 140 mM LiCl.

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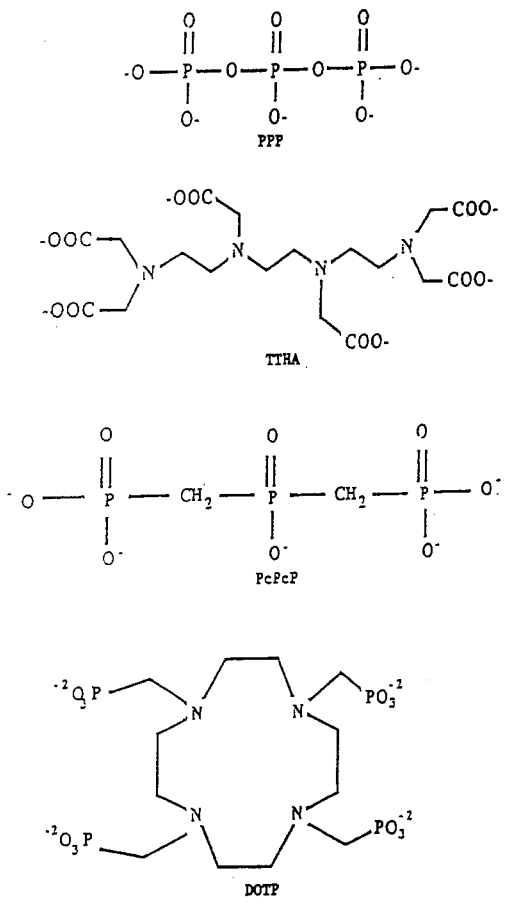

Figure 1. Structures of lanthanide shift reagent ligands used in this study. See Materials and Methods for the meanings of the abbreviations used.

$Dy(DOTP)^{5-}$. The concentration of NaCl used depended on the concentration of LiCl and shift reagent present in the suspension medium (Table I).

SEM and Coulter Counter Measurements. A 0.05-mL amount of each RBC suspension was placed on a 10 × 10 mm cover slip. After the RBCs were allowed to settle for 20 s, they were covered with an isotonic solution containing 3% glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.5 and allowed to fix for 3–4 h. They were then rinsed thoroughly in the above buffer solution.[25] Postfixation was done in 1% OsO_4 at pH 7.5. After dehydration in ethanol, critical point drying was performed in liquid CO_2 with a Polaron E-2000 critical point dryer. The specimens were then sputter coated with gold–palladium (60% gold, 40% palladium) alloy to a thickness of approximately 30 nm in argon atmosphere by using a Hummer VI sputtering system from Anatech Ltd. Photographs were taken with an ISI SX-30 scanning electron microscope (SEM) operated at 15 kV. Size measurements of RBCs were performed on SEM pictures by using the image analyzing system Bio-Quant II, interfaced with an Apple IIe computer. SEM data for each entry in Tables II and III were obtained on six samples. About 8–10 pictures were taken for each sample.

Cell volume measurements were obtained by using a Coulter counter, Model ZM from Coulter Electronics (Hialeah, FL). Prior to Coulter counter (CC) measurements, the cell suspension medium was subjected to 1:25 000 dilutions with the same isotonic Na^+ and choline suspension media described below for Li^+ transport measurements. The data reported take into account this dilution factor.

Hypophosphite Ion Preparation. Hypophosphite was added to cell suspensions as an isotonic solution prepared by first adjusting the pH of the solution to 7.5 with NaOH and then diluting the solution with deionized water to give an osmolarity of 300 mosM.[19]

NMR and AA Measurements. ^7Li, ^{19}F, and ^{31}P NMR measurements were made on a Varian VXR 300-MHz spectrometer (Palo Alto, CA), equipped with a 10-mm multinuclear probe, operating at resonance frequencies of 116.5, 282.2, and 121.4 MHz, respectively. The RBC samples were not spun during the acquisition to minimize settling of RBCs. To acquire the ^7Li NMR data in the presence of shift reagents, a 45° pulse, an acquisition time of 1 s, a spectral width of 8000 Hz, and a delay of 6.5 s were used in the absolute intensity mode. In the absence of shift reagents, ^7Li NMR data were accumulated with the modified inversion recovery (MIR) pulse sequence described in the accompanying paper. To record the ^{31}P NMR spectra, a 45° pulse was used to scan a spectral width of 10 000 Hz with an acquisition time of 1.5 s and a delay time of 7.5 s. Line broadening of 5 Hz was used in both ^7Li and ^{31}P NMR experiments.

Atomic absorption (AA) analysis was performed on a Perkin-Elmer spectrophotometer (Norwalk, CT) equipped with a graphite furnace. AA determination of Na^+–Li^+ exchange rates was obtained by a modification of a published procedure.[21] The washed Li^+-loaded cells were suspended in isotonic Na^+ media (solutions B, D, F, H, or J; Table I) or choline media (solutions C, E, G, I, or K; Table I). The rate of Li^+ transport measured in Na^+ medium is made up of two components: the Na^+–Li^+ exchange and the leak pathway. In contrast, the rate measured in the choline medium represents the Li^+ leak. Thus, the reported rates of Na^+–Li^+ exchange were obtained by subtracting the measured rates in the choline media from those in the Na^+ media.[21]. In general, $MgCl_2$ is used instead of choline for determination of Li^+–Na^+ exchange rates in RBCs.[21] However, for transport studies involving $Dy(PPP)_2^{7-}$ and $Dy(PcPcP)_2^{7-}$, $MgCl_2$ could not be used due to precipitation of the shift reagent.[13-15] We have previously ruled out any specific effect of choline on Li^+ rates in RBCs by measuring comparable rates by atomic absorption in Mg^{2+}- or choline-containing media (see accompanying paper).

Statistical Data Analysis. The statistical significance of the effect of shift reagents and Li^+ ion on RBC diameters and on ion transport rates was tested by using a student t test. The confidence levels are indicated as footnotes to the tables. Correlation coefficients were used to compare the AA and NMR data or the two membrane potential determinations.

Results

Effect of Lanthanide Shift Reagents on Shape and Size of Li^+-Free RBCs. SEM pictures depicted in Figure 2b–d show the effect of three negatively charged shift reagents, $Dy(PPP)_2^{7-}$, $Dy(DOTP)^{5-}$ and $Dy(TTHA)^{3-}$, at concentrations typically used in metal NMR experiments, on the morphology of Li^+-free RBCs. At these low concentrations even the most highly charged shift reagents do not have a noticeable effect on the shape and size of RBCs, which appear as discocytes. However, at higher concentrations of $Dy(PPP)_2^{7-}$ and $Dy(TTHA)^{3-}$ these shift reagents have a noticeable effect, producing shrunken RBCs having numerous cytoplasmic projections (see Figure 2e,f). SEM pictures for the other two shift reagents, 5 mM $Tm(PPP)_2^{7-}$ and 3 mM $Dy(PcPcP)_2^{7-}$, showed behavior similar to that of 3 mM $Dy(PPP)_2^{7-}$ (data not shown). Data on the average diameters and volumes of RBCs obtained by SEM and Coulter counter (CC) measurements, respectively, are given in Table II. Both data sets indicate that shift reagent treatment does not change the average RBC size.

Size measurements of RBCs after preparation for SEM must be interpreted with caution. Because of fixation, dehydration, and drying, shrinkage of cells and fixation-related artifacts are to be expected. In the pictures presented here a diameter of about 7.6 μm was measured in RBC controls (NaCl-treated cells). The specific effects of different shift reagent treatments were interpreted relative to the control samples. In order to confirm that the SEM measurements were indeed valid, we also observed RBCs by using a phase contrast microscope prior to fixing. The shapes of cells observed before and after fixing were essentially the same. This procedure confirmed that there were no qualitative errors due to fixation of red cells.

Effect of Li^+ and Dy^{3+} on Shape and Size of RBCs. In order to differentiate the effects of the anionic shift reagents from those of the cations on the morphology of RBCs, the specific effect of the Li^+ ion was further investigated because of its unusual hydration properties.[25] Figure 3 shows the effect of the presence and absence of Li^+ in the suspension medium on the shape and size of RBCs. The intracellular Li^+ concentration in sample 3a was measured by atomic absorption to be 0.48 mM, and thus, it is representative of the deformations that RBCs from bipolar patients may be subject to during lithium therapy.[22] As the concentration of Li^+ increases from 1.5 to 140 mM in the incubation medium, the tendency for cell swelling increases in RBCs. Table III shows that the average diameters and volumes of Li^+-loaded cells (measured by both SEM and CC, respectively) are significantly larger than those found in Li^+-free RBCs.

Some of the shift reagents used, in particular the triphosphate complexes,[26,27] are subject to hydrolytic instability. As a result,

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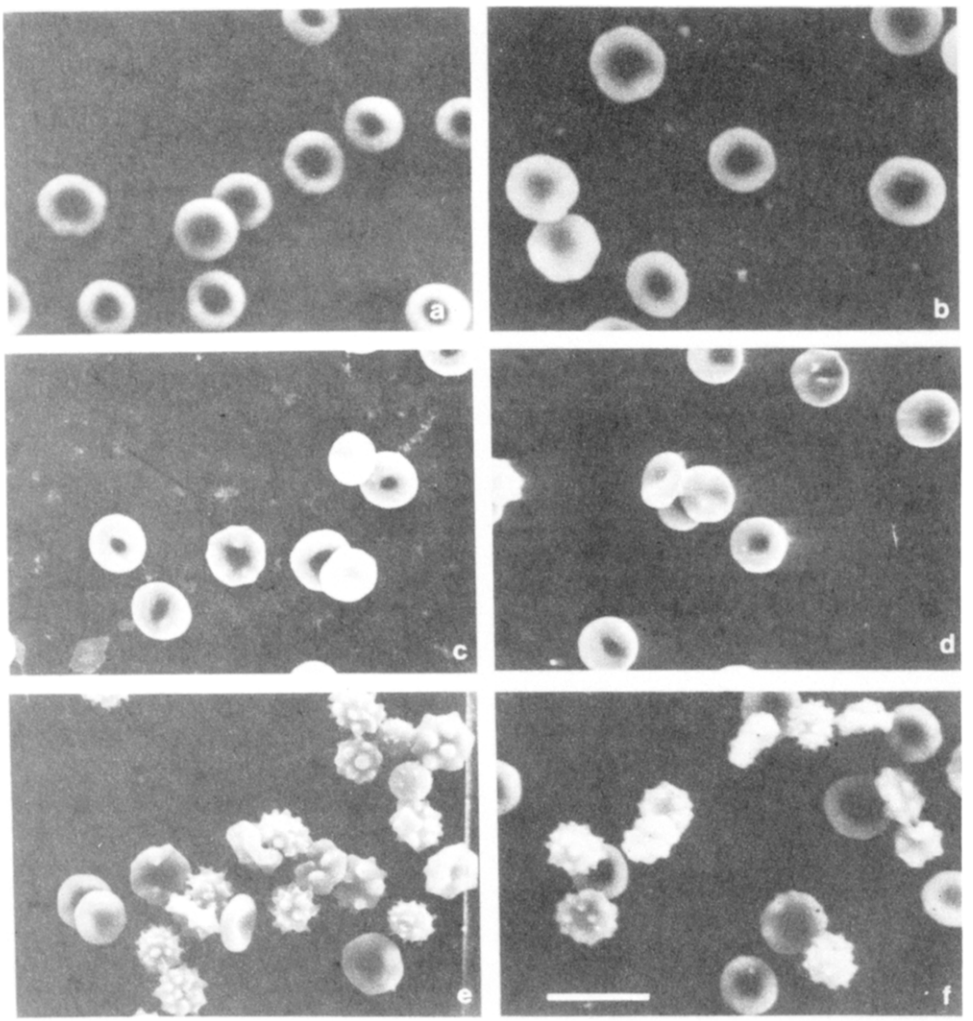

Figure 2. Comparison of SEM pictures of (a) control Li^+-free RBCs and (b) 3 mM $Dy(PPP)_2^{7-}$, (c) 5 mM $Dy(DOTP)^{5-}$, (d) 10 mM $Dy(TTHA)^{3-}$, (e) 15 mM $Dy(PPP)_2^{7-}$, and (f) 20 mM $Dy(TTHA)^{3-}$ treated Li^+-free RBCs. The osmolarity was adjusted to 300 ± 5 mosM with sucrose. The pH values of all the samples were maintained at 7.5. All micrographs are magnified 1500 times. The scale bar equals 10 μm. The suspension media were the same as for Table II.

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some Dy^{3+} ion could diffuse into the RBCs and, thus, exert an effect on the cell morphology. On the basis of the fact that the dissociation constants for the Dy^{3+} and Tm^{3+} complexes of the aforementioned ligands are all below −3 (in log K units),[17,28,29] the effect of 0.06 mM of $DyCl_3$ on the size and shape of RBCs was also investigated (Table III). (This is the estimated maximum concentration of free Dy^{3+} that could be present at these chelate concentrations.) $DyCl_3$, at least at these low concentrations, has a negligible effect on the morphology of RBCs, which is in good agreement with previous literature reports.[30,31]

Effect of Lanthanide Shift Reagents on Na^+–Li^+ Exchange by ^{7}Li NMR Spectroscopy. To find out whether the presence of shift reagents in the cell suspension had a significant effect on cation transport in RBCs, we investigated the effect of LSRs on Na^+–Li^+ exchange in RBCs by ^{7}Li NMR spectroscopy using a single pulse sequence. Intracellular Li^+ concentrations were calculated from the peak area of the intracellular Li^+ NMR resonance (see accompanying paper). Table IV shows a comparison of Li^+ transport rates in Na^+ and choline media and difference (Na^+–Li^+ exchange) rates for Li^+-loaded RBCs determined by AA and ^{7}Li NMR methods. The NMR measurements were carried out on intact RBC suspensions, while the AA measurements required cell lysis prior to chemical analysis. Thus, AA measurements were also made on RBC samples that had been pretreated with LSR, since the determination of intracellular Li^+ concentration by the ^{7}Li NMR method required the incorporation of a shift reagent in the suspension medium. The shift reagents $Dy(PPP)_2^{7-}$, $Dy(PcPcP)_2^{7-}$, $Dy(DOTP)^{5-}$, and $Tm(PPP)_2^{7-}$ effected a significant enhancement in the measured rates of RBC Li^+ transport in both Na^+ and choline media and on the calculated difference Na^+–Li^+ exchange rates. In contrast, no significant effect was observed with $Dy(TTHA)^{3-}$. The significant correlation (r = 0.998) between ^{7}Li NMR and AA exchange rate data indicates that the reported shift reagent effects on ion transport rates are independent of the technique being used for monitoring Li^+ transport. The fact that the effect of shift reagents on RBC Li^+ transport rates is present in Na^+ and to a lesser extent in choline media (Table IV) suggests that these shift reagent effects are not just present in nonphysiological choline media. These observations are in agreement with the known higher affinity of the triphosphate type shift reagents[13,15,37] for metal cations relative to $Dy(TTHA)^{3-}$. Thus, it can be concluded from these data that the larger the negative charge on the shift reagent, the larger the rate of Na^+–Li^+ exchange. Moreover, the effect of $Dy(PPP)_2^{7-}$ on the rate of Na^+–Li^+ exchange reported in the accompanying paper is not specific to the triphosphate shift reagent but it occurs with most shift reagents with high negative charges.

Effect of Lanthanide Shift Reagents on RBC Membrane Potential. The effect of lanthanide shift reagents on the RBC

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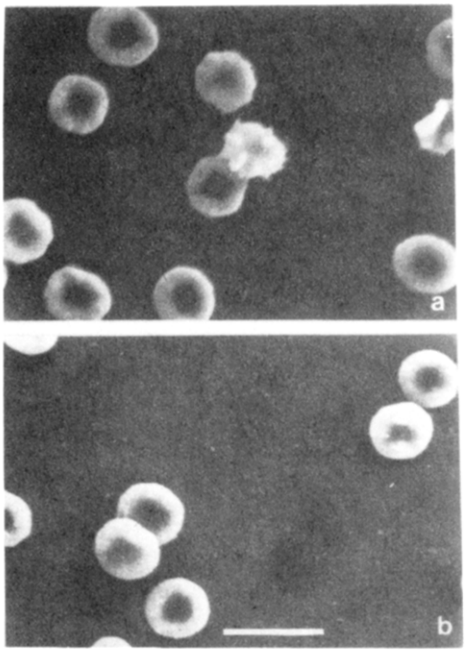

Figure 3. SEM pictures obtained for (a) RBCs incubated for 1 h and suspended in 5 mM LiCl and (b) control Li^+-free RBCs. The intracellular Li^+ concentration in sample a was measured by AA and found to be 0.48 mM. The loading and resuspension procedures are described in Materials and Methods. The pH was maintained at 7.4, and the osmolarity of all samples was maintained at 300 ± 5 mosM. Magnification and SEM conditions were the same as for Figure 2.

Table IV. Comparison of Li^+ Transport and Difference (Na^+–Li^+ Exchange) Rates [mmol of Li^+/(L of RBCs h)] of Li^+-Loaded RBCs Obtained in the Presence and Absence of Shift Reagents by AA and ^7Li NMR Spectroscopy

sample[a]	n	type of rate	rate: AA	rate: NMR
control	12	sodium	0.48 ± 0.04	
		choline	0.29 ± 0.03	
		difference	0.19 ± 0.04	
7 mM $Dy(TTHA)^{3-}$	3	sodium	0.51 ● 0.02	0.51 ± 0.02
		choline	0.30 ± 0.01	0.29 ± 0.02
		difference	0.21 ± 0.02	0.22 ± 0.02
5 mM $Dy(DOTP)^{5-}$	6	sodium	0.58 ± 0.02	0.59 ± 0.02
		choline	0.33 ± 0.01	0.33 ± 0.02
		difference	0.25 ± 0.01	0.26 ± 0.02
3 mM $Dy(PPP)_2^{7-}$	12[b]	sodium	0.64 ± 0.03	0.66 ± 0.02
		choline	0.37 ± 0.02	0.38 ± 0.01
		difference	0.27 ● 0.04	0.28 ± 0.02
3 mM $Dy(PcPcP)_2^{7-}$	12[b]	sodium	0.63 ± 0.01	0.66 ● 0.03
		choline	0.35 ± 0.02	0.35 ± 0.02
		difference	0.28 ± 0.02	0.29 ± 0.03

[a] The Na^+ suspension for Li^+-loaded RBC control samples was medium B (Table I), while the choline suspension was made with medium C. The following letters indicated in parentheses correspond to the Na^+ and choline media, respectively, used for the shift reagents: $Dy(TTHA)^{3-}$ (D and E), $Dy(DOTP)^{5-}$ (F and G), $Dy(PPP)_2^{7-}$ (H and I), and $Dy(PcPcP)_2^{7-}$ (J and K). [b] The difference between Na^+–Li^+ countertransport rates measured in the presence and absence of 3 mM $Dy(PPP)_2^{7-}$ or 3 mM $Dy(PcPcP)_2^{7-}$ is significant up to a 99% confidence level. The difference in Li^+ transport rates observed with the other two shift reagents is not statistically significant.

membrane potential, V_m, was measured by ^{31}P NMR spectroscopy.[19] The measurement of V_m involved incorporation of hypophosphite ion in the cell suspension. Hypophosphite ion crosses the cell membrane via the anion-exchange protein, and the intra- and extracellular populations of the ion give rise to separate ^{31}P NMR resonances.[32] The relative areas of the two peaks correspond to the relative sizes of the intra- and extracellular hypophosphite populations, $r(H_2PO_2^-)$. It was assumed[19] that for RBCs the membrane permeability of Cl^- is much larger than that of either K^+ or Na^+ and that $r(Cl^-) = r(H_2PO_2^-)$. Li^+-free RBCs in isotonic suspension medium consistently yielded a membrane potential of around −11 mV (Table V). This value is in good agreement with that obtained by others.[9,19,33,34] Li^+-loaded RBCs suspended in an isotonic suspension medium also yielded a membrane potential around −10 mV (n = 3), thereby indicating that Li^+ loading does not significantly change the RBC membrane potential. Thus, the effect of highly negatively charged shift reagents on RBC membrane potential (vide infra) is a specific effect independent of Li^+ loading of RBCs.

Table V. Effect of Various Shift Reagents on the Membrane Potential of Li^+-Free RBCs Obtained by ^{31}P and ^{19}F NMR Spectroscopy

sample[a]	$r(H_2PO_2^-)$[b]	V_m/mV: ^{31}P NMR[b]	V_m/mV: ^{19}F NMR[c]
RBC control	0.65 ± 0.04	−11.42 ± 0.54 (n = 12)	−11.26 ± 0.32 (n = 3)
RBCs treated with[c] 7 mM $Dy(TTHA)^{3-}$	0.67 ± 0.03	−10.28 ± 0.54 (n = 6)	−10.32 ± 0.27 (n = 3)
RBCs treated with 5 mM $Dy(DOTP)^{5-}$	0.78 ± 0.05	−6.38 ± 0.46 (n = 6)	−6.46 ± 0.32 (n = 3)
RBCs treated with[d] 5 mM $Dy(PPP)_2^{7-}$	0.92 ± 0.02	−2.14 ± 0.56 (n = 6)	−1.98 ± 0.36 (n = 3)

[a] For ^{31}P NMR determinations, the suspension medium for the Li^+-free RBC control was 130 mM NaCl, 15 mM sodium hypophosphite, 10 mM glucose, and 10 mM TrisCl, at pH 7.4. For ^{19}F NMR determinations, the suspension medium consisted of 154 mM NaCl, 10 mM HEPES pH 7.4, 10 mM glucose, 2 mM sodium trifluoroacetate, and 1.5 mM trifluoroacetamide. For the shift reagent containing samples, aliquots of isotonic solutions of $Dy(TTHA)^{3-}$ (70 mM), $Dy(DOTP)^{5-}$ (50 mM), and $Dy(PPP)_2^{7-}$ (30 mM) were added to obtain the desired concentration of shift reagent. [b] The relative distribution of the intra- and extracellular hypophosphite populations, $r(H_2PO_2^-)$, and the membrane potential, V_m, were calculated according to the equations[19] $r(H_2PO_2^-) = (I_i/I_o)[(1 - H_t)/\alpha H_t]$ and $V_m = RT/F \ln r(H_2PO_2^-)$, where I_i and I_o are the areas of the intra- and extracellular $H_2PO_2^-$ ^{31}P NMR peaks, H_t is the hematocrit (0.45), α is the gravimetrically determined fractional water volume of the RBC,[34] R is the gas constant, T is the absolute temperature, and F is the Faraday constant. [c] $p > 0.001$ for RBCs treated with 7 mM $Dy(TTHA)^{3-}$ relative to RBC control without shift reagent. [d] The effect of 3 mM $Tm(PPP)_2^{7-}$ on RBC membrane potential was briefly studied and was found to be similar (−1.98 ± 0.39, n = 3) to that of 5 mM $Dy(PPP)_2^{7-}$. [e] To calculate V_m from the ^{19}F NMR method, the intensities of the intra- and extracellular trifluoroacetate, I(TFA), and trifluoroacetamide, I(TFM), were incorporated into the Nernst equation:[20] $V_m = \{RT[I_i(TFA)I_e(TFM)]\}/F[I_e(TFA)I_i(TFM)]$.

In experiments involving shift reagents, an aliquot of the isotonic shift reagent solution was added to the isotonic RBC suspension so that the osmolarity of the samples did not change upon shift reagent addition. Of the shift reagents studied, $Dy(PPP)_2^{7-}$ and $Dy(DOTP)^{5-}$ induced significant changes in the RBC membrane potential (Table V). Addition of 5 mM $Dy(PPP)_2^{7-}$ to the RBC suspension caused the RBC membrane potential to change from −11 to −2 mV. $Dy(DOTP)^{5-}$ gave an intermediate value for the membrane potential. By contrast, $Dy(TTHA)^{3-}$ had a marginal effect, which was not statistically significant (Table V). Incorporation of the shift reagent in the suspension medium, via NaCl replacement, also caused the membrane potential to become more positive (from −11.42 ± 0.54 mV (n = 12) for control RBC suspensions to −9.52 ± 0.63 (n = 6) for RBCs treated with 7 mM $Dy(TTHA)^{3-}$, to −5.10 ± 0.72 (n = 6) for RBCs treated with 5 mM $Dy(DOTP)^{5-}$, and to +0.56 ± 0.08 (n = 9) for RBCs treated with 5 mM $Dy(PPP)_2^{7-}$). Thus, replacement of Cl^- in the suspension medium by shift reagent had a marginally larger effect on the RBC membrane potential than isotonic addition of shift reagent solutions.

The intracellular pH was monitored by the ^{31}P NMR resonance of inorganic phosphate and was found to be unchanged by the addition of shift reagents or hypophosphite. The RBC membrane

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potentials in suspensions containing shift reagents were maintained over a 4-h period and within this time did not readjust toward the values obtained in RBC suspensions not containing shift reagents. Hence, the changes in RBC membrane potential upon addition of shift reagents are shift reagent specific and not due to intracellular pH or time-dependent ion distribution fluctuations.

The membrane potential of shift reagent treated RBC was also determined by an independent ^{19}F NMR method.[20] To determine V_m (Table V), distributions of sodium trifluoroacetate and trifluoroacetamide across the RBC membrane were measured. The V_m values obtained by the ^{19}F NMR method correlated significantly ($r = 0.996$) with those obtained by the ^{31}P NMR method using hypophosphite.

Discussion

Discriminating between the two pools of lithium in Li^+-loaded RBCs by ^7Li NMR spectroscopy can be accomplished with the help of several shift reagents (Figure 1). Because of the high negative charge on some of these shift reagents, the amount of extracellular Li^+ (and other cations) bound to them is significant. Thus, these reagents may change the distribution of Li^+ (and of other cations) across the RBC membrane as it is apparent from the different ratios of intra- and extracellular Li^+ obtained in Li^+-loaded RBC suspensions in the presence of different shift reagents (see accompanying paper). During ion transport measurements (Table IV), this effect is complicated by the fact that the amount of Li^+ bound to the shift reagent will vary, and thus, the increasing amounts of extracellular cation bound to the shift reagent will cause an increase in ion transport rates. We found that this problem of complexation is virtually eliminated when a shift reagent with a lower negative charge like $Dy(TTHA)^{3-}$ is used, since the Li^+ transport rates in the presence and absence of $Dy(TTHA)^{3-}$ are essentially the same (see Table IV). This observation is in agreement with previous reports[13-15,17,37] that showed that the shift reagents used in this study, except $Dy(TTHA)^{3-}$, have relatively high affinities for divalent and monovalent cations such as Ca^{2+}, Mg^{2+}, Li^+, Na^+, and K^+.

Studies on the effect of cell impermeable shift reagents on RBC membrane potential by ^{31}P and ^{19}F NMR spectroscopy suggested that the extent to which RBC membrane potential is altered depends on the charge of shift reagent (Table V). The maximum changes in membrane potential were observed for $Dy(PPP)_2^{7-}$. $Dy(TTHA)^{3-}$ had no appreciable effect on RBC membrane potential, while $Dy(DOTP)^{5-}$ caused an intermediate change. The cell impermeable anionic shift reagents $Dy(PPP)_2^{7-}$ and $Dy(DOTP)^{5-}$ caused a reduction in extracellular $[Cl^-]$, thereby inducing an increase in V_m. A similar observation on the effect of the RBC impermeable anion citrate on membrane potential was reported.[19] This behavior follows from the fact that permeable Cl^- ions are passively distributed across the RBC membrane according to a Gibbs–Donnan equilibrium. Immediately after the addition of isotonic shift reagent solutions to RBC suspensions the media $[Cl^-]$ concentrations are expected to change. For ^{31}P NMR membrane potential measurements, the suspension medium Cl^- concentration was 140 mM before shift reagent addition and it changed to 135 mM with 3 mM $Dy(PPP)_2^{7-}$, 126 mM with 7 mM $Dy(TTHA)^{3-}$, and 141 mM with 5 mM $Dy(DOTP)^{5-}$. For ^{19}F NMR measurements, the starting medium $[Cl^-]$ concentration was 154 mM before isotonic addition of shift reagent and it changed to 148 mM with 3 mM $Dy(PPP)_2^{7-}$, 139 mM with 7 mM $Dy(TTHA)^{3-}$, and 154 mM with 5 mM $Dy(DOTP)^{5-}$. If nothing else happened, this would cause the largest change in membrane potential for $Dy(TTHA)^{3-}$, which was not observed. Clearly, a chloride, and to a smaller extent cation, redistribution takes place. The steady state reached would depend on the affinities of Na^+ and Li^+ ions for the shift reagent anions, which are known to be very different for $Dy(TTHA)^{3-}$ relative to the other shift reagents studied,[15,37] and for intracellular binding sites.

It is important to note that the Gibbs–Donnan equilibrium controls the RBC membrane potential only at equilibrium, since Cl^- permeability is orders of magnitude greater than cation permeability. The membrane potential would be zero if there were no impermeant anions present. The large concentration of the intracellular impermeant anion hemoglobin in RBCs leads to the Cl^- gradient and hence the pH and electropotential gradients. Isotonic addition of an impermeant anion (shift reagent) to the suspension medium would have the effect of increasing the membrane potential, making it more positive, as observed. If one suddenly changes the composition of the RBC suspension medium, the membrane potential is expected to follow a partial ionic equilibrium in which the instantaneous Cl^- distribution generates the potential. However, that Cl^- distribution may be continuously changing as slower anions and very slow cations redistribute themselves. Cl^- would be redistributed to maintain electroneutrality. The V_m values measured with RBC suspensions containing shift reagents did not change over a 4-h period, suggesting that within this time a measurable slow Cl^- or cation redistribution is not occurring with RBC suspensions containing shift reagents. Cation and pH fluxes probably occurred upon addition of shift reagents to the RBC suspension media. However, these fluxes were too fast to be detected by these NMR methods. The effect that a polyvalent chelating anion, such as the negatively charged shift reagents used in this study, might have on membrane potential clearly depends on the degree to which it is complexed to cations. This complexation effectively reduces the negative charge on the shift reagents. The reported effects of shift reagents on RBC membrane potential are not expected to be the same for other cell systems with considerably lower Cl^- permeability and very active cation channels.

Our SEM and CC results showed that the typical concentrations of lanthanide shift reagents used in ion transport studies have very little effect on the shape and size of Li^+-free RBCs (Table II). It has been proposed[12] that anionic lipophilic drugs intercalate mainly into lipids in the exterior half of the bilayer and thereby induce cells to crenate. The lanthanide shift reagents used in this study are inorganic complexes with high negative charges. Shift reagents are impermeable to the RBC membrane, since the NMR resonances corresponding to the two metal ion pools never collapse into one. The probability of these shift reagents intercalating into the lipids in the exterior half of the bilayer and expanding the exterior layer relative to the cytoplasmic portion is very unlikely. Preferential entry of the shift reagents into the outer leaflet of the membrane is not possible due to repulsion between negative charges on the shift reagents and the RBC membrane.

Our findings on the effect of varying concentrations of Li^+ on the shape and size of RBCs are in good agreement with another study.[25] The main difference between the two studies is that Zimmermann and Soumpasis[25] looked at the effect of Li^+ incubation at large concentrations (140 mM) only. We found that RBCs that contained an intracellular Li^+ level of 0.49 mM LiCl (a typical concentration for patients taking lithium carbonate) as a result of a previous 1-h incubation with 1.5 mM LiCl exhibited considerable hypertrophy (cell swelling) comparable to that obtained with 140 mM LiCl incubation (Figure 3 and Table III). The unusual hydration properties of Li^+ in part could account for hypertrophy and diminished biconcavity of Li^+-loaded RBCs. Among the alkali metals, Li^+ has a large hydration sphere because of its charge density. Li^+ coordinates with four, five, or six water molecules. The hydrated radius of Li^+ is 3.40 Å, while that of Na^+ is 2.80 Å. As the concentration of LiCl is increased in Li^+-treated RBCs, the concentration of intracellular Li^+ increases. The Li^+ ions that partially replace intracellular Na^+ and K^+ ions in Li^+-loaded RBCs are much larger in size (due to their high hydration energy) and are likely to cause hypertrophy. In Li^+-loaded RBCs, some of the intracellular Li^+ is bound while most of it presumably exists as hydrated ion. As the concentration of intracellular Li^+ increases, the amount of free intracellular Li^+ ions also increases, causing the cells to undergo hypertrophy. An alternative explanation for the hypertrophy observed upon Li^+ incubation may be due to increased water permeation of the RBC membrane to water.[35]

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Noninvasive metal NMR methods, based on the different relaxation properties of the two ion pools, have recently been proposed by us (see accompanying paper) and others[36] for the investigation of Li^+ and K^+ transport in biological systems. The alternative methods, which are based on a modified inversion recovery (MIR) pulse sequence, do not involve the use of shift reagents. However, the MIR method may not be applicable to certain biological systems and other nuclei, such as $^{23}Na^+$ transport, where a clear distinction in T_1 relaxation times for the intra- and extracellular compartments may not be present. Interestingly, the transmembrane Na^+ distribution ratio ($[Na^+]_{RBC}/[Na^+]_{plasma}$) removed by ^{23}Na NMR in the presence of 3 mM $Dy(PPP)_2^{7-}$ was different from that obtained from a NMR approach based on a difference in T_2 relaxation times for intra- and extracellular RBC Na^+ ions.[38] In those cases where no difference exists between T_1 or T_2 values for intra- and extracellular metal ion resonances, the shift reagent method may continue to be the only alternative despite its limitations.

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Registry No. $Dy(PPP)_2^{7-}$, 81868-53-3; $Tm(PPP)_2^{7-}$, 89031-43-6; $Dy(TTHA)^{3-}$, 91264-39-0; $Dy(PcPcP)_2^{7-}$, 129102-69-8; $Dy(DOTP)^{5-}$, 115701-67-2; Li, 7439-93-2; Na, 7440-23-5.

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Contribution from the Department of Chemistry, University of Queensland, Brisbane, Australia 4072

Reactions of the *cis*-Diamminediaquaplatinum(II) Cation with 2-Aminomalonic Acid and Its Homologues, Aspartic and Glutamic Acids. Rearrangements of Metastable Complexes with Carboxylate-Bound Ligands to N,O-Chelates and Formation of Di- and Trinuclear Complexes[1]

Trevor G. Appleton,* John R. Hall, Donald W. Neale, and Craig S. M. Thompson

Received March 20, 1990

Reactions of *cis*-$Pt(NH_3)_2(H_2O)_2^{2+}$ (**1**) with 2-aminomalonic acid ($amalH_2$), aspartic acid ($aspH_2$), and glutamic acid ($gluH_2$) have been studied by multinuclear (^1H, ^{13}C, ^{195}Pt, ^{15}N) NMR spectroscopy. With $aspH_2$ or $gluH_2$, **1** gives initially a complex in which the ligand is bound through only one carboxylate group, *cis*-$Pt(NH_3)_2(LH_2\text{-}O)(H_2O)^{2+}$. At pH <2, the α-carboxyl group is bound predominantly, but at pH 4–5, both carboxylate groups are involved to a similar extent. Over 2–3 days at pH 1.5, a complex with five-membered N,αO-chelate ring is formed, $Pt(NH_3)_2(LH\text{-}N,\alpha O)^+$. Reaction of aminomalonate with **1** gives initially a complex $Pt(NH_3)_2(amalH\text{-}O,O')^+$, with a six-membered chelate ring. Over 2–3 days at pH 1.5, this complex converts to $Pt(NH_3)_2(amalH\text{-}N,O)^+$, with a five-membered ring. In solutions sufficiently acidic to protonate the uncoordinated carboxyl group, decarboxylation then occurs over several days to give the glycinate complex $Pt(NH_3)_2(gly\text{-}N,O)^+$. The carboxylate group that is not part of the five-membered chelate ring in each of the three complexes $Pt(NH_3)_2(L\text{-}N,O)$ is able to coordinate to platinum from excess **1**. With aspartic and glutamic acids, one or two $Pt(NH_3)_2(L)$ moieties can coordinate to one diammineplatinum(II) cation, to give $[\{Pt(NH_3)_2(L)\}\{Pt(NH_3)_2(H_2O)\}]^{2+}$ or $[\{Pt(NH_3)_2(L)\}_2\{Pt(NH_3)_2\}]^{2+}$, respectively. One $Pt(NH_3)_2(amal)$ molecule can react with **1** to give $[\{Pt(NH_3)_2(amal)\}\{Pt(NH_3)_2(H_2O)\}]^{2+}$, which is in equilibrium with $[\{Pt(NH_3)_2\}_2(amal)]^{2+}$, in which the carboxylate group involved in a five-membered N,O-chelate ring with one Pt atom also bridges to the second Pt atom to complete a six-membered O,O′-chelate ring.

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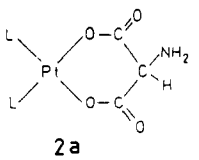
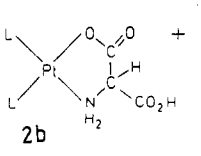
Introduction

Gandolfi et al.[2] recently described the preparation of a series of complexes *cis*-$PtA_2(amal)$, where A is an amine ligand and $amalH_2$ is 2-aminomalonic acid, by reaction of *cis*-$[PtA_2(H_2O)_2]SO_4$ with Ba(amal). Significant antitumor activity was claimed for some of the compounds. They considered only the O,O′-chelate structure **2a** for their complexes, with the role of



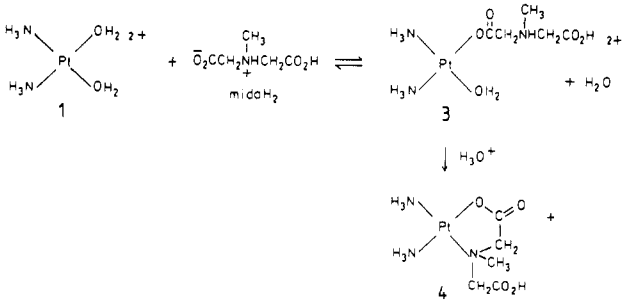
2a



2b

the amine group primarily to provide enhanced solubility for complexes of a substituted malonate ligand. However, N,O-chelation is a well-established coordination mode (e.g., in Co(III) complexes[3]) for 2-aminomalonate and derivatives. We have shown by multinuclear NMR[4] spectroscopy that (methylimino)diacetic acid ($midaH_2$) reacts with *cis*-$Pt(NH_3)_2(H_2O)_2^{2+}$ (**1**) to give

Scheme I



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