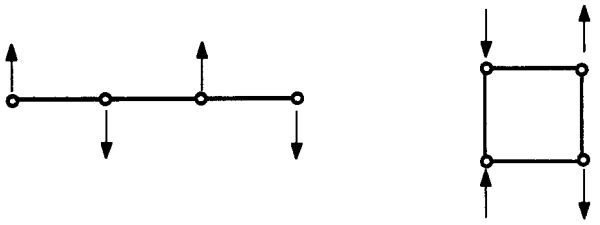
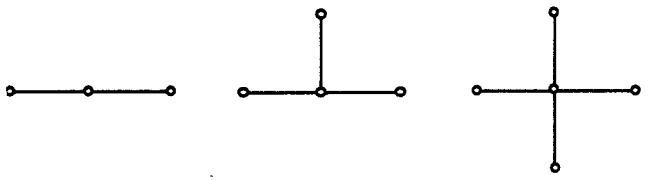


This quite simple topological approach could be formalized in terms of graph theory.[32]

The best design for polymetallic complexes containing n ions is the graph where one point has a degree equal to $n - 1$ and all the others have a degree equal to 1. This rule leads to the following graphs for n = 3-5:



The challenge for the chemist is to find real molecules having these topologies. In this respect, inorganic compounds offer the unique possibility of choosing the spin value for each point of the graph by changing the nature of the interacting ions. For $[Cu_3Ni]$ and $[Cu_3Mn]$ the smaller $S = {}^1/_2$ spins are located outside with the largest spin at the center, yielding low-spin multiplicities overall. But if one could synthesize a molecule with a reverse of this arrangement, a high-spin ground state would be reached. We have already had some success along this line in the case of trinuclear species with the synthesis of a linear $Mn^{II}Cu^{II}Mn^{II}$ complex having a $S = {}^9/_2$ ground state.[33]

To conclude, molecular topology is a very important factor determining the magnetic properties of polynuclear complexes with more than two metal ions. It is significant that the actual geometry does not govern the spin structure. For instance, the three-dimensional compounds studied in this article and the planar trimethylenemethane biradical have the same topology and consequently the same spin structure. Thus it is possible to tune the magnetic properties of the polynuclear complex by controlling the topology and the nature of the ions in interaction. This approach is particularly promising for the synthesis of high-spin molecules.

Safety Note. Perchlorate salts of metal complexes with organic ligands are potentially explosive. In the syntheses described here we used only small amounts of material (the preparations were carried out at the millimole scale) and the starting perchlorate salt was an aquo complex. The dilute solutions were handled with great caution and evaporated slowly at room temperature.[34] When noncoordinating agents are required, every attempt should be made to substitute anions such as the fluoro sulfonates for the perchlorates.

Acknowledgment. This work was partially supported by the "Proyecto Prioritario Hispano-Francês No. 52" and the Comisiôn Interministerial de Ciencia y Technologia (Proyecto PB88-0490).

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Comparison of Li^+ Transport and Distribution in Human Red Blood Cells in the Presence and Absence of Dysprosium(III) Complexes of Triphosphate and Triethylenetetraminehexaacetate[1]

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Discrimination between intra- and extracellular Li^+ pools in Li^+-loaded human red blood cells (RBCs) was achieved by two distinct ^7Li NMR methods. One NMR method involved the incorporation in the RBC suspension of cell-impermeable shift reagents, either $Dy(PPP)_2^{7-}$ (dysprosium(III) triphosphate) or $Dy(TTHA)^{3-}$ (dysprosium(III) triethylenetetraminehexaacetate), and recording a standard one-dimensional FT-NMR spectrum of the $^7Li^+$ nucleus. The other NMR approach took advantage of the different relaxation properties of the two Li^+ pools and involved a modified inversion recovery (MIR) pulse sequence. We investigated the effect of $Dy(PPP)_2^{7-}$ and $Dy(TTHA)^{3-}$ on the transmembrane Li^+ distribution ratio ($[Li^+]_{RBC}/[Li^+]_{plasma}$) and on the rates of Na^+-Li^+ countertransport in Li^+-loaded human RBC by the two NMR techniques and by atomic absorption (AA) spectrophotometry, an invasive approach commonly used in clinical studies. The Li^+ transport parameters measured in the absence of shift reagents by MIR and AA or in the presence of $Dy(TTHA)^{3-}$ by ^7Li NMR spectroscopy or AA correlated significantly. However, the Li^+ transport rates measured in the presence of $Dy(PPP)_2^{7-}$ by ^7Li NMR spectroscopy or AA were higher than those measured in the presence of $Dy(TTHA)^{3-}$ or in the absence of shift reagents; the Li^+ RBC transmembrane ratios measured in the presence of the triphosphate shift reagent were higher than those measured in the two other suspension media under the same conditions. In contrast, the Na^+ distribution ratios measured in the presence of $Dy(PPP)_2^{7-}$ by ^{23}Na NMR spectroscopy or AA were lower than those measured in the presence of $Dy(TTHA)^{3-}$ or in the absence of shift reagents. Although both ^7Li NMR methods have distinct advantages over AA, such as visualization of Li^+ transport and no requirement for cell lysis, the incorporation of $Dy(PPP)_2^{7-}$ in the cell suspension changed the Li^+ transport rates and ratios in RBCs and must be used with caution.

Introduction

Lithium salts are the preferred drugs in the treatment and maintenance of both manic and depressive episodes in psychiatric patients with bipolar affective disorders (or manic-depressive disorders as they were formerly called).[2] Lithium has also been used in a variety of other psychiatric and medical conditions,

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

medium ref	composition	purpose
A	150 mM NaCl, 5 mM Na_2HPO_4, pH 7.4	RBC washing buffer
B	150 mM LiCl, 10 mM glucose, 10 mM TrisCl, pH 7.5	Li^+ loading buffer
C	150 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 10 mM TrisCl, pH 7.5	Na^+ medium for Li^+ transport rates without shift reagents
D	112.5 mM choline chloride, 10 mM glucose, 85 mM sucrose, 0.1 mM ouabain, 10 mM TrisCl, pH 7.5	choline medium for Li^+ transport rates without shift reagents
E	70 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 7 mM $Na_7Dy(PPP)_2$·3NaCl, 10 mM TrisCl, pH 7.5	Na^+ medium for Li^+ transport rates with $Dy(PPP)_2^{7-}$
F	84.5 mM choline chloride, 10 mM glucose, 0.1 mM ouabain, 7 mM $Na_7Dy(PPP)_2$·3NaCl, 10 mM TrisCl, pH 7.5	choline medium for Li^+ transport rates with $Dy(PPP)_2^{7-}$
G	119 mM NaCl, 10 mM glucose, 0.1 ouabain, 7 mM $Na_3Dy(TTHA)$, 10 mM TrisCl, pH 7.5	Na^+ medium for Li^+ transport rates with $Dy(TTHA)^{3-}$
H	91.5 mM choline chloride, 10 mM glucose, 0.1 mM ouabain, 7 mM $Na_3Dy(TTHA)$, 10 mM TrisCl, pH 7.5	choline medium for Li^+ transport rates with $Dy(TTHA)^{3-}$
I	140 mM NaCl, 1.5 mM LiCl, 10 mM glucose, 10 mM TrisCl, pH 7.5	medium for RBC ion ratios without shift reagents
J	70 mM NaCl, 1.5 mM LiCl, 7 mM $Na_7Dy(PPP)_2$·3NaCl, 10 mM glucose, 10 mM TrisCl, pH 7.5	medium for RBC ion ratios with $Dy(PPP)_2^{7-}$
K	119 mM NaCl, 1.5 mM LiCl, 7 mM $Na_3Dy(TTHA)$, 10 mM glucose, 10 mM TrisCl, pH 7.5	medium for RBC ion ratios with $Dy(TTHA)^{3-}$

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

including treatment of low white blood cell counts resulting from cancer chemotherapy and conditions caused by the Herpes simplex virus.[2]

Despite the important pharmacological action of lithium, the mechanism(s) for its biological action(s) remains (remain) uncertain. However, several main hypotheses have been proposed on the basis of biochemical,[3-5] genetic,[6-8] or bioinorganic[9,10] studies. A cell membrane anomaly has been reported for bipolar patients leading to a membrane abnormality hypothesis for bipolar disorders.[11] This hypothesis is based on studies of Li^+ transport in red blood cells (RBCs) of bipolar patients.[12,13] The importance of studying Li^+ transport in RBCs is 3-fold. First, RBCs have many ion transport systems similar to those of neurons[12] and, thus, they may be suitable for testing a link between bipolar disorders and cell membrane abnormality. Second, Li^+ transport in these cells can be more easily characterized because of their ready availability and simple morphology. Third, abnormalities in Li^+ transport across RBC membranes have been reported in families of bipolar patients.[13] Although lithium has no therapeutic effect in hypertension, it is interesting to note that Li^+ transport abnormalities have also been reported in RBCs from hypertensive patients.[14]

Li^+ transport in biological systems has been traditionally studied by atomic absorption and flame spectrophotometries.[13,14] In this report, we compare two ^7Li NMR spectroscopic methods for the measurement of Na^+–Li^+ countertransport rates and transmembrane Li^+ distribution in normal RBCs to the method of atomic absorption (AA) spectrophotometry. Several NMR-based methods have been reported that do or do not require the use of shift reagents in order to resolve intra- and extracellular cation pools.[15-18] The two ^7Li NMR techniques used here are variations of methods previously employed for studying related membrane transport phenomena.[15-18] In this report, we show that the highly negatively charged shift reagent $Dy(PPP)_2^{7-}$ disturbs Li^+ ion distribution in cell suspensions and, thus, it must be used with caution. By contrast, a shift reagent at the same concentration but with a lower negative charge $Dy(TTHA)^{3-}$ does not significantly change Li^+ transport properties. In the accompanying paper, we show that the shift reagent effects reported here for $Dy(PPP)_2^{7-}$ and $Dy(TTHA)^{3-}$ are general.

All the studies described here were carried out in Li^+-loaded RBCs from healthy donors. A comparison of Li^+ transport rates in packed RBCs from bipolar patients, hypertensives, and matched normotensive controls by ^7Li NMR methods was also investigated in our laboratory.[19]

Methods

Packed RBCs from healthy donors were supplied by the Chicago Chapter of Life Source. The compositions of all suspension media used in this investigation are given on Table I. The RBCs were washed three times by centrifugation at 2000*g* for 10 min with medium A at 4 °C and were separated from the plasma and buffy coat by aspiration. Li^+ loading of RBCs was achieved by incubating packed RBCs with medium B at 50% hematocrit and 37 °C for 3 h.[20] Typically the intracellular Li^+ concentration after this incubation procedure was 1.0 mM as measured by atomic absorption. Immediately before NMR measurements, the cells were washed by centrifugation with one of the media C–K prior to the final resuspension at 13% hematocrit in the same medium. The exact composition of the RBC suspension media varied depending on whether or not the experiment involved the use of shift reagents (Table I). The osmolarity of all RBC suspensions was adjusted with sucrose and measured to be approximately 300 mosM with a Wescor vapor-pressure osmometer (Wescor Inc., Logan, UT). The shift reagent dysprosium(III) triethylenetetraminehexaacetate, $Dy(TTHA)^{3-}$, was prepared from dys-

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prosium hydroxide and the acidic form of the ligand according to published procedures.[15] Dysprosium(III) triphosphate, $Dy(PPP)_2^{7-}$, was prepared from dysprosium chloride and sodium triphosphate.[15]

For determination of Na^+–Li^+ countertransport rates in control RBCs in the absence of shift reagents, the Li^+-loaded cells were suspended in isotonic Na^+-rich (medium C) or choline-rich (medium D) suspension media. To determine the effect of $Dy(PPP)_2^{7-}$ on Li^+ rates, media E and F were used; media G and H were used for rate measurements in the presence of $Dy(TTHA)^{3-}$. Ouabain, a plant steroid, inhibited any Li^+ transport through Na^+,K^+-ATPase.[13,14] The choline-suspension medium allows the determination of the contribution of the leak pathway toward Li^+ efflux.[20] In general, $MgCl_2$ is used instead of choline.[14] However, $MgCl_2$ could not be used for transport studies involving $Dy(PPP)_2^{7-}$ due to precipitation of the shift reagent.[15] For shift reagent containing samples, less NaCl or choline was used in the medium (Table I). The rate of Li^+ transport measured in the Na^+ medium is made up of two components: the Na^+–Li^+ countertransport and the leak pathways. Thus, the reported rates of Na^+–Li^+ countertransport were obtained by subtracting the measured rates in the Na^+ and choline media.[14]

To determine transmembrane Li^+ or Na^+ distributions ([RBC]/[plasma]) under steady-state conditions in RBCs,[21] Li^+-loaded cells were suspended at 37 °C for 4 h. For the NMR experiments not requiring the use of shift reagents, medium I was used; for experiments involving shift reagents, media J and K were used instead (Table I).

^7Li and ^{23}Na NMR measurements were made at 116.5 and 79.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer (Palo Alto, CA) equipped with a multinuclear probe and a variable-temperature control unit set at 37 °C. T_1 (spin–lattice relaxation time) measurements of the $^7Li^+$ NMR signals were obtained by the inversion recovery method. T_1 measurements of intra- or extracellular Li^+ were performed on packed Li^+-loaded RBCs or Li^+-containing suspension medium, respectively. To test whether the presence of a Li^+ pool has an effect on the relaxation of the other Li^+ compartment (see Results), T_1 measurements of intra- or extracellular Li^+ were also carried out on Li^+-loaded RBCs suspended in a Li^+-free (choline) medium or on Li^+-free RBCs suspended in a Li^+-containing medium. Since Li^+ influx is extremely slow (with a half-life of approximately 14 h),[22] the T_1 value obtained with Li^+-free RBCs suspended in a Li^+-containing medium represents the extracellular Li^+ contribution. However, there is an appreciable amount of Li^+ efflux from Li^+-loaded RBCs suspended in a choline medium (vide infra). Thus, the T_1 value obtained for Li^+-loaded RBCs in a non-Li^+ medium was analyzed in terms of two components in order to isolate the T_1 parameter for intracellular Li^+. All NMR spectra were measured in the absolute intensity mode without sample spinning by using 10-mm tubes. The Li^+ concentrations reported are based on relative peak areas of intra- and extracellular $^7Li^+$ NMR signals (vide infra). The integrated peak areas under the $^{23}Na^+$ resonances were converted into Na^+ concentrations after taking into account the 20% invisibility of the intracellular $^{23}Na^+$ signal.[23]

To monitor changes in cell volume during metal ion transport, hematocrit (using a IEC MB centrifuge microhematocrit, Needham Hts, MA) and Coulter counter (obtained with a Coulter Electronics Model ZM instrument, Hialeah, FL) measurements were taken at the same time intervals as for the NMR experiments. For the Coulter counter measurements, the same Na^+, choline, and Mg^{2+} suspension media described above were used to perform a 1:20000 dilution. The reported cell volume data take into account this dilution factor.

AA studies were carried out on a Perkin-Elmer 5000 spectrophotometer (Norwalk, CT) with graphite furnace. AA determinations of Na^+–Li^+ exchange rates and transmembrane Li^+ or Na^+ distributions were adapted from procedures described in refs 14 and 21, respectively. To initiate Li^+ efflux, Li^+-loaded RBCs were added to each of two sample tubes containing either isotonic Na^+-rich media (solutions C, E, or G, Table I) or choline-rich media (solutions D, F, or H) to a 0.13 final hematocrit and incubated at 37 °C in a water bath. The 500-μL aliquots were placed at 15-min intervals in precooled culture tubes. The aliquots were centrifuged at 2000*g* for 4 min at 4 °C; the supernatants were collected and after appropriate dilution analyzed for Li^+ on an AA spectrophotometer. Li^+ standards (0.2–42 μg/mL Li^+) were prepared in both Na^+ or choline media and calibration curves constructed accordingly. The Na^+–Li^+ countertransport rates were obtained as for NMR methods from the difference in the measured rates in the two media. In contrast to the NMR methods of measurement of Na^+–Li^+ transport, which were based on intracellular Li^+ concentrations, the AA values are based on supernatant Li^+ concentrations. Thus, no cell washing was required for the AA determinations.

Samples for AA determinations of RBC/plasma Li^+ or Na^+ ratios were prepared as described above for NMR measurements. The supernatant was separated by centrifugation, diluted, and analyzed for extracellular [Li^+]. The pelleted cells were washed three times with medium D before 100 μL of packed cells was lysed by addition of 3 mL of water. Intracellular [Li^+] was then analyzed by AA spectrometry.

LiCl, $DyCl_3$, $Dy(OH)_3$, glucose, sodium triphosphate (recrystallized three times from 40% ethanol), triethylenetetraminehexaacetic acid, and D_2O (99.8%) were supplied by Aldrich (St. Louis, MO). TrisCl, Triton X-100, ouabain, and gramicidin were from Sigma (St. Louis, MO).

Results

^7Li NMR Method Involving Shift Reagents. Intra- and extracellular Li^+ pools are not resolved in the ^7Li NMR spectrum of RBC suspensions not containing shift reagent.[22,24] Chemical shift separation of the two $^7Li^+$ resonances was previously reported by us[24] for a small concentration (3 mM) of the highly negatively charged shift reagent, $Dy(PPP)_2^{7-}$. Clear separation of the NMR signals corresponding to the two pools was also achieved by the addition of 7 mM $Dy(TTHA)^{3-}$ to the suspension medium.[25] The higher charge on the triphosphate reagent presumably leads to a larger number of lithium ions being bound to $Dy(PPP)_2^{7-}$ than to $Dy(TTHA)^{3-}$. pH and metal competition studies[25] have shown that $Dy(TTHA)^{3-}$ is the most promising shift reagent (among those tested thus far) for $^7Li^+$ NMR transport studies in that it interacts weakly with the lithium ion and yet it produces relatively large shifts that are independent of pH and are less subject to competition from monovalent and divalent cations such as Na^+, K^+, Ca^{2+}, and/or Mg^{2+}. In all RBC suspensions containing membrane-impermeable shift reagents, the shifted lithium NMR signal is assigned as being extracellular. The membrane-impermeability property of the shift reagents is presumably due to their high negative charge and the related decreased lipid solubility in RBC membrane phospholipids.

A comment is in order regarding the relaxation properties of the intra- and extracellular lithium NMR resonances. The T_1 values for the extracellular $^7Li^+$ signal in the presence and absence of shift reagent were 0.1 and 16.5 s, respectively, while the T_1 value for the intracellular $^7Li^+$ signal was 4.9 s.[24] Since the spin–lattice relaxation rates for the two lithium resonances were very different in magnitude, the ^7Li NMR spectra recorded in the presence of shift reagents were acquired in such a way that the relative peak areas of the two measured NMR resonances reflect the relative amounts of the two lithium pools. This was accomplished by employing a flip angle of 45° and a repetition rate of 7.5 s. With these settings, one can ensure that in each spectrum the $^7Li^+$ resonance with the longer relaxation time undergoes full relaxation between successive radio frequency pulses.

^7Li NMR Method Involving a Modified Inversion Recovery Pulse Sequence. By using a modified inversion recovery (MIR) method previously reported for $^{39}K^+$ NMR studies,[16] we were also able to differentiate between the two pools of lithium in RBCs (Figure 1). The pulse sequence takes advantage of the large difference in T_1 values for the intra- and extracellular $^7Li^+$ ions (5.4 ± 0.4 and 16.5 ± 0.5 s, n = 3, respectively). The standard one-dimensional ^7Li NMR spectrum (D–60°–AQ) of the RBC suspension depicted in Figure 1A contains only one signal that represents the overlapped intra- and extracellular $^7Li^+$ NMR resonances. A 60° flip angle and a delay D between single pulses of 60 s resulted at least in 95% relaxation of both lithium signals.

Figure 1B shows the ^7Li NMR spectrum of the intracellular lithium resonance obtained by the MIR method (D_1–180°–D_2–60°–AQ). Figure 2 shows the effect of varying D_2 on the $^7Li^+$ resonance intensities of Li^+-free RBCs in Li^+ medium, Li^+-loaded RBCs in Li^+-free medium, and Li^+-loaded RBCs in Li^+-containing suspension medium. At D_2 = 11.5 s, the extracellular $^7Li^+$ signal for Li^+-free RBCs in a Li^+-containing medium (triangles) is nulled,

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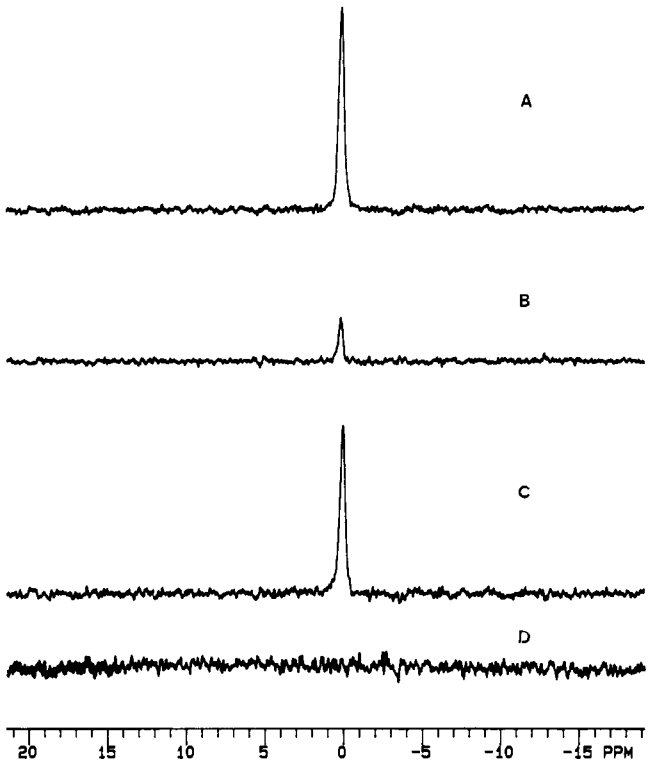

Figure 1. (A) ^7Li NMR (116.5 MHz, 37 °C) spectrum of Li^+-loaded control RBCs in a suspension containing 3.5 mM LiCl, 5 mM KCl, 140 mM NaCl, 10 mM glucose, and 50 mM HEPES, at pH 7.5. The hematocrit was 13%. 17% D_2O was present for field frequency lock. A single pulse sequence (D–60°–AQ) was employed, consisting of a repetition rate D of 60 s and a flip angle of 60° before spectral acquisition AQ of 1 s. The spectrum represents the two overlapped intra- and extracellular lithium pools. (B) ^7Li NMR spectrum of the same RBC suspension as in (A) except that a MIR pulse sequence (D_1–180°–D_2–60°–AQ) was used. The values of D_1 and D_2 were 60 and 11.5 s, respectively. The spectrum represents the intracellular $^7Li^+$ resonance. (C) The difference spectrum of parts A and B. The spectrum represents the extracellular $^7Li^+$ resonance. (D) ^7Li NMR spectrum of the Li^+-containing suspension medium (no RBCs), using the same MIR method as in (B). A total of eight scans were taken for each spectrum with a total accumulation time of approximately 9.7 min except for spectrum A, which took 8 min.

while the intracellular $^7Li^+$ resonance for Li^+-loaded RBCs suspended in a non-Li^+-containing medium (diamonds) has reached 81% of its maximum intensity. Intracellular Li^+ in packed Li^+-loaded RBCs has a T_1 value (5.4 ± 0.4 s, n = 3) identical with that of intracellular Li^+ from Li^+-loaded RBCs in a Li^+-containing medium (4.9 ± 0.5 s, n = 3). Similarly, Li^+ in the suspension medium alone has a T_1 value (17.0 ± 0.5 s, n = 3) identical with that of extracellular Li^+ from Li^+-free RBCs in a Li^+-containing medium (16.5 ± 0.5 s, n = 3). Therefore, all extracellular Li^+ in a Li^+-loaded RBC suspension is expected to be at a null in the MIR experiment for D_2 = 11.5 s (Figures 1B,D and 2, squares curve). The signal observed under the conditions specified in Figure 1B is therefore due to intracellular Li^+ only and not to a combination of intra- and extracellular signals. At a delay D_2 of 11.5 s and after a 60° pulse was applied, the magnetization component of extracellular $^7Li^+$ along the y axis is vanishingly small and its resonance disappears. This conclusion is confirmed by the absence of a signal in ^7Li NMR spectra of the Li^+-containing suspension medium with the same modified pulse sequence (Figure 1D). The difference spectrum depicted in Figure 1C represents the extracellular lithium pool.

The delay time D_2 for suppression of the extracellular $^7Li^+$ resonance and the relaxation time T_1 of the extracellular Li^+ are dependent on the nature of the suspension medium being used. Thus, for the experiments below using sodium and choline suspension media these parameters were remeasured. The T_1 (17.5 ± 0.50 s, n = 12, and 16.5 ± 0.50, n = 12) and D_2 values (11.5 ± 0.10, n = 12, and 11.5 ± 0.10, n = 12) were virtually the same in the choline- and salt-containing media. Good temperature control is also important for the MIR technique to work properly, since T_1 and consequently D_2 are temperature dependent.

Packed RBCs loaded with Li^+ to 0.2, 0.5, and 1.0 mM intracellular Li^+ concentrations gave T_1 values of 4.5 ± 0.5, 5.2 ± 0.4, and 5.4 ± 0.4 s, respectively. Thus, the intracellular T_1 value was found to be slightly dependent on intracellular Li^+ concentration as well as hematocrit concentration. Therefore, all the measurements reported here were carried out at the same initial intracellular Li^+ concentration, 1.0 mM, and at the same hematocrit, 13%. Under these conditions, the T_1 values of the intra- and extracellular Li^+ ions did not change significantly during Li^+ transport. The value of D_2 was not adjusted during Li^+ transport experiments. This was a minor source of error, since good agreement was obtained between the rates measured by MIR and AA (vide infra).

Visibility of the ^7Li NMR Resonance. Before converting the signal intensities obtained in the NMR experiments into concentrations, it is important to establish whether or not the observed $^7Li^+$ resonances reflect the entire population of the two lithium pools. This is because the $^7Li^+$ nucleus has a quadrupole moment[26] and, as a result, quadrupolar broadening could render part of the lithium signal invisible in the ^7Li NMR experiment. We subjected Li^+-loaded RBCs at 13% hematocrit suspended in an isotonic Li^+-free medium (medium D) to hemolysis. Cell hemolysis was achieved by including 10% Triton X-100 in the RBC suspension medium. Addition of $Dy(TTHA)^{3-}$ to the RBC suspension did not result in separation of two resonances, confirming that the medium was indeed Li^+ free. The total suspension volumes were the same before and after hemolysis. Thus, the intensities of the $^7Li^+$ resonances in the two experiments could be directly compared, since there was no need for a dilution correction with this cell hemolysis procedure. The RBC membrane was totally solubilized by the detergent, and thus, any Li^+ complexed to membrane phospholipids will also be measurable by this procedure. Treatment with Triton X-100 presumably disrupts specific metal ion–membrane interactions, and thus, it may ensure 100% NMR visibility of Li^+ ions. It was found that the intensity of the $^7Li^+$ NMR resonance did not change after cell hemolysis, indicating that lithium is 100% (98 ± 2, n = 5) visible in the NMR experiment. Li^+ concentrations from three RBC suspensions that had been loaded with different Li^+ levels were compared by AA and ^7Li NMR methods. The RBCs were found to contain 0.29, 0.50, and 0.90 mM Li^+ by AA and 0.31, 0.49, and 0.88 mM Li^+ by NMR. Determinations were done in triplicates, and the values obtained by the two methods are in very good agreement. The good agreement between intracellular Li^+ concentrations in Li^+-loaded RBCs measured by the two methods confirms 100% NMR visibility of intracellular Li^+ ions.

Similar experiments carried out on intracellular Na^+ by ^{23}Na NMR[23,27] spectroscopy have shown that the intracellular sodium pool in RBCs is only 80% visible in the ^{23}Na NMR experiment. However, addition of the Na^+ ionophore gramicidin resulted in depolarization of RBCs suspended in a non-Na^+ medium and rendered the Na^+ resonance 100% visible.[27] We have confirmed these $^{23}Na^+$ NMR observations in RBCs. The visibility of the $^{23}Na^+$ NMR resonance was 80% (80 ± 5, n = 3). The $^{23}Na^+$ NMR results were interpreted as evidence for the existence of a subpool of intracellular Na^+ ions experiencing quadrupolar broadening large enough to cause the disappearance of the corresponding resonance.[23,27] The existence of this sodium pool is dependent upon the presence of a negative membrane potential, as demonstrated by the effect of gramicidin on the intensity of the intracellular Na^+ resonance.[27] The NMR-invisible Na^+ ions may be interacting with negatively charged phospholipids on the

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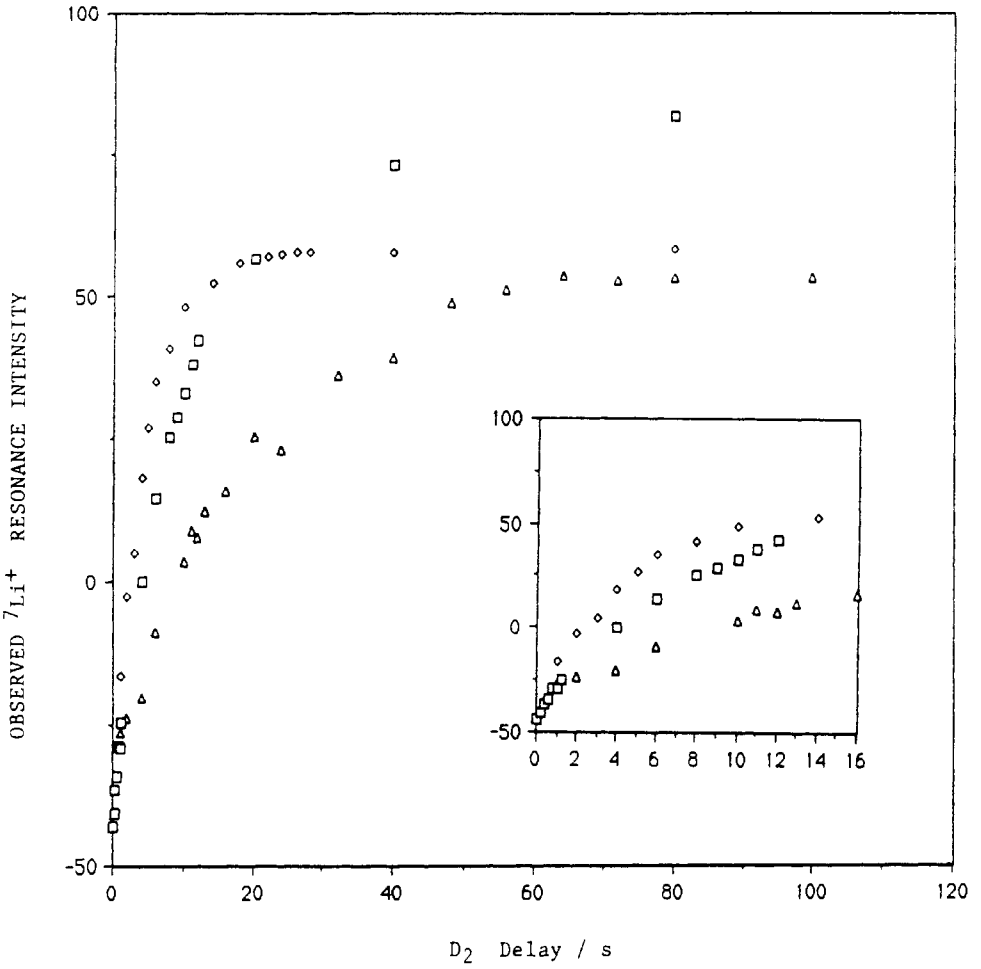

Figure 2. Plot of $^7Li^+$ resonance intensities of Li^+-loaded RBCs suspended in a non-Li^+-containing medium (diamonds), Li^+-free RBCs suspended in a Li^+-containing medium (triangles), and Li^+-loaded RBCs in a Li^+-containing medium (squares) as a function of delay time D_2, employing the MIR pulse sequence. The other acquisition parameters are as for Figure 1B. The Li^+-free suspension was medium D (Table I). The Li^+-containing suspension medium was the same as for Figure 1A.

cytoplasmic side of the RBC membrane, such as phosphatidyl serine, and must be in chemical exchange with free intracellular Na^+ ions. Although a subpool of intracellular Li^+ ions may also be present in RBCs, it does not lead to partial NMR invisibility. Several reasons may account for the different NMR visibility properties of Li^+ and Na^+ resonances. Ion competition studies[23] indicated that the binding sites of Li^+ and Na^+ to the RBC membrane are probably different. Moreover, the lower quadrupole moment[26] of the ^7Li nucleus (relative to that of the ^{23}Na nucleus) could also result in smaller quadrupolar broadening for membrane-bound Li^+ ions. This relaxation mechanism may not be large enough to cause invisibility of the intracellular ^7Li NMR resonance. However, the presence of bound Li^+ ions would be expected to result in shorter relaxation times for the intracellular $^7Li^+$ resonance in RBCs as observed (vide supra).

It is important to note that the question of ^{23}Na NMR visibility is controversial.[23] However, the good agreement between AA- and NMR-determined transmembrane Na^+ ratio (Table II) indicates that the conclusions from this study are not limited by this controversy. The different relaxation behavior of the intracellular $^{23}Na^+$ and $^7Li^+$ resonances in RBCs and the site of interaction of these metal cations with the cytoplasmic side of the RBC membrane are however beyond the scope of this paper.

Measurement of the Rates of the Na^+–Li^+ Countertransport in RBCs and Transmembrane Li^+ Distribution by ^7Li NMR Spectroscopy and Atomic Absorption. Cell volume changes during the time course of Na^+–Li^+ countertransport measurements by NMR spectroscopy and AA were analyzed by Coulter counter and hematocrit determinations for Li^+-loaded RBCs with an initial intracellular Li^+ concentration of 1.0 mM. During the time course of Li^+ transport in both Na^+- and choline-containing media, the hematocrit reading was constant at 0.13 ± 0.01 (n = 3) and the mean cell volume determined by the Coulter counter was 89 ± 0.2 fL (n = 3). Thus, there are no significant changes in cell volume during the 60-min period that rates of Na^+–Li^+ countertransport are being monitored by either NMR or AA methods. The observed intracellular Li^+ peak areas for the RBC suspensions, A_{obs}, are corrected, A, for total cell volume according to eq 1, where

$$A = (A_{obs}A_0)/A_{IN} \quad (1)$$

A_{IN} represents the MIR-determined peaks areas of intracellular Li^+ for standard RBC suspensions, containing AA-determined intracellular Li^+ in the 0.1–3.0 mM concentration range and A_0 represents the one-pulse NMR determined peak areas of Li^+ standard isotonic solutions (no RBCs) containing AA-determined intracellular Li^+ at the same Li^+ concentrations. The actual Li^+ concentration corresponding to A was extrapolated from a calibration curve of Li^+ resonance peak areas against Li^+ concentrations in millimolar units in suspension media alone.

The delay D_2 between pulses in the MIR experiment is sufficient to provide for only 81% relaxation of the magnetization of the intracellular Li^+ pool prior to the 60° excitation pulse. The observed intracellular Li^+ peak areas, A_{IN} and A_{obs}, represent only 81% of the total peak areas, and thus, this is the origin of the correction factor 0.81 in eqs 2 and 3, where f_{IN} represents the

$$A = A_{obs}/0.81f_{IN} \quad (2)$$

$$f_{IN} = A_{IN}/0.81A_0 \quad (3)$$

NMR-determined hematocrit (fraction of cells per volume of sample) within the portion of the NMR tube for which a sample signal is being collected. The hematocrit needs to be determined directly by MIR, f_{IN}, because at the hematocrit used in our experiments there is an appreciable amount of cell sedimentation.

Table II. Comparison of Transmembrane Li^+ and Na^+ Ratios ([RBC]/[plasma]) from Control RBCs Obtained by AA and Two NMR Methods, One Involving the Use of Shift Reagents (SR) and the Other a Modified Inversion Recovery Pulse Sequence (MIR)[a,b]

	AA			NMR		
		with SR			with SR	
	no SR $n = 12$	$Dy(TTHA)^{3-}$ $n = 3$	$Dy(PPP)_2^{7-}$ $n = 12$	MIR $n = 12$	$Dy(TTHA)^{3-}$ $n = 3$	$Dy(PPP)_2^{7-}$ $n = 12$
			A. Li^+ Ratios			
intact RBCs				0.35 ± 0.02	0.36 ± 0.02	0.48 ± 0.03
lysed RBCs	0.36 ± 0.02	0.35 ± 0.02	0.48 ± 0.03		0.35 ± 0.03	0.45 ± 0.01
			B. Na^+ Ratios			
intact RBCs				c	0.041 ± 0.001	0.027 ± 0.004
lysed RBCs	0.042 ± 0.004	0.041 ± 0.004	0.026 ± 0.004	c	0.043 ± 0.002	c

[a] A student t test was applied to the data, and the difference for Li^+ or Na^+ ratios measured in the presence or absence of 7 mM $Dy(PPP)_2^{7-}$ is significant up to a 99.9% confidence level. [b] Aliquots of the same blood sample were analyzed by the three methods. The composition of the suspension media used in the absence of shift reagents (medium I), in the presence of $Dy(PPP)_2^{7-}$ (medium J), and in the presence of $Dy(TTHA)^{3-}$ (medium K) are indicated in Table I. [c] Not determined.

However, the protocol given above corrects for cell sedimentation during the NMR experiments. The intracellular Li^+ peak areas obtained by MIR (Figure 1B) are therefore underestimated by approximately 20%, while the extracellular Li^+ peak areas obtained by spectral subtraction (Figure 1C) are overestimated. Thus, the MIR procedure outlined above (eq 1) for determining intracellular Li^+ concentrations is self-correcting, since the final value of A is independent of the correction factor (by combining eqs 2 and 3, we obtain eq 1).

Table II shows a comparison of transmembrane Li^+ or Na^+ distributions in normal RBCs determined by AA and the two NMR methods. NMR experiments are generally carried out on intact RBC suspensions, while the AA measurements require cell lysis prior to analysis. Thus, for purposes of comparison, ^7Li NMR measurements were carried out on both intact RBC suspensions and lysed RBCs (as for AA determinations). Since the determination of intracellular Li^+ concentration by one of the NMR methods required the incorporation of a shift reagent in the suspension medium, AA measurements were also taken for RBC samples that had been treated with shift reagent.

The values obtained for transmembrane Li^+ and Na^+ distributions in control RBCs by AA and NMR spectroscopy involving either the MIR pulse sequence or the use of the shift reagent $Dy(TTHA)^{3-}$ correlated significantly ($r = 0.976$). The good agreement between AA and MIR data also indicates that the equations outlined above for determination of Li^+ concentrations by the MIR method are valid. However, the Li^+ ratios obtained in the presence and absence of 7 mM $Dy(PPP)_2^{7-}$ are quite different from those obtained by AA or the NMR method in the presence of 7 mM $Dy(TTHA)^{3-}$. These observations are in agreement with the known higher affinity of $Dy(PPP)_2^{7-}$ (K = 740 M^{-1}), relative to $Dy(TTHA)^{3-}$ ($K < 50$ M^{-1}), for the Li^+ ion.[25] The steady-state transmembrane Li^+ and Na^+ ratios (RBC cation concentration/suspension medium cation concentration) for normal RBCs measured in the presence of triphosphate reagent are respectively higher and lower than those measured in its absence. Thus, the addition of $Dy(PPP)_2^{7-}$ to the suspension medium leads to more binding of extracellular Na^+ ions (since they are present at a 100-fold excess over Li^+ ions), which in turn may cause the efflux of intracellular Na^+ ions. A 3-mM concentration of $Dy(PPP)_2^{7-}$ was also tried leading to Li^+ and Na^+ ratios (data not shown) different from those obtained with 7 mM $Dy(PPP)_2^{7-}$, suggesting that competition between extracellular Na^+ and Li^+ ions for the triphosphate shift reagent is significant. More extracellular Na^+ ions are bound to $Dy(PPP)_2^{7-}$ presumably because they are present in large excess relative to extracellular Li^+ ions and the affinities of Na^+ and Li^+ ions for $Dy(PPP)_2^{7-}$ are of the same order of magnitude.[25] The efflux of intracellular Na^+ ions is partially compensated by an influx of extracellular Li^+ ions leading to higher values of transmembrane Li^+ ratios in Li^+-loaded RBCs in the presence of the triphosphate shift reagent.

From the changes in intracellular Li^+ peak areas observed by the MIR method when Li^+-loaded RBCs from healthy donors are suspended in either a Na^+- or choline-containing medium, the intracellular Li^+ concentrations at different time intervals (Figure 3) were calculated according to eq 1. By subtracting the slope of the curve obtained in the choline medium from that obtained in the Na^+ medium, we determined the rate of Na^+–Li^+ countertransport in Li^+-loaded RBCs.[14] Table III shows a comparison of Li^+ transport rates in Na^+ and choline media as well as difference rates (Na^+–Li^+ countertransport) for Li^+–loaded RBCs determined by AA and the two ^7Li NMR methods. Once again, only the use of the shift reagent $Dy(PPP)_2^{7-}$ caused significant increases in the rates of Na^+–Li^+ exchange in control RBCs. Moreover, the rates of Li^+ transport measured in MIR and AA in the absence of triphosphate shift reagent correlated significantly ($r = 0.982$), indicating the validity of eq 1 for determining Li^+ concentrations from MIR data. The effect of $Dy(PPP)_2^{7-}$ on the rates of Li^+ transport is present in both Na^+ and choline media E and F, thus ruling out any specific effect of choline (Table III); this is smaller in the choline medium F than in the Na^+ medium E presumably because of the lower Na^+ content in medium F. To rule out any specific effect of choline,[28] Na^+–Li^+ counter-

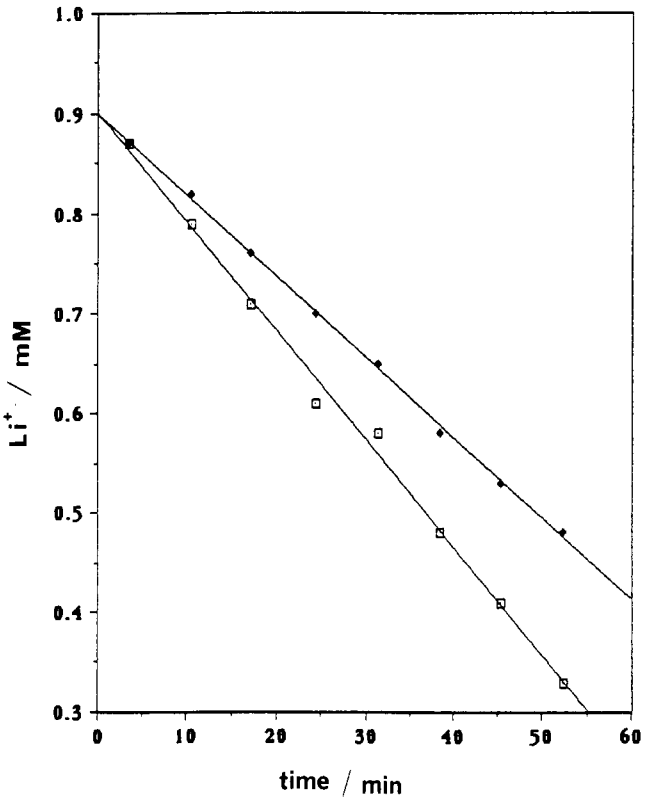

Figure 3. Time dependence of intracellular Li^+ concentration in Na^+ (open squares) and choline (closed diamonds) media. The time intervals correspond to the midpoints of the accumulation periods. ^7Li NMR spectra were recorded by using the MIR method described in Figure 1B. The intracellular Li^+ concentrations were calculated according to eq 1.

Table III. Comparison of Li^+ Transport Rates in Na^+ and Choline Media and Difference (Na^+–Li^+ Countertransport) Rates [mmol of Li^+/(L of RBCs h)] Obtained by AA and Two ^7Li NMR Methods, One Involving the Use of Shift Reagents (SR) and the Other a Modified Inversion Recovery Pulse Sequence (MIR)[a]

		rate		
technique	type of rate	no SR $n = 12$	7 mM $Dy(PPP)_2^{7-}$ $n = 12$	7 mM $Dy(TTHA)^{3-}$ $n = 3$
AA	sodium	0.49 ± 0.03	0.68 ± 0.04	0.51 ± 0.02
	choline	0.28 ± 0.02	0.34 ± 0.03	0.30 ± 0.02
	difference	0.21 ± 0.03	0.34 ± 0.04	0.21 ± 0.02
MIR	sodium	0.48 ± 0.02		
	choline	0.26 ± 0.02		
	difference	0.22 ± 0.02		
SR	sodium		0.66 ± 0.02	0.50 ± 0.01
	choline		0.33 ± 0.02	0.27 ± 0.01
	difference		0.33 ± 0.02	0.23 ± 0.01

[a] The compositions of the sodium and choline suspension media (C and D) used for measurements in the absence of shift reagents are listed on Table I. For $Dy(PPP)_2^{7-}$- and $Dy(TTHA)^{3-}$-containing samples, the Na^+ media E and G, and the choline media F and H, were used instead. A student t test shows that the data on the difference between Na^+–Li^+ countertransport rates measured in the presence or absence of 7 mM $Dy(PPP)_2^{7-}$ is significant up to a 99.9% confidence level.

transport rates were also measured in a Mg^{2+} medium by atomic absorption, by MIR, and with the aid of $Dy(TTHA)^{3-}$. In all three cases, the difference rates measured in a Mg^{2+} medium (0.21 ± 0.03 mmol of Li^+/(L of RBCs h), $n = 3$) were in good agreement with those determined in a choline medium (Table III).

All the values reported in Tables II and III for shift reagent containing samples were measured under isotonic conditions by replacing some of the salt in the suspension media for shift reagent and readjusting the osmolarity to 300 ± 10 mosM with sucrose. Thus, the effect of $Dy(PPP)_2^{7-}$ on the RBC rates and ratios is a specific shift reagent effect rather than a general osmolarity effect. In fact, we verified that direct addition of 3 mM $Dy(PPP)_2^{7-}$ to the RBC suspension medium caused the transmembrane Li^+ ratio to increase to 0.81 (rather than 0.48). By contrast, direct addition of 7 mM $Dy(TTHA)^{3-}$ to the RBC suspension did not cause any significant change in the transmembrane Li^+ ratio. Since direct addition of the triphosphate shift reagent results in a 30 mosM increase in the osmolarity of the medium, water presumably leaked from the cells, resulting in abnormally high intracellular and low extracellular Li^+ concentrations. Transmembrane Li^+ ratios are likely to be overestimated if a shift reagent is added, rather than incorporated, in the RBC suspension medium. Thus, whenever using NMR shift reagents to study ion distribution in cell suspensions, one must ensure that the osmolarity of the suspension medium and the ion distribution across the cellular membrane are the same in the presence and absence of a shift reagent.

Discussion

Several applications of both ^1H and ^7Li NMR techniques to the problems of bipolar disorders and related lithium therapy have appeared in the literature.[22,24,25,28-34] In this paper, we report on the application of two different ^7Li NMR methods to the measurement of Na^+–Li^+ exchange (or countertransport) rates in RBCs (Table III) and transmembrane Li^+ ratios (Table II). Comparison of the results to those obtained by the standard AA method was also carried out. The data measured by MIR and AA in the absence of shift reagent correlated significantly, and so did the data measured by the shift reagent method and AA in the presence of shift reagent. The good agreement with AA measurements ensured the validity of the two NMR methods.

The discrimination between the two lithium pools in Li^+-loaded RBCs by ^7Li NMR spectroscopy with the help of the shift reagent $Dy(PPP)_2^{7-}$ had been previously reported.[22,24,25] Because of the high negative charge of this shift reagent, the amount of extracellular Li^+ (and other cations) complexed to $Dy(PPP)_2^{7-}$ is significant and it changes the distribution of Li^+ ions and other cations across the RBC membrane. During ion transport this complexity is exacerbated by the fact that the amount of Li^+ complexed to the shift reagent will vary, and thus, it will be difficult to correct for the effects of complexation during an ion transport experiment. The effect of the shift reagent $Dy(PPP)_2^{7-}$ on the measured transmembrane Li^+ ratios and rates of Na^+–Li^+ countertransport in Li^+-loaded RBCs is apparent from Tables II and III. The effect of $Dy(PPP)_2^{7-}$ is not just present in choline media, since it was observed in Na^+ suspension media to an even greater extent (Table III). The fact that both the ^7Li NMR method employing $Dy(PPP)_2^{7-}$ and AA in the presence of the same shift reagent show Li^+ transport and distribution properties in RBCs different from those measured by ^7Li MIR, ^7Li NMR spectroscopy with $Dy(TTHA)^{3-}$, and AA in the absence of shift reagent indicates that $Dy(PPP)_2^{7-}$ has a specific effect on ion transport in RBCs. The triphosphate shift reagent should therefore be used with caution in biological NMR transport studies. We found that this problem of complexation is eliminated when a shift reagent with a lower negative charge, like $Dy(TTHA)^{3-}$, is used. The absence of an effect of $Dy(TTHA)^{3-}$ on Li^+ distribution and transport in Li^+-loaded RBC suspensions might suggest that it is a superior shift reagent for ion transport studies. However, it was shown recently that both $Dy(TTHA)^{3-}$ and $Dy(PPP)_2^{7-}$ display significant renal toxicity.[35]

A noninvasive ^7Li NMR method based on a MIR pulse sequence[16] is reported here. This technique does not suffer from the limitations introduced by AA and the use of shift reagents in ^7Li NMR spectroscopy in that there is no sample destruction, potential ion binding to cell membranes during washing steps, or effects on transmembrane ion distribution. Potential drawbacks of the MIR technique are the dependence of the T_1 and D_2 parameters on hematocrit, temperature extent of RBC Li^+ loading, and nature of suspension medium. For different experimental conditions, controls such as those shown in Figure 2 must be carried out. Atomic absorption and flame photometry involve invasive procedures in that physical separation of RBCs and suspension medium or plasma are required prior to chemical analysis. The need for separation of intra- and extracellular compartments in the AA method could potentially lead to errors related to nonspecific ion binding to membranes and additional ion transport during blood processing. However, the good agreement between the AA and MIR data reported here indicates that the previously published AA data were indeed reliable. By use of the AA approach, initial information on Li^+ transport is however lost as a result of the additional centrifugation steps required. For batch processing of a very large number of samples, AA continues to be the preferred method, since each individual measurement takes a few seconds as opposed to approximately 10 min for MIR (see Figure 1). However, the MIR method reported here has the ability of providing additional new information about Li^+ interactions with RBC membrane components and metabolites.[19]

The reason for the difference in T_1 values for the intra- and extracellular Li^+ pools, which constitutes the basis for the MIR method, will be investigated in the future. The short intracellular

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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.

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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