1993

Nmr Study of Na+-H+ and Na+-Li+ Exchange in Human Erythrocytes

Suilan Mo
Loyola University Chicago

LOYOLA UNIVERSITY OF CHICAGO

NMR STUDY OF Na^+-H^+ AND Na^+-Li^+ EXCHANGE IN HUMAN ERYTHROCYTES

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

BY

SUILAN MO

CHICAGO, ILLINOIS

January 1993

ACKNOWLEDGMENTS

I am grateful to my mentor Dr. Duarte Mota de Freitas for the excellent supervision. I am also very thankful to Dr. Duarte Mota de Freitas for the intellectual stimulation, encouragement, moral and financial support that he gracefully provided during this study. He helped postulate innovative research problems of biological interest.

My grateful appreciation to Dr. Kenneth Olsen and Dr. Daniel Graham for their excellent suggestions during the course of this work. I thank Dr. Leslie W. Fung for helping me join the graduate program at Loyola.

I sincerely thank my labmates Qinfen Rong, Aida Abraha, Lisa Wittenkeller and Dr. Elizabeth Dorus for their encouragement and constructive criticisms.

I gratefully acknowledge the support of faculty, staff and all the graduate students in the Chemistry Department. I thank the Graduate School for a Teaching Assistant Fellowship.

Special thanks to the Raffensperger family. I appreciate their great help and support during a difficult time in my life.

Finally, I am very thankful to the omniscient Lord for all of these blessings.

To my dear parents

TABLE OF CONTENTS

LIST OF PUBLICATIONS

Mota de Freitas, D., Rong, Q., Mo, S.: Reinvestigation of the Effects of Hematocrit on Li^+ Uptake and on ^7Li NMR T_1 Values in Human Erythrocyte Suspension. Magn. Reson. Med. (Accepted)

Mota de Freitas, D., Abraha, A., Rong, Q., Mo. S., Wittenkeller, L.: Elucidation of Transport Mechanisms for Alkali Cations in Human RBC by Metal NMR. J. Inorg. Biochem. 43: 386, 1991.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	atomic absorption
CPMG	Carl-Purcell-Meiboom-Gill
δ	chemical shift
$\Delta\nu_{1/2}$	linewidth at half height
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DMSO	dimethylsulfoxide
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
Ht	hematocrit
MeP	methylphosphonic acid
MES	2-[N-morpholino]ethanesulfonic acid
MIR	modified inversion recovery
MOPS	3-[N-morpholino]propanesulfonic acid
mOsm	mmol/kg
NMR	nuclear magnetic resonance
PPP	triphosphate
RBCs	red blood cells
T_1	spin-lattice relaxation time
T_2	spin-spin relaxation time
TTHA	triethylenetetraminehexaacetate
Tris	tris-(hydroxymethyl)-aminomethane

CHAPTER I

INTRODUCTION

1. Na^+-H^+ Exchange in RBC

The Na^+-H^+ exchange ion transport pathway is the exchange of intracellular H^+ with Na^+ present in the plasma or in the suspension medium. Na^+-H^+ exchange plays an important role in regulation of intracellular pH (pH_i), cell volume and transmembrane ion transport (1). A cellular acidification increases the activity of the exchanger, thus allowing cells to exclude the excess of protons and to recover their initial pH. Because of its relatively alkaline pH_i dependence, the Na^+-H^+ exchange system of most excitable cells catalyzes a significant uptake of Na^+ at a physiological pH_i value. The Na^+-H^+ exchange system may or may not be involved in volume regulation, depending on the cell type. In human red blood cells, the Na^+-H^+ exchange system is activated in response to exposure to hyperosmolar solutions and contributes to volume regulation. Activation of Na^+-H^+ exchange has also been proposed to be a signal of cellular differentiation. The activity of the exchanger appears to be modulated by several important biological agents, including hormones, growth factors, and tumor-promoting agents (1). Na^+-H^+ exchange may provide a mechanism for raising blood pressure by increasing intracellular Na^+ concentration or pH resulting in hypertension (2). The mean rate of Na^+-H^+ exchange in red blood cells (RBCs) from hypertensive patients is higher than the mean rate measured in RBCs from normotensive controls (3).

Lifton et al. (4) have cloned a gene encoding a RBC membrane protein effecting

amiloride-sensitive Na^+-H^+ exchange. They mapped the gene locus to the short arm of chromosome 1 (1p).

Amiloride, which is an inhibitor of Na^+-H^+ exchange, blocked about 60% of Na^+ transport when there was a pH gradient, but showed small inhibitory effects in the absence of pH gradients (5). This Na^+-H^+ transport pathway was also sensitive to the amiloride analog 5-N-methyl-N-butyl-amiloride (5). In the absence of Na^+ gradient, an outward H^+ gradient can drive a net amiloride-sensitive Na^+ uptake (5). Intracellular Ca^{2+} can also activate Na^+ transport which was inhibited to 30-60% by amiloride (6). At the condition of abolished proton gradient ($pH_{in}=pH_{out}$) across the membrane, isosmotic and hyperosmotic media also stimulate Na^+-H^+ exchange (7). The stoichiometry of Na^+ and H^+ fluxes by the Na^+-H^+ exchanger suggests a 1:1 coupling of Na^+:H^+ fluxes over wide ranges of Na^+ and proton concentrations (1). The activation of net transport by Na^+ in the extracellular solution suggests that only a single binding site may be kinetically resolved (1). The internal H^+ stimulates the net influx of Na^+ with sigmoidal kinetics. Aronson et al. (8) proposed that the action of a modifier site alters the kinetics of activation because the dependence of net flux on internal $[H^+]$ had a slope which was greater than 1, suggesting kinetics with an order larger than first order. The kinetic information on Na^+-H^+ exchange suggest two possible models: one model is a random-binding simultaneous model, and this varies between cell types; another hypothesis is a modifier site on the cell membrane (1).

Amiloride-sensitive Na^+-H^+ transport is activated by cytoplasmic lithium (9). The flux due to external Li^+ has a stoichiometry of 1:1 and is sensitive to amiloride (9). Amiloride acts as a competitive inhibitor vs external Li^+ and Na^+ acts as a competitive inhibitor of net Li^+ influx. Conversely, external Li^+ acts as a competitive inhibitor of net Na^+ influx (10). All the information suggests that Na^+, Li^+ and amiloride share a single external transport site.

Canessa et al. (11) investigated the kinetic effects of internal and external pH on Na^+-H^+, Li^+-H^+ and Na^+-Li^+ exchange. These three exchange modes have highly asymmetric affinities $H^+ > > Li^+ > Na^+$. They are activated by internal H^+ with a high "Hill coefficient" (n=2.5), and are competitively inhibited by external H^+. Cell Li^+ activated Na^+-H^+ exchange and was transported by Na^+-Li^+ exchange at a lower rate than internal H^+ (11).

2. Na^+-Li^+ Exchange in RBC

The Na^+-Li^+ ion exchange transport pathway in RBCs refers to the exchange of intracellular Li^+ in Li^+-loaded RBCs with Na^+ present in the plasma or in the suspension medium. Canessa et al. (12) found that treated or untreated hypertensive patients had a significantly higher rate of RBC Na^+-Li^+ exchange than normotensive individuals. The rate of RBC Na^+-Li^+ exchange is consistently higher in hypertensives and in normotensives having a family history of hypertension than in normotensives who have no family history of hypertension (13). An autosomal gene locus encodes a polypeptide chain of the Na^+-Li^+ exchange protein (14). This gene has not been mapped but it is known that it is located at one of the 22 pairs of nonsex autosomal chromosomes of the total 23 pairs of human chromosomes.

The kinetics of Na^+-Li^+ exchange or countertransport in human RBCs was studied (15,16). A "ping-pong" model was proposed for the mechanism of Na^+-Li^+ countertransport in RBC. The intracellular Na^+ or Li^+ (Na^+_i or Li^+_i) bound to a carrier or an active site on the inner side of cell membrane was translocated to the outer side of cell membrane and then released to the outside of the membrane. The ion translocation through the membrane was the rate-limiting step. There are other Li^+ pathways in RBC, such as the leak pathway, the ouabain-sensitive sodium pump, and the chloride-dependent Na^+-K^+ cotransport system. All of these transport pathways are also used by the Na^+ ion. The Li^+ ion can also enter the RBC

by the band 3 protein; this transport pathway is, however, not shared by Na^+ (17).

Na^+-Li^+ countertransport rates in RBCs from essential hypertensive patients are higher than in RBCs from normotensive controls (18-20). Because Na^+-H^+ exchange also has higher mean rates in hypertensive patients than in normotensive controls, and the Na^+-H^+ exchange is significantly correlated with Na^+-Li^+ exchange (21), an important question arises. Are these two transport pathways mediated via the same transport protein or not?

3. The Importance of Na^+-H^+ and Na^+-Li^+ Exchange in Hypertension

When a person has diastolic blood pressure higher than 90 mmHg, he or she is considered to be hypertensive. When there is no any other medical cause for high blood pressure, the condition is defined as essential hypertension; otherwise it is referred to as secondary hypertension. The Na^+-H^+ transport rate is defined as the Na^+ influx in acid-loaded RBC suspensions at two different pH values. Study of hypertensive patients with insulin dependent diabetes mellitus (IDDM) showed that Na^+-H^+ exchange and Na^+-Li^+ exchange were significantly correlated in hypertensive, normotensive diabetics and controls (3). Na^+-H^+ exchange rates were 78 ± 28 mmol/L cell/hr in hypertensive IDDM, 50 ± 21 in normotensive IDDM, and 55 ± 24 in controls. Li^+-Na^+ exchange rates were 0.37 ± 0.13 mmol/L cell/hr in hypertensive IDDM, 0.27 ± 0.10 in normotensive IDDM, 0.25 ± 0.11 in controls (3). These studies suggested that Na^+-H^+ and Na^+-Li^+ transport may be operated by the same exchange protein in the RBC membrane. Evidence exists, however, that Na^+-H^+ and Na^+-Li^+ transport may be effected by different proteins. Amiloride is an inhibitor of human RBC Na^+-H^+ exchange (5,6), but not of Na^+-Li^+ exchange (22). A study of Na^+-Li^+ countertransport with aging erythrocytes showed that the transport rate increased with aging blood; this increase was due to a phloretin insensitive component not present in young erythrocytes (23). The increase

of Na^+-Li^+ countertransport rate in essential hypertensive patients was mainly due to an increased phloretin sensitive component. Amiloride had no effect upon Na^+-Li^+ exchange in cells of any age (23). Amiloride inhibited Na^+-H^+ exchange for rabbit RBCs and brush border vesicles (24,25). In contrast, amiloride inhibited both Na^+-H^+ and Na^+-Li^+ exchange in bovine vascular smooth muscle (26). Whereas Na^+-Li^+ exchange is readily observable in human RBC at physiological conditions (12,27), Na^+-H^+ exchange can only be activated under special conditions, such as acid loading or calcium activation (5,6), suggesting that the Na^+-Li^+ countertransport was not a mode of action of the Na^+-H^+ exchange in RBCs.

Na^+-Li^+ exchange rates were lower in RBC from manic-depressive patients receiving lithium carbonate than in normal individuals (17, 28). Na^+-H^+ exchange rates have not been measured in RBCs from manic-depressive patients, and it is not known what is the involvement, if any, of Na^+-H^+ exchange in the etiology of manic-depression.

Based on these arguments, a molecular level study of Na^+-H^+ and Na^+-Li^+ exchange pathways in human RBCs is required to decide whether these two transport mechanisms occur via the same membrane protein or not.

4. Techniques for Measurement of Metal Ion Transport

Measurements of Na^+-Li^+ countertransport in human RBC suspensions have been obtained using NMR and atomic absorption (AA) methods. The rate of Na^+-Li^+ exchange determined using a special NMR method, a modified inversion recovery (MIR) pulse sequence, was significantly correlated with the measurements made by AA (28,29). The rate of Na^+-Li^+ exchange for 10 hypertensive patients (0.53 ± 0.02 mmol/L cell/hr) was significantly higher than the mean rates for 10 matched normal controls (0.22 ± 0.02 mmol/L cell/hr) (29). Similar NMR and AA measurements were obtained for psychiatric patients receiving lithium carbonate

and normal controls (28); that study showed that the rates of Na^+-Li^+ countertransport from 14 psychiatric patients on lithium therapy were significantly lower than the rates from 14 normal controls (0.12 ± 0.03 mmol/L cell/hr for patients and 0.22 ± 0.03 mmol/L cell/hr for controls).

Lanthanide shift reagents have been used to distinguish the intra- and extracellular pools of alkali-metal ions (30-34). $Dy(PPP)_2^{7-}$ gives larger upfield chemical shifts than the downfield shifts induced by $Dy(TTHA)^{3-}$. Whereas the alkali metal NMR shifts afforded by $Dy(PPP)_2^{7-}$ are dependent on pH and subject to competition by other metal cations, those afforded by $Dy(TTHA)^{3-}$ are not (35). Because of its high negative charge, the shift reagent $Dy(PPP)_2^{7-}$ increases the ionic transport rates and changes the transmembrane ion distribution and membrane potential (32,33). The Li^+ transport rates measured in the presence of $Dy(PPP)_2^{7-}$ by ^7Li NMR 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 ($[Li^+]_{RBC}/[Li^+]_{plasma}$) measured in the presence of triphosphate shift reagent were higher than those measured in the two other suspension media under the same conditions (32,33). These effects are related to the mode of binding of alkali metal cations to $Dy(PPP)_2^{7-}$ (31). Shift reagents with a lower negative charge such as $Dy(TTHA)^{3-}$ do not affect the Li^+ transport rate and membrane potential (32,33).

Studies on ^7Li relaxation time measurements of Li^+-loaded RBCs have been conducted (36,37). The spin-lattice relaxation time (T_1) of ^7Li in Li^+-loaded RBCs with approximately 1.5 mM intracellular Li^+ concentration was significantly longer (5.6 ± 0.4 s) than the spin-spin relaxation time (T_2) (0.09 ± 0.02 s) (n=3). Li^+-loaded ghosts with 1.5 mM Li^+ concentration also have longer T_1 (11.1 ± 0.90 s) than T_2 values (0.17 ± 0.02 s) (n=3). Slow motions contribute only to T_2, whereas components of motion at the resonance frequency contribute to both T_1 and T_2 (38). The relaxation times for a 1.5 mM LiCl aqueous solution whose viscosity

was adjusted to that of intracellular RBC (5 cP) with glycerol were 1.0 ± 0.1 s for T_1 and 0.8 ± 0.1 s for T_2 (36,37). Thus, the high intracellular viscosity decreased both T_1 and T_2 values but was not responsible for the difference between T_1 and T_2 values observed in Li^+-loaded RBCs. Because the same relaxation behavior was observed in RBC ghosts and intact RBCs, this difference between T_1 and T_2 for intracellular Li^+ was attributed to the binding of Li^+ to the RBC membrane. This proves that Li^+ binds to the RBC membrane and ^7Li NMR relaxation times are a good probe to detect Li^+ binding information to the RBC membrane.

Canessa, M. (39) studied Na^+-H^+ transport in RBC using AA. The transport rates had large interindividual differences in red cells of normal subjects (10-40 mmol/L cell/hr). ^{23}Na NMR studies on Na^+ binding to human RBC membrane were conducted by several groups (40-42). Evidence for K^+ competition with Na^+ binding sites on erythrocyte membrane was demonstrated by a decrease of the sodium line width in the presence of K^+ (40). The NMR longitudinal relaxation rate ($1/T_1$) of ^{23}Na was studied on unsealed human RBC ghosts from hypertensive patients and normotensive controls in the presence and absence of ouabain (41,42). Na^+ had tighter binding with normotensives and weaker binding with hypertensives; using James-Noggle plots, hypertensives gave binding constants of the order of 100 M^{-1} ($n=2$) and normotensives had relatively higher binding constants in the range of 500-1000 M^{-1} ($n=7$). By adding 5 mM ouabain, which is an inhibitor for $(Na^+ + K^+)$-ATPase, the ^{23}Na T_1 was increased and the binding constant was about 2000 M^{-1} (42). At lower $[Na^+]$ ranges, Na^+ binds to the membrane protein $(Na^+ + K^+)$-ATPase. As $[Na^+]$ increases (>5 mM), Na^+ started to bind to other membrane proteins combined with lipids (41).

The NMR visibility of intracellular Na^+ in RBC is about 80% (43), whereas that of intracellular Li^+ is 100% (32). Therefore, the visibility factor of 0.8 must be taken into account in the Na^+ transport measurements by ^{23}Na NMR of human RBC suspensions.

CHAPTER II

STATEMENT OF THE PROBLEM

The primary goal of this project is to study the binding of Na^+ and Li^+ ions to human RBC membrane using nuclear magnetic resonance relaxation methods to advance the understanding of Na^+-H^+ and Na^+-Li^+ transport in human RBCs.

AA studies of Na^+-Li^+ transport in human RBC suspensions were previously performed (18,28,29,39). Also AA measurements of Na^+-H^+ transport in human RBC suspensions have been conducted (39). A shortfall of the AA method is that separation of cells from the suspension medium and lysis of the intracellular compartment is required prior to AA analysis. An ionic concentration adjustment during sample processing could occur. The initial ion transport information may also be lost under these conditions. In addition to being invasive, these techniques are time consuming. The AA method loses vital information on ion binding to membrane, the nature of ion binding sites, and changes in membrane potential.

The NMR method used in this study enables detection of Na^+ and Li^+ concentrations and pH changes while transport occurs. The NMR measurements are performed on the same blood sample without interrupting ion transport. Concentrations and pH values are obtained from intact viable cells. The NMR relaxation measurements provide a detailed molecular understanding of the factors that control the rates of transmembrane ion transport. Such an NMR investigation of the Na^+-H^+ transport pathway in human RBCs had never been conducted before.

1. Characterization of the Na^+ and Li^+ Binding Sites in Human RBC Membranes

According to Canessa's work (11), the three ions of interest H^+, Li^+ and Na^+ have the following relative affinities in red blood cells: $H^+ > > Li^+ > Na^+$, and the interaction of these ions with the two sides of the RBC membrane is asymmetric. I propose to study the ion competition mechanism using ^7Li and ^{23}Na NMR measurements of human RBC membrane samples at different pH values.

2. Characterization of the Effect of Inhibitors on Ion Transport

Previous studies showed that amiloride, which is an inhibitor for Na^+-H^+ transport, had no effect on Na^+-Li^+ countertransport (22) and phloretin, which is an inhibitor for Na^+-Li^+ exchange, had no effect on Na^+-H^+ transport (23). Inhibition effects may not show up in ion transport assays because of their low sensitivity. A NMR relaxation time study was designed to study the competition between metal ions and inhibitors for the human RBC membrane.

3. Na^+-H^+ Transport in Human RBCs by ^{23}Na and ^{31}P NMR

I propose to use ^{23}Na NMR spectroscopy to monitor Na^+ flux through the Na^+-H^+ exchange pathway in human RBC suspensions by using the shift reagent $Dy(TTHA)^{3-}$.

The indicator methylphosphonate (MeP) was used to monitor intracellular pH (44-46). I propose to use MeP to detect pH changes during the course of Na^+-H^+ exchange in human RBC suspensions.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1. Reagents

Lithium chloride (LiCl), dysprosium chloride ($DyCl_3$), triethylenetetraamine hexacetic acid (TTHA), deuterium oxide (99.8% D_2O), sucrose, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), choline chloride and methyl sulfoxide (DMSO) were supplied by Aldrich (Wisconsin). HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], Tris-(hydroxymethyl)aminomethane (Tris), bumetanide, amiloride, ouabain, MES (2-[N-morpholino]ethanesulfonic acid), MOPS (3-[N-morpholino]propanesulfonic acid), DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid), nystatin, phloretin and albumin (bovine fraction V) were supplied by Sigma Chemical Company (St. Louis, MO). Methazolamide was supplied by Lederle Laboratories Division of the American Cyanamide Company (Pearl River, NY). n-butyl phthalate from Fisher Scientific Company (Fairlawn, NJ). Methylphosphonic acid was from Alfa Products (Ward Hill, MA). All chemicals were used as received.

1.2. Blood samples

Fresh red blood cells were supplied by the Chicago Chapter of Life Source. Blood samples from hypertensive patients were obtained through Loyola University Medical Center. The protocols for experiments involving human blood were approved by the Institutional

Review Board for the Protection of Human Subjects (Loyola University Medical Center).

2. Sample Preparation

2.1. Preparation of acid-loaded RBCs (39)

The intracellular pH of 5.8 in RBCs was reached by incubating the cells at 10% Ht in acid-loading solution (170 mM KCl, 40 mM sucrose, 10 mM glucose, 20 mM Tris-MES buffer, pH=5.6, 0.1 mM ouabain, 0.01 mM bumetanide, 0.15 mM $MgCl_2$, 350 mOsm) for 10 minutes in a 37 °C water bath. I then added 1.0 ml of acid-loading solution containing DIDS and methazolamide to lock the cell pH. The final concentration of DIDS was 0.2 mM and methazolamide was 0.5 mM. The cell suspensions were kept at 37 °C for an additional 30 minutes. These acid-loaded cells were washed at 4 °C four times with 5 volumes of acid-washing solution (170 mM KCl, 40 mM sucrose, 0.15 mM $MgCl_2$).

The intracellular pH was checked by lysing 0.2 ml of packed RBCs in 2 ml of double distilled water allowing 5 minutes to reach equilibrium. The acid-loading solution was made fresh and I checked the pH before cell loading. The inhibitors were made fresh daily and added to acid-loading solutions before cell loading.

2.2. Preparation of sodium-modified RBCs (39)

Nystatin, a channel-forming ionophore, was used to bring cell sodium concentration to the desired level. One volume of washed packed RBCs was added at 4 °C to five volumes of nystatin containing solutions (150 mM KCl, 40 mM sucrose, 40 μg/ml nystatin for removing cell sodium, or 150 mM NaCl, 40 mM sucrose, 40 μg/ml nystatin for increasing cell sodium) and kept in the dark at 4 °C for 20 minutes. The suspension was then replaced by loading solution without nystatin and incubated at 4 °C for another 10 minutes. Nystatin was removed

by adding 5 volumes of nystatin washing solution (150 mM KCl, 40 mM sucrose, 10 mM glucose, 10 mM potassium phosphate buffer, pH=7.4, 1g/liter albumin) to the cells. The cells were incubated at 37 °C for 10 minutes. After four times washing with nystatin washing solution, nystatin was completely removed. Final cell sodium concentration was checked by atomic absorption or NMR spectroscopy. Nystatin must be added fresh daily.

2.3. Preparation of lithium-loaded RBCs (18)

Washed RBCs were added to lithium loading solution (150 mM LiCl, 20 mM glucose, 20 mM Tris-MES buffer, pH=7.4) at 10% Ht and incubated at 37 °C for 3 hours. Cells were washed four times by choline washing solution (112 mM choline chloride, 85 mM sucrose, 10 mM glucose, 10 mM Tris-MES buffer, pH=7.4). The final cell lithium concentration was determined by atomic absorption.

2.4. Preparation of CO and deoxy RBC

The CO form of RBC was prepared by bubbling washed RBC with CO gas for 30 minutes. The deoxy form of RBC was prepared by bubbling RBC with N_2 gas for 30 minutes. These gas bubbled RBCs were used within half an hour after preparation, and were characterized using ^{31}P NMR spectroscopy (44,45).

2.5. Preparation of RBC membrane (47)

Blood was washed by 5 volumes of PBS (150 mM NaCl, 5 mM KPO_4) three times at 6,686 g for 5 minutes. Packed washed RBCs were then lysed by 5H8 (5 mM HEPES-Tetramethylammonium hydroxide pentahydrate, pH=8.0) at a ratio of approximately 1:40 but not higher than 1:20. The lysate was centrifuged at 26,746 g for 10 minutes. The supernatant

was aspirated and the remaining membrane was washed two more times until cloudy white.

2.6. Preparation of shift reagent (48)

Dysprosium (III) triethylenetetraamine hexacetate ($DyTTHA^{3-}$) was prepared from dysprosium hydroxide and H_6TTHA. For preparing 5 ml of 0.125 M stock solution of $Na_3DyTTHA$, 0.2356 g of $DyCl_3$ was first dissolved in 3 ml water. H_6TTHA powder (0.3090 g) was then dissolved in $DyCl_3$. I titrated 1.5 ml of 2.5 M NaOH in 30 μl aliquots while stirring. The final pH was less than 8.0 and the solution was clear. To exclude sodium from the shift reagent preparation, tetramethylammonium hydroxide was used to titrate instead of sodium hydroxide.

2.7. Membrane digestion for protein concentration analysis (49)

Membrane made from RBC was diluted four times to fit into a standard curve. The Bradford stock solution was purchased from Bio-Rad. The working buffer (425 ml distilled water, 15 ml 95% ethanol, 30 ml 88% phosphoric acid, 30 ml Bradford stock solution) which was used to digest membrane was also added to the blank.

3. Instrumentation

3.1. NMR spectrometer

^{23}Na, ^{7}Li and ^{31}P measurements were made at 79.4, 116.5 and 121.4 MHz respectively on a Varian VXR-300 NMR spectrometer. The instrument was equipped with a 10 mm multinuclear probe and a variable temperature unit. All blood and membrane samples were used at 37 °C. Packed Na^+ modified cells were put into 10 mm NMR tubes to measure T_1 and T_2 values without spining, whereas membrane samples were studied spining. Field-

frequency locking and shimming on D_2O present in the aqueous media was done spining every time before blood sample measurements.

For Na^+-H^+ transport measurements, each ^{23}Na spectrum was obtained with 200 transients, with a 60° degree pulse width (19 μs for the 10 mm low frequency probe; 22 μs for the 10 mm high frequency probe) and line broading of 2 Hz. Each ^{31}P spectrum was obtained with 1,200 transients, a 60° degree pulse width (13 μs) and line broading of 1 Hz.

A standard single pulse sequence was employed to obtain the NMR spectra of transport measurements. Spin-lattice relaxation time (T_1) measurements were performed using the inversion recovery method; spin-spin relaxation time (T_2) measurements were done by the Carl-Purcell-Meiboom-Gill method (38). The pulse sequences used for the NMR measurements are shown in Figure 1.

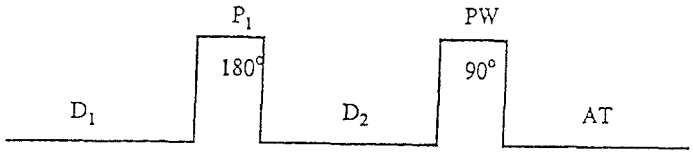
3.2. Atomic absorption spectrophotometer

AA studies were performed on a Perkin-Elmer spectrophotometer, model 5000, equipped with a graphite furnace.

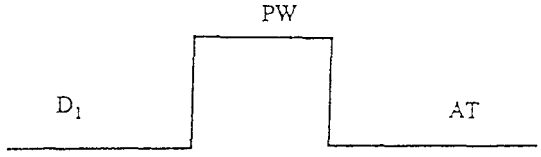
3.3. UV-Visible spectrophotometer

UV-Visible studies were conducted on an IBM Instruments Inc. spectrophotometer, model 9420. Hemoglobin and protein concentration measurements were conducted using Method 2 (single wavelength measurement). Hemoglobin concentration was measured at 415 nm (50) and protein concentration at 595 nm (49). For hemoglobin concentration measurements, I used double distilled water as a blank, whereas the blank for protein concentration measurement was the Bradford reagent plus 5H8.

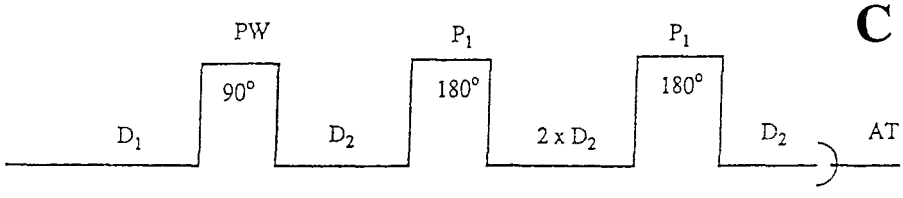
Figure 1: NMR pulse sequences for standard and T_1 and T_2 measurements. (A) Standard single pulse sequence. (B) Inversion recovery pulse sequence for T_1 measurements. (C) Carl-Purcell-Meiboom-Gill pulse sequence for T_2 measurements. AT is the acquisition time. PW is the pulse width (it can be 90°, 60° or 45°) in the standard single pulse sequence. P_1 is the 180° pulse width, and D_1 and D_2 are the delay times between the radiofrequency pulses.



A



B



C

3.4. Osmometer

The osmolarity of the suspension medium for all the RBC samples was checked by a Wescor Vapor Pressure Osmometer (Wescor Inc., Logan, UT) and adjusted to 300±10 mOsm with sucrose. The osmometer was checked every month with a 290 mOsm standard solution.

3.5. Centrifuge

Blood processing and unsealed membrane were prepared at 4 °C using a Beckman J2-21 refrigerated centrifuge equipped with the fixed angle rotors JA-10 and JA-20. A Savant refrigerated centrifuge, model HSC 10000, was also used for washing and separating RBCs.

4. Data Analysis

4.1. Na^+-H^+ transport by atomic absorption (39)

Acid loaded cells (pH_{in} around 5.7) were added to Na^+ influx suspension media at pH=6.0 or at pH=8.0 (150 mM NaCl, 10 mM glucose, 10 mM buffer, Tris-MES for pH 6 or Tris-MOPS for pH=8.0, 0.15 mM $MgCl_2$, 0.1 mM ouabain, 0.01 mM bumetanide, around 350 mOsm, ouabain and bumetanide made fresh daily). Transport experiments were conducted at 37 °C and 2% hematocrit. For pH 6.0, 250 μl duplicates were taken at 1 and 20 minutes. For pH 8.0, 250 μl duplicates were taken at 1, 5 and 11 minutes. These samples were pipetted into prechilled 1 ml centrifuge tubes containing 0.6 ml sodium free solution (80 mM choline chloride, 82 mM KCl, 0.25 mM $MgCl_2$, 10 mM Tris-MOPS buffer, pH 7.4) and 0.3 ml dibutyl phthalate. The samples were spun at 10,519 g for 1 minute. The solution and oil were aspirated and the top part of the tube was wiped to avoid sodium contamination. Double distilled water (1 ml) was added to the tube to lyse the packed red blood cells. These tubes were kept in the refrigerator overnight at 4 °C. Before optical density and AA measurements,

cell lysates were centrifuged for 10 minutes at 2,078 g to lyse the remaining cells. Cell lysate (40 μl) was added to 3.96 ml double distilled water and the optical density representing hemoglobin concentration was measured by UV-visible spectroscopy at 415 nm. A standard was made by pipetting 500 μl packed red blood cells to 9.5 ml choline wash solution to measure the hematocrit. Twenty μl of this solution was added to 2 ml of water to measure the optical density. The cell sodium concentration was calculated using the equation (39):

$$[Na]\ (mmol/L) = [Na]_{lys}(\mu M) \times OD_{stand}/(Ht_{stand} \times OD_{lys} 1000), \quad (1)$$

where $[Na]_{lys}$ and OD_{lys} were the sodium concentration and optical density of cell lysate measured by AA and UV-Visible, respectively, and Ht_{stand} and OD_{stand} were the hematocrit and optical density of the standard sample, respectively.

The Na^+-H^+ transport rate was obtained by subtracting the sodium influx rate at pH 6.0 from that at pH 8.0.

4.2. Na^+-Li^+ transport by atomic absorption (18)

Li^+ loaded red blood cells (600 μl) were added to 5.4 ml NaCl suspension (150 mM NaCl, 10 mM glucose, 10 mM buffer pH 7.4, 0.1 mM ouabain) or to 5.4 ml choline chloride suspension (112.5 mM choline chloride, 10 mM glucose, 10 mM buffer pH=7.4, 0.1 mM ouabain). At 15, 30, 45, 60 and 75 minutes, 1 ml aliquots were taken from either the NaCl or the choline suspensions and centrifuged at 10,519 g for 2 minutes. Supernatants were kept to measure lithium concentration. The Na^+-Li^+ transport rate was obtained from the equation shown below:

$$Rate = Slope_{NaCl} \times (1-Ht_{NaCl})/Ht_{NaCl} - Slope_{Choline} \times (1-Ht_{Choline})/Ht_{Choline} \quad (2)$$

4.3. Na^+-H^+ transport by ^{23}Na and ^{31}P NMR

Na^+ influx

Acid loaded RBCs were added to Na^+ influx suspensions at pH 6.0 or at pH 8.0 (129 mM NaCl, 7 mM $Na_3DyTTHA$, 0.15 mM $MgCl_2$, 10 mM glucose, 10 mM buffer, Tris-MES for pH 6.0 or Tris-MOPS for pH 8.0, 0.1 mM ouabain, 0.01 mM bumetanide). The ^{23}Na NMR measurements were taken immediately after mixing cells with suspensions at 20% Ht. All RBC samples were run unlocked and without spining to avoid cell settling. The ^{23}Na spectra were the average of 5 minute acquisitions with 5 minute intervals up to an hour.

The intracellular Na^+ concentration was obtained from the equation shown below:

$$[Na^+]_{in} = \{[Na^+]_{out0} \times (1\text{-}Ht) + [Na^+]_{in0} \times Ht\} / \{Ht \times (1 + 1/0.8R)\}, \quad (3)$$

where $[Na^+]_{in0}$ is the intracellular Na^+ concentration at time 0 minute, obtained from AA measurements of packed, acid-loaded RBCs, $[Na^+]_{out0}$ is the starting concentration of Na^+ in the suspension, R is the ratio of peak areas for intracellular and extracellular $^{23}Na^+$ NMR resonances during the course of the transport experiment. The visibility factor for the intracellular Na^+ NMR resonance, 0.8, was taken into account in the calculation (43).

Na^+ efflux

Na^+ loaded RBCs were added to efflux suspensions at pH 6.0 or at pH 8.0 (129 mM KCl, 7 mM $DyTTHA^{-3}$ in tetramethylammonium form, 0.15 mM $MgCl_2$, 10 mM glucose, 10 mM buffer, Tris-MES for pH 6.0 or Tris-MOPS for pH 8.0, 0.1 mM ouabain, 0.01 mM bumetanide). The ^{23}Na NMR measurements were the same for Na^+ influx. The intracellular Na^+ concentration was calculated from the equation shown below:

$$[Na^+]_{in} = [Na^+]_{in0} / (1 + 1/0.8R), \quad (4)$$

where the symbols have the same meaning as for equation (3). The derivation of equations (3) and (4) is given in Appendices I and II.

H^+ transport (45)

The intracellular pH indicator methyl phosphonate (MeP) was loaded into the RBCs at the beginning of acid loading by incorporating 10 mM MeP in the acid loading solution. These acic-loaded and MeP-loaded RBCs were added to Na^+ influx suspensions at pH 6.0 or at pH 8.0. The ^{31}P NMR measurements were taken from packed RBCs at 0 min and 30 min for both pH 6.0 and pH 8.0.

CHAPTER IV

RESULTS

1. ^{23}Na NMR Relaxation Time Measurements of Intact RBCs

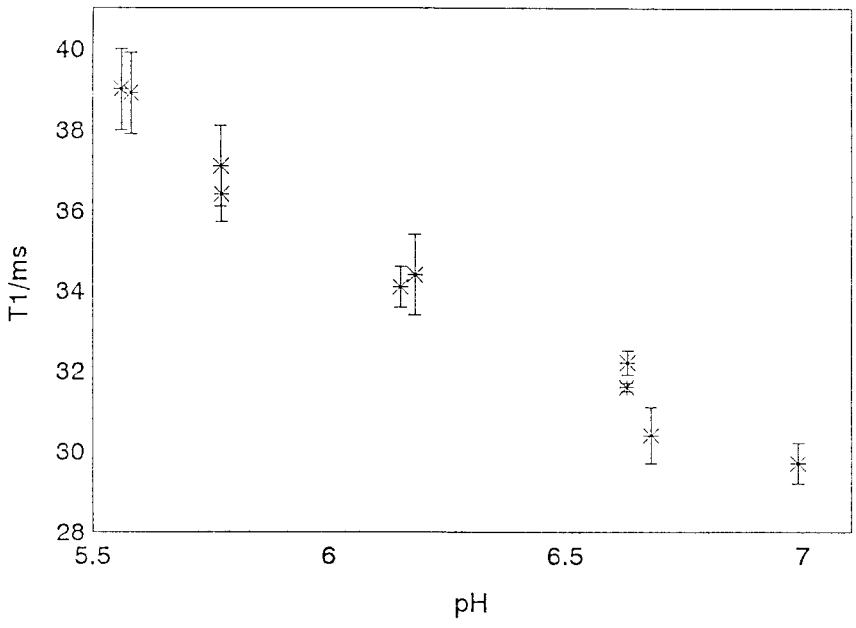
^{23}Na NMR relaxation times are a weighted average of the relaxation times of free and bound Na^+. If competition occurs among H^+, Li^+ and Na^+ for binding to the RBC membrane transport protein, by increasing $[H^+]$ and $[Li^+]$ the amount of free Na^+ should increase causing the relaxation times of ^{23}Na to increase.

Packed RBCs were loaded at different pH_{in} values, and ^{23}Na relaxation times were measured (Figure 2, Table 1). A small difference in relaxation times existed between the two blood samples studied. The different protein concentrations in RBC membranes or the different intracellular Na^+ concentrations may be responsible for the small differences in relaxation times. For both blood samples, the T_2 values (Figure 2B) were significantly shorter than the T_1 values (Figure 2A), indicating that ^{23}Na NMR relaxation mesurements are sensitive to Na^+ binding in RBCs.

As expected, by decreasing pH_{in} H^+ competed with Na^+ for binding sites in RBCs. More free Na^+ resulted in higher ^{23}Na relaxation times. Since $[Na^+]_{in}$ did not change markedly within each experiment, the observed changes in relaxation times were due to different redistributions of Na^+ ions between bound and free forms in the two blood samples. From the data above, one might conclude that intracellular Na^+ concentration may change the ^{23}Na NMR relaxation values. Further studies of the effect of intracellular Na^+ concentration on ^{23}Na NMR

Figure 2. pH dependence of ^{23}Na NMR relaxation times in intact RBCs. (A) ^{23}Na T_1 relaxation times vs cell pH. (B) ^{23}Na T_2 relaxation times vs cell pH. Packed RBCs were loaded at different pH_{in} values by conducting acid loading in suspension media of various pH values (see Methods). The relaxation time measurements were made in packed RBCs without spining. Only the values for the first blood sample (Table 1) are plotted.

A


40
38
36
34
32
30
28
T1/ms
5.5
6
6.5
7
pH

B

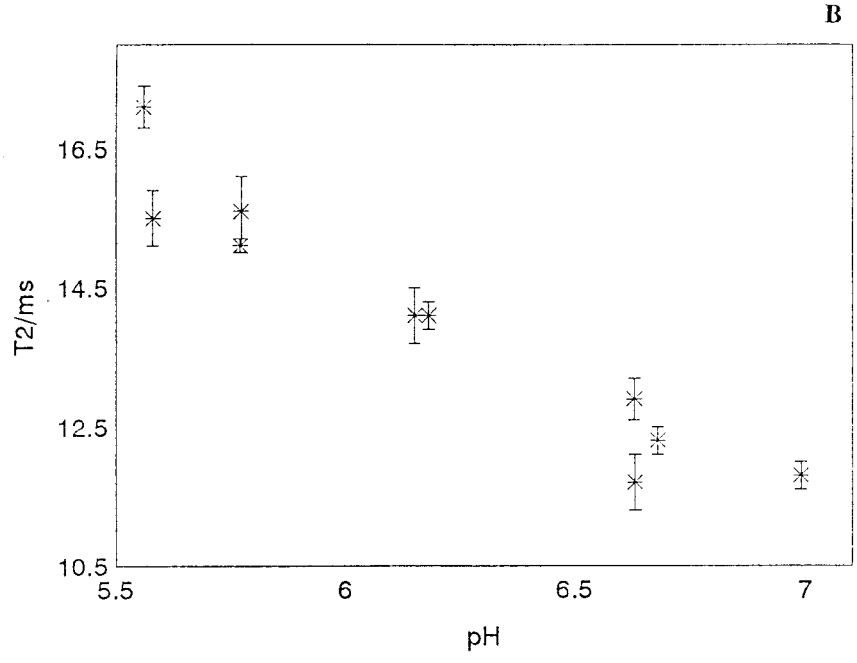

16.5
14.5
12.5
10.5
T2/ms
5.5
6
6.5
7
pH

Table 1. Dependence of ^{23}Na NMR relaxation times on intracellular pH. Same conditions as for Figure 2.

pH	T_1/ms	T_2/ms	$[Na^+]_{in}$/mM
First blood sample			
5.56	39.0±1.0	17.1±0.3	10.8
5.58	38.9±1.0	15.5±0.4	10.5
5.77	37.1±1.0	15.1±0.1	12.8
5.77	36.4±0.7	15.6±0.5	12.5
6.15	34.1±0.5	14.1±0.4	13.2
6.18	34.4±1.0	14.1±0.2	10.4
6.63	31.6±0.1	12.9±0.3	12.0
6.63	32.2±0.3	11.7±0.4	12.0
6.68	30.4±0.7	12.3±0.2	11.0
6.99	29.7±0.5	11.8±0.2	11.8
Second blood sample			
5.58	36.3±2.0	16.8±0.7	6.18
5.76	36.6±1.0	16.8±0.5	6.23
5.97	33.4±1.0	13.7±0.4	5.98
5.83	35.1±1.0	14.6±0.6	6.67
6.21	33.0±0.7	13.0±0.9	5.16
6.59	30.4±0.8	13.3±0.4	6.72
6.76	29.1±0.6	11.2±0.3	6.49
6.71	28.0±0.2	9.77±0.4	7.67

relaxation times (Figure 3, Table 2) show that in a very large intracellular Na^+ concentration range, the relaxation times did not change significantly with Na^+ concentration. However, the relaxation times differed at two different intracellular pH values (7.4 and 5.8). In the Na^+ concentration range from 1 mM to 22 mM, the relaxation times were higher in pH 5.8 cells than in pH 7.4 cells as shown before. Because the physiologic intracellular Na^+ concentration is between 6 mM and 10 mM (51), ^{23}Na NMR relaxation times are sensitive to competition between Na^+ and H^+ for binding sites in RBC for physiologically relevant intracellular Na^+ concentration and pH values.

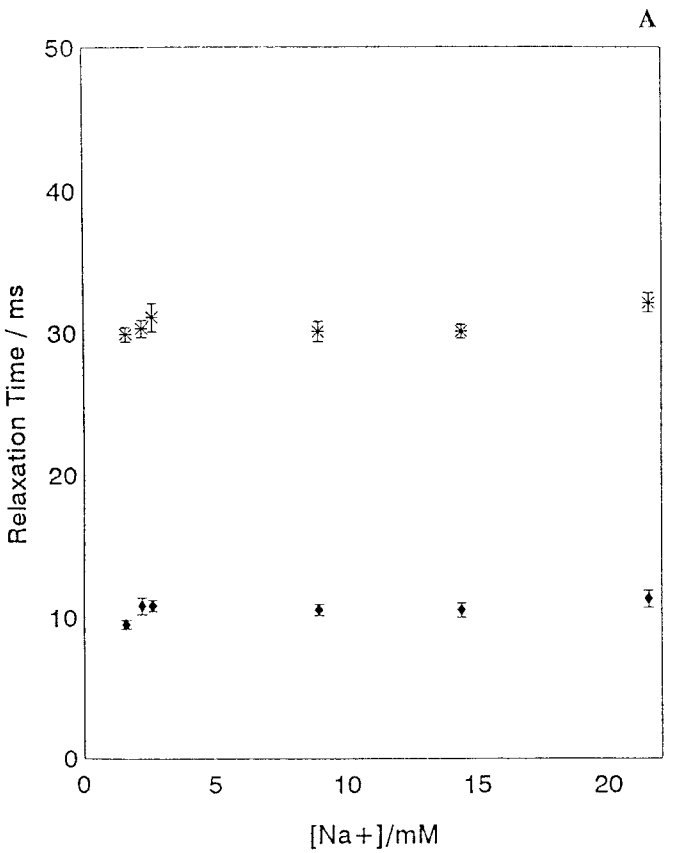
By changing cell pH, hemoglobin will have a different distribution between deoxy and oxy forms due to the Bohr effect (52). The deoxy form of hemoglobin has a paramagnetic center (high spin Fe^{2+}) which may cause the $^{23}Na^+$ NMR resonance to broaden and give a lower relaxation time. The ^{23}Na NMR relaxation times of RBCs in three different oxygenated forms were measured (Table 3). The N_2 bubbled RBCs have predominantely the deoxy form of hemoglobin; the CO bubbled RBCs provide a more stable analog of the oxy form. The ^{23}Na NMR relaxation times did not change appreciably in the three forms. Thus, the pH-induced changes in ^{23}Na relaxation times reported above are not caused by the magnetic properties of hemoglobin. The ^{31}P NMR spectra of these cells were taken to insure accuracy of preparation (Figure 4, Table 4). The N_2 bubbled RBCs which have the paramagnetic hemoglobin center have all ^{31}P peaks broadened and subject to downfield chemical shifts (45). The CO bubbled RBCs are diamagnetic with sharp and upfield ^{31}P NMR resonances (45).

2. ^{23}Na and ^{7}Li NMR Relaxation Time Measurements of Unsealed Membrane

2.1. Dependence of ^{23}Na NMR relaxation time values on sodium concentration

The protein which operates Na^+-H^+ transport is located in the RBC membrane, making

Figure 3. Dependence of ^{23}Na NMR relaxation times on intracellular sodium concentration in packed sodium-modified RBCs. The Na^+ modified RBCs were prepared using nystatin (see Methods). For pH 5.8 cells, the RBCs were loaded in the acid-loading solution (see Methods) after nystatin treatment. In (A) the stars represent T_1 values, whereas the diamond symbols denote T_2 measurements for an intracellular pH of 7.4. In (B) the stars represent T_1 whereas the diamonds show T_2 values for pH 5.8 cells.


A
Relaxation Time / ms
50
40
30
20
10
0
0
5
10
15
20
[Na+]/mM

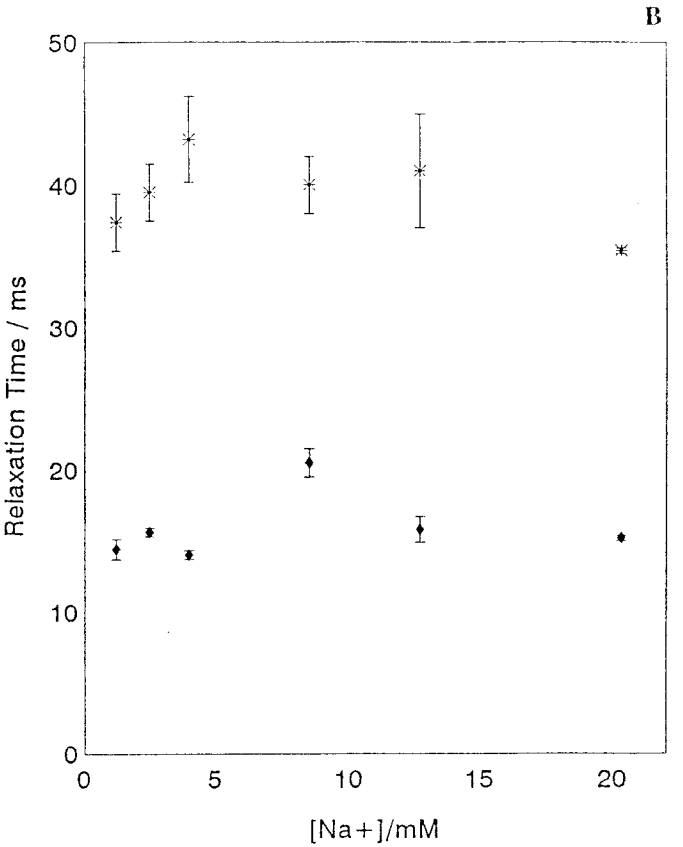

B
Relaxation Time / ms
50
40
30
20
10
0
0
5
10
15
20
[Na+]/mM

Table 2. Dependence of ^{23}Na NMR relaxation times on intracellular sodium concentration in packed sodium-modified RBCs. Same conditions as for Figure 3. n=1.

$[Na^+]_{in}$/mM	T_1/ms	T_2/ms
A) pH_{in}=7.4		
1.6	29.9±0.5	9.5±0.3
2.2	30.3±0.6	10.8±0.6
2.6	31.1±1.0	10.8±0.4
8.9	30.1±0.7	10.5±0.4
14.4	30.1±0.5	10.5±0.5
21.5	32.1±0.7	11.3±0.6
B) pH_{in}=5.8		
1.2	37.4±2.0	14.4±0.7
2.5	39.5±2.0	15.6±0.3
4.0	43.2±3.0	14.0±0.3
8.5	40.0±2.0	20.5±1.0
12.7	41.0±4.0	15.8±0.9
20.3	35.4±0.2	15.2±0.2

Table 3. ^{23}Na NMR relaxation times of packed intact RBCs in various oxygenation states.

	T_1/ms	T_2/ms	$[Na^+]_{in}$/mM	pH_{in}
RBC	30.1±0.3	11.3±0.6	11.4	6.75
	30.1±0.5	11.8±0.6	11.4	6.72
N_2 RBC	33.0±1.0	10.8±0.6	12.1	6.75
	31.4±0.7	12.2±0.5	12.1	6.74
CO RBC	29.1±0.2	10.0±1.0	11.8	6.75
	30.7±0.3	10.7±0.7	11.8	6.72

Figure 4. ^{31}P NMR spectra of packed RBCs in various oxygenation states. (A) CO bubbled, packed RBCs. (B) Packed RBCs. (C) N_2 bubbled, packed RBCs.

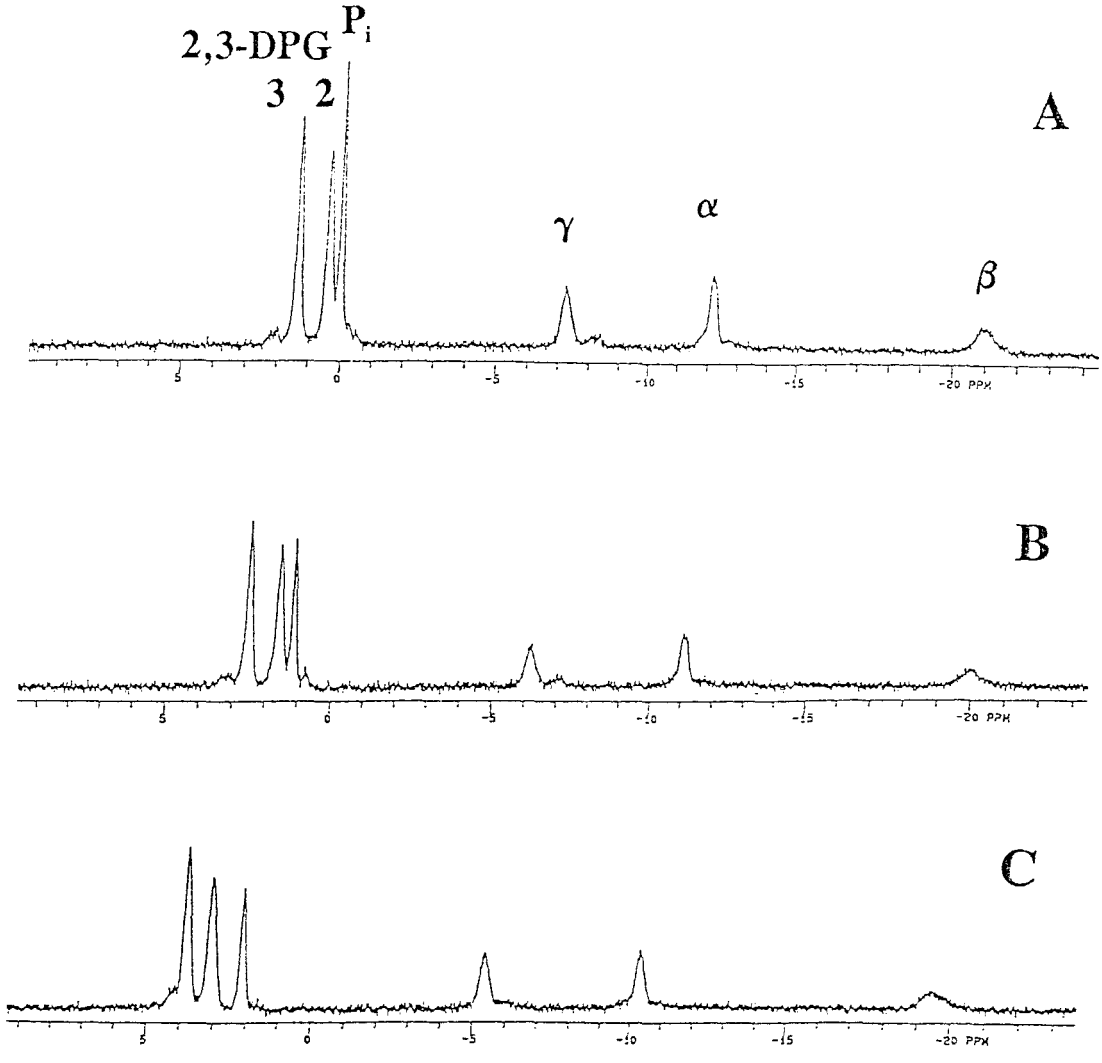

2,3-DPG
3
2
P_i
A
γ
α
β
5
0
-5
-10
-15
-20 PPM
B
5
0
-5
-10
-15
-20 PPM
C
5
0
-5
-10
-15
-20 PPM

Table 4. ^{31}P NMR chemical shifts and linewidths for RBCs in different oxygenation states. $\Delta\nu_{1/2}$ is the linewidth at half height for the α, β and γ phosphate resonances of ATP, $\delta_{Pi\text{-}\beta}$ is the chemical shift separation between the P_i and the β phosphate resonances of ATP.

	$\delta_{Pi\text{-}\beta}$/ppm	$\Delta\nu_{1/2}(\gamma)$/Hz	$\Delta\nu_{1/2}(\alpha)$/Hz	$\Delta\nu_{1/2}(\beta)$/Hz
CO RBC	21.023	43.84	36.81	65.93
RBC	21.156	39.49	39.34	53.35
N_2 RBC	21.551	39.62	43.10	73.15

the cell membrane a likely binding site for Na^+. A large number of Na^+ binding sites are present in intact RBCs. To investigate the putative binding of Na^+ to the Na^+-H^+ exchange protein, a ^{23}Na NMR relaxation study of purified RBC membrane was conducted. Unsealed RBC membranes were made from blood from Life Source (see Methods). The relaxation times increased as Na^+ concentration increased at both pH 8.0 and pH 6.5 (Figure 5, Table 5). The number of binding sites for Na^+ in the membrane samples was constant; with an increase in Na^+ concentration, more free Na^+ having a higher relaxation time existed leading to an increase in the observed relaxation times.

2.2. Dependence of ^{23}Na and ^{7}Li relaxation time on pH of membrane preparation

The binding sites in RBC membrane for Na^+ have the relative affinity $H^+ > Li^+ > > Na^+$ (11). In membrane preparations with various pH values, the ^{7}Li relaxation times increased as the pH of the membrane sample decreased (Figure 6, Table 6). All three samples show that when the pH of the membrane samples is increased, the ^{7}Li T_1 values decrease. The T_1 differences among samples may be caused by the different amount of membrane protein. The duplicates in sample 2 indicate that these measurements are reproducible with an intraindividual coefficient of variation of 13% (Table 6). As Na^+ binds much more weakly than Li^+, the pH-induced changes of ^{23}Na relaxation times were smaller than those observed for ^{7}Li (Figure 7, Table 7). Both samples show that as the pH of the membrane samples decreased, the relaxation times increased. After adding Li^+ to the samples, the ^{23}Na T_1 increased further. The T_1 values are different in the two samples. This may again be caused by different protein concentrations in the membrane samples (Table 7). By adding LiCl to the Na^+ membrane samples, Li^+ replaced bound Na^+ which resulted in higher ^{23}Na T_1 values (Figure 7, Table 7).

Figure 5. Dependence of ^{23}Na T_1 values in unsealed membrane on Na^+ concentration at pH 8.0 (open squares) and pH 6.5 (diamonds). Unsealed membranes were made by 5H8 from blood from Life Source. Aliquots from a 0.5 M NaCl stock solution were added to 2.5 ml membrane to obtain the final Na^+ concentration in the range 2 mM to 37 mM. Before NMR measurements, pH 6.5 membrane samples were treated with 10 mM Tris-MES buffer, pH=5.7. The pH of the membrane preparation was confirmed with a pH meter after relaxation time measurements. Each point is the average of three measurements. The protein concentrations of the membrane samples were 6.5 ± 0.4 mg/ml for the pH 8.0 samples and 6.4 ± 1.0 mg/ml for the pH 6.5 samples.

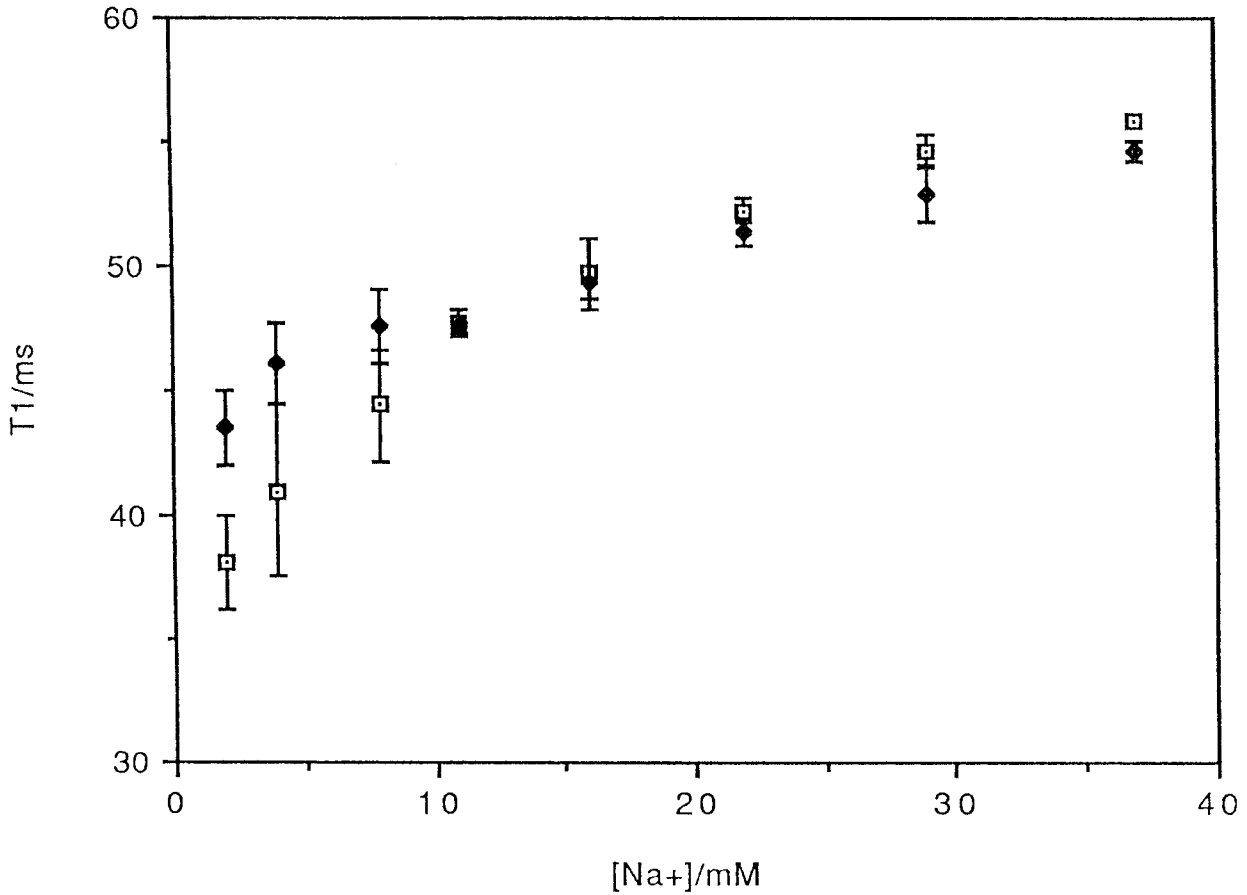

60
50
40
30
T1/ms
0
10
20
30
40
[Na+]/mM

Table 5. Dependence of ^{23}Na T_1 values in unsealed membrane on Na^+ concentration. Same conditions as for Figure 5. n=3.

$[Na^+]$/mM	T_1/ms			AVG
A) pH=8.0				
2.0	36.4±2.0	37.7±1.0	40.2±0.8	38.1±1.9
4.0	38.3±0.3	39.7±1.0	44.9±1.0	41.0±3.5
8.0	43.0±0.6	43.2±0.7	46.9±0.7	44.4±2.2
11.0	47.3±0.6	48.4±0.6	47.4±0.8	47.7±0.6
16.0	48.1±0.5	50.6±0.7	50.5±0.4	49.7±1.4
22.0	51.8±0.7	52.7±0.5	52.1±0.4	52.2±0.5
29.0	55.2±0.2	53.8±0.5	54.8±0.5	54.6±0.7
37.0	55.6±0.4	55.8±1.0	56.1±0.5	55.8±0.3
B) pH=6.5				
2.0	45.1±6.0	42.2±1.0	43.2±2.0	43.5±1.5
4.0	44.3±2.0	46.9±2.0	47.1±2.0	46.1±1.6
8.0	49.2±0.3	47.5±0.7	46.2±0.7	47.6±1.5
11.0	47.7±0.4	47.4±0.7	47.3±0.8	47.5±0.2
16.0	49.5±0.3	48.6±0.3	49.9±0.5	49.3±0.7
22.0	51.8±0.4	51.1±0.4	50.9±0.5	51.3±0.5
29.0	53.0±0.5	54.0±0.5	51.7±0.2	52.9±1.2
37.0	54.4±0.2	55.0±0.4	54.3±0.6	54.6±0.4

Figure 6. pH dependence of ^{7}Li NMR relaxation times in membrane samples. Unsealed membranes were added to buffers of different pH at 10 mM concentration to obtain different pH values. The pH of each sample was confirmed after each relaxation time measurement. Sample 3 in Table 6 is plotted.

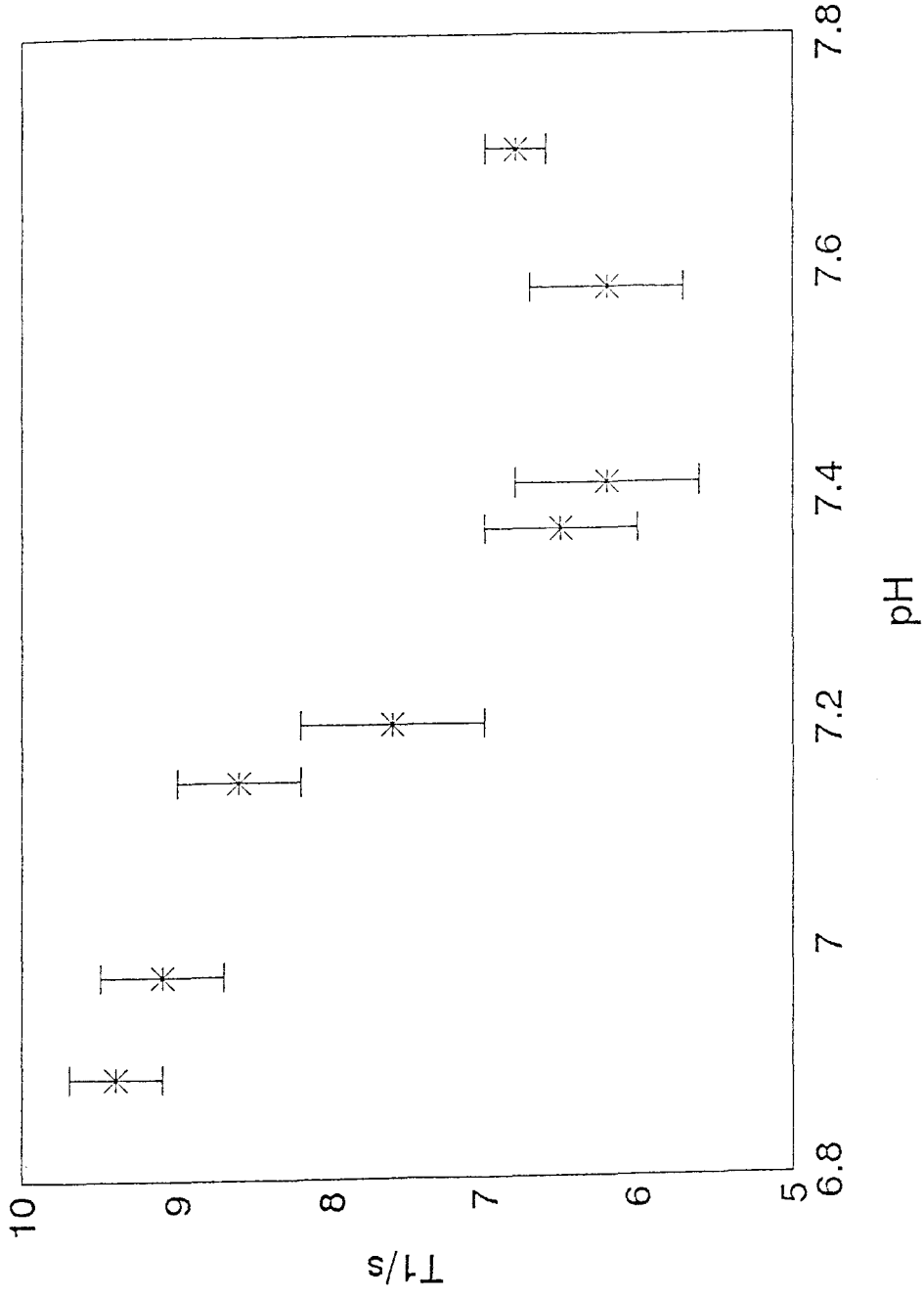

10
9
8
7
6
5
T1/s
6.8
7
7.2
7.4
7.6
7.8
pH

Table 6. pH dependence of ^{7}Li NMR relaxation times in membrane samples. Four separately prepared membranes were used. Same conditions as for Figure 6.

Membrane pH	T_1/s		[Protein]/mg/ml	
Sample 1				
6.05	7.9±0.3		8.13	
6.49	8.1±0.4		6.80	
6.92	7.0±0.5		6.41	
7.30	6.6±0.6		6.13	
7.59	6.3±0.4		5.52	
8.01	4.5±0.3		6.46	
Sample 2				
7.71	9.2±0.5	8.4±0.7	7.25	7.37
7.55	9.7±0.3	10.7±0.4	6.71	7.42
7.08	12.0±0.7	9.9±0.8	6.79	7.21
6.77	9.7±1.1	8.0±0.4	8.84	8.91
6.13	13.0±1.0	12.2±1.7	7.71	6.54
Sample 3				
7.70	6.8±0.2		6.43	
7.58	6.2±0.5		5.79	
7.41	6.2±0.6		8.05	
7.37	6.5±0.5		6.95	
7.20	7.6±0.6		6.65	
7.15	8.6±0.4		7.35	
6.98	9.1±0.4		7.95	
6.89	9.4±0.3		8.15	

Figure 7. pH dependence of ^{23}Na NMR relaxation times in membrane samples. A 3 mM NaCl was added to each membrane sample to obtain the ^{23}Na T_1 values (stars). After these measurements, 3 mM LiCl were added to the same membrane samples and the ^{23}Na T_1 were remeasured (diamonds). Sample 2 in Table 7 is plotted here.

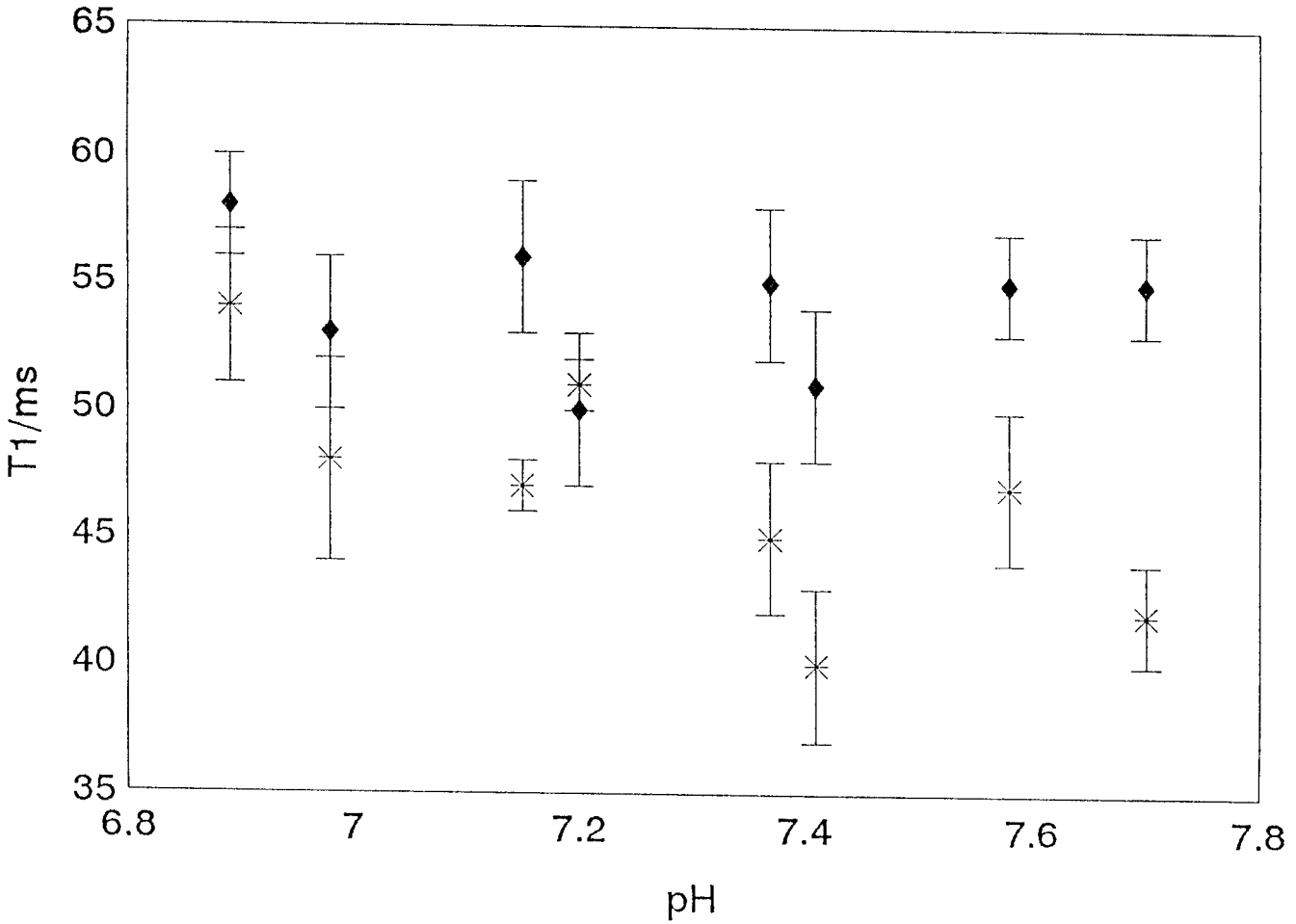

65
60
55
50
45
40
35
T1/ms
6.8
7
7.2
7.4
7.6
7.8
pH

Table 7. pH dependence of ^{23}Na NMR relaxation times in membrane samples. Two separately preparaed membranes were used. Same conditions as for Figure 7.

Membrane pH	T_1/ms (without Li^+)	T_1/ms (with Li^+)	[Protein]/mg/ml
Sample 1			
8.01	37±1	41±3	6.46
7.59	41±3	43±2	5.52
7.30	46±3	50±5	6.13
6.92	45±1	47±5	6.41
6.49	44±3	47±3	6.80
6.05	49±4	45±2	8.13
Sample 2			
7.70	42±2	55±2	6.43
7.58	47±3	55±2	5.79
7.41	40±3	51±3	8.05
7.37	45±3	55±3	6.95
7.20	51±1	50±3	6.65
7.15	47±1	56±3	7.35
6.98	48±4	53±3	7.95
6.89	54±3	58±2	8.15

2.3. ^{23}Na and ^{7}Li NMR relaxation time measurements in the presence and absence of transport inhibitors

Amiloride is an inhibitor of Na^+-H^+ transport (5) and phloretin is an inhibitor of Na^+-Li^+ transport (22). If the inhibitors bind to the RBC membrane transport protein, by adding inhibitors to Na^+- or Li^+-treated membranes, the ^{23}Na and ^{7}Li relaxation times should increase as the inhibitor concentration increase. As expected, amiloride caused increases on both ^{23}Na (Figure 8, Table 8) and ^{7}Li (Figure 9, Table 9) NMR relaxation times. The changes in T_1 values in Table 8 are smaller than the changes in Table 9, because Na^+ binds more weakly to the membrane than Li^+ (11). Amiloride could bind to Na^+ or Li^+ instead of competing with the Na^+ or Li^+ binding sites on the membrane. The data without membrane (Tables 8 and 9) are control experiments for 3 mM Na^+ or 3 mM Li^+ in 5H8, which is the buffer system used for the membrane study. The relaxation times with and without amiloride showed no significant difference, indicating there is no specific interaction between Na^+ or Li^+ and amiloride. Phloretin, in the same concentration range as for amiloride, caused increases on ^{7}Li NMR relaxation times (Figure 10, Table 10) but had no significant effect on ^{23}Na NMR relaxation times (Table 11). This indicates that the effect of phloretin on the metal ion binding to the RBC membrane is smaller than that of amiloride. To test if phloretin bound metal ions, control experiments were done by measuring the ^{23}Na and ^{7}Li relaxation times in the buffer with and without phloretin. There was no significant change in the relaxation times (Tables 10 and 11) indicating that there is no significant binding of Na^+ or Li^+ to phloretin.

3. Na^+-H^+ Transport Measurement by ^{23}Na and ^{31}P NMR

3.1. Na^+ influx measurement by ^{23}Na NMR

Using AA, the Na^+-H^+ transport rate was measured in acid loaded cells (39). The

Figure 8. ^{23}Na T_1 measurements for membrane samples in the presence of amiloride. A 2.5 ml unsealed membrane with 3 mM NaCl is placed in a 10 mm NMR tube for the T_1 measurement. The 500 mM amiloride solution (DMSO solvent) was added to the tube to obtain the T_1 values with inhibitors. The protein concentrations of the two membrane samples were 8.82 and 9.36 mg/ml.

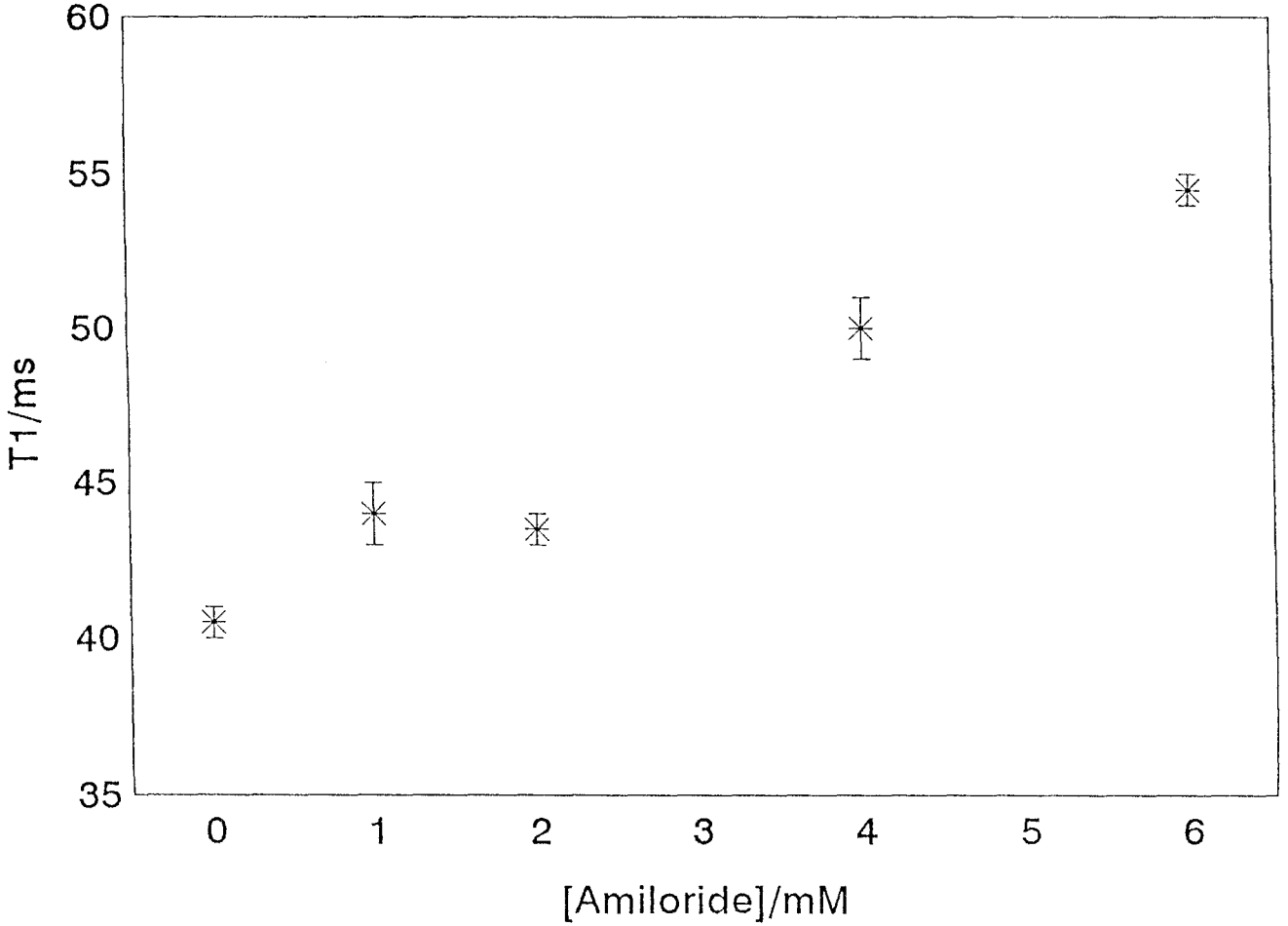

60
55
50
45
40
35
T1/ms
0
1
2
3
4
5
6
[Amiloride]/mM

Table 8. ^{23}Na T_1 measurements for membrane samples in the presence of amiloride. Same conditions as for Figure 8. The values in the table below have the unit of ms.

A: with membrane				
0 mM A	1 mM A	2 mM A	4 mM A	6 mM A
41±1	45±3	44±1	49±2	55±2
40±1	43±3	43±2	51±2	54±1
B: without membrane				
71±3				70±2
69±1				70±2

Figure 9. ^{7}Li T_1 measurements for membrane samples in the presence of amiloride. A 2.5 ml unsealed membrane with 3 mM LiCl is in the 10 mm NMR tube. The 500 mM amiloride stock solution in DMSO solvent was added to the NMR tube after the first measurement without inhibitors. The membranes in this experiment have protein concentrations of 8.9 and 9.0 mg/ml.

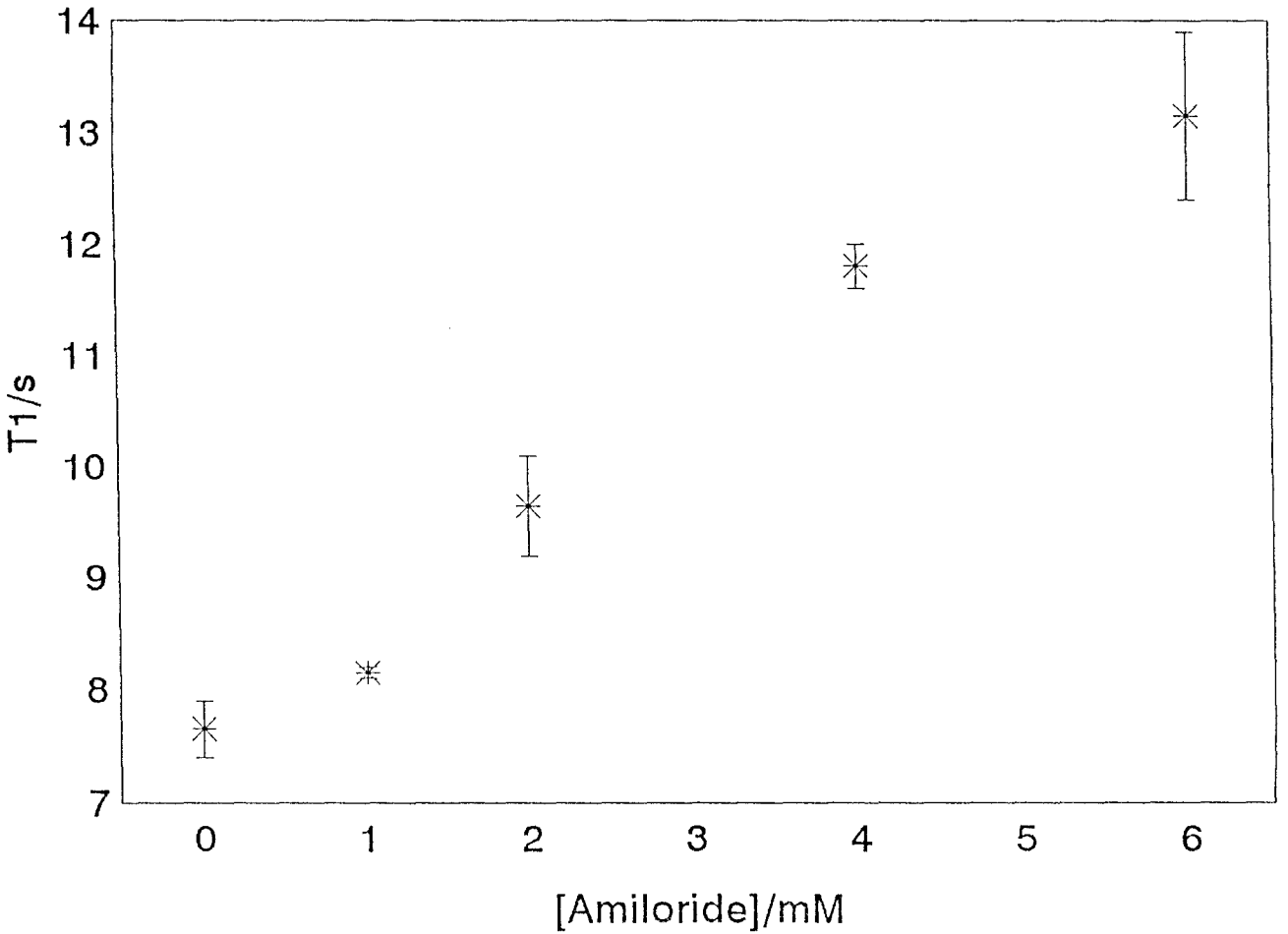

14
13
12
11
10
9
8
7
T1/s
0
1
2
3
4
5
6
[Amiloride]/mM

Table 9. ^{7}Li T_1 measurements for membrane samples in the presence of amiloride. Same conditions as for Figure 9. The data in this table have the unit of s.

0 mM A	1 mM A	2 mM A	4 mM A	6 mM A
A: with membrane				
7.9±0.6	8.1±0.4	9.2±0.7	12.0±0.5	12.4±0.7
7.4±0.4	8.2±0.5	10.1±0.8	11.6±0.4	13.9±0.4
B: without membrane				
22±1				21±1
21±2				23±1

Figure 10. Effect of phloretin on ^{7}Li NMR relaxation times in unsealed membrane. A phloretin stock solution of 200 mM in DMSO was added to a 2.5 ml membrane sample with 3 mM LiCl. The protein concentrations of these two samples were 9.3 and 9.2 mg/ml.

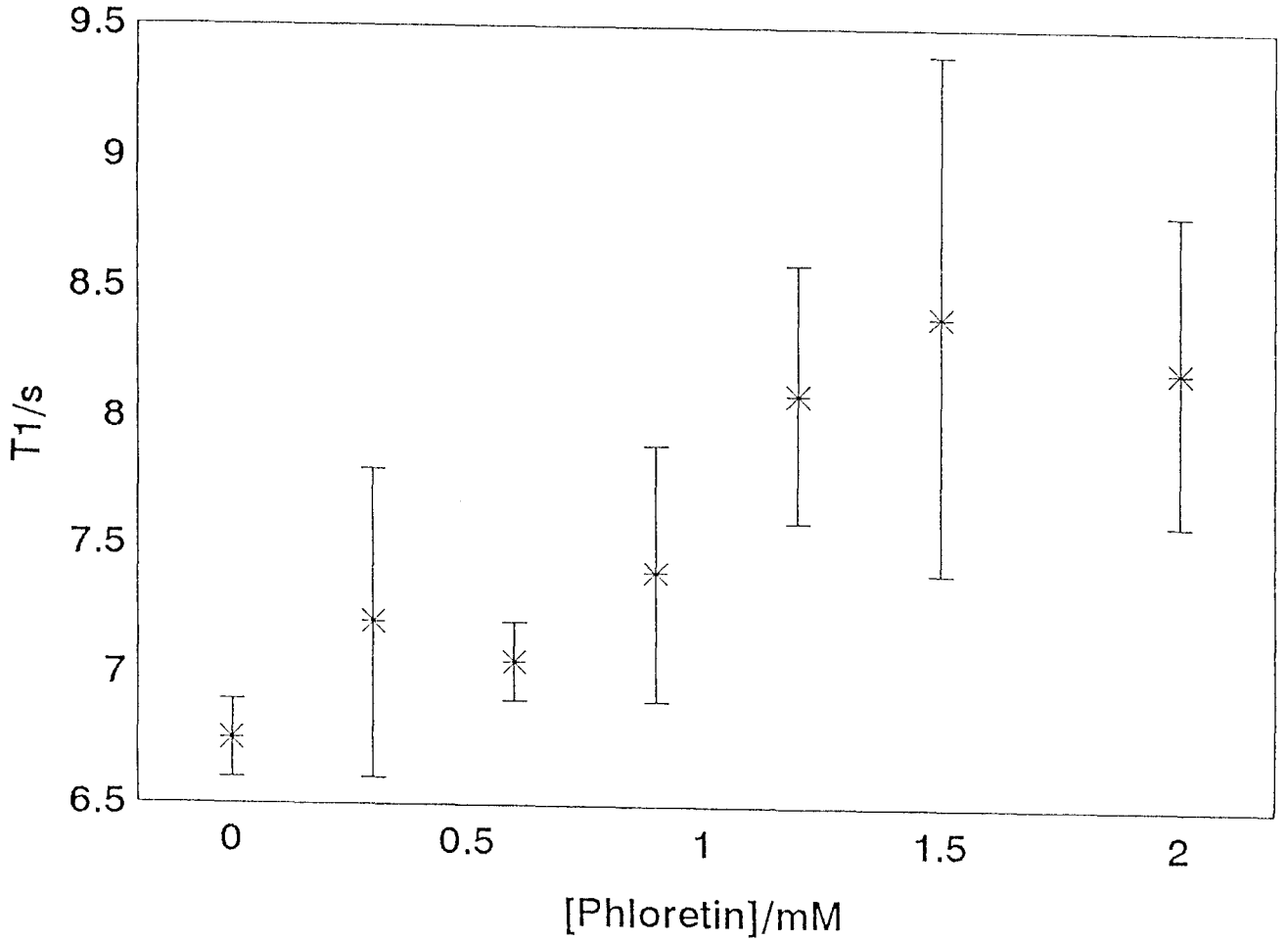

9.5
9
8.5
8
7.5
7
6.5
T1/s
0
0.5
1
1.5
2
[Phloretin]/mM

Table 10. Effect of phloretin on ^{7}Li NMR relaxation times in unsealed membrane. Same conditions as for Figure 10. For the control experiment, 2 mM phloretin was added to 2.5 ml 5H8 buffer with 3 mM LiCl. The data in this table have units of s.

0 mM P	0.3 mM P	0.6 mM P	0.9 mM P	1.2 mM P	1.5 mM P	2.0 mM P
A: with membrane						
6.9±0.3	7.8±0.7	6.9±0.4	7.9±0.6	8.6±0.4	9.4±0.7	8.8±0.8
6.6±0.4	6.6±0.4	7.2±0.9	6.9±0.4	7.6±0.6	7.4±0.8	7.6±0.4
B: without membrane						
21±2						23±1
22±1						20±2

In order to compare the effects of phloretin and amiloride, I tested the competition effect of phloretin (up to a concentration of 6 mM) with and without membrane. The protein concentration in this membrane sample was 8.5 mg/ml.

0 mM P	1 mM P	2 mM P	4 mM P	6 mM P
A: with membrane				
6.8±0.2	8.8±0.7	9.2±0.3	9.6±0.6	9.0±0.3
B: without membrane				
21±2				21±1
22±1				20±2

Table 11. Effect of phloretin on ^{23}Na NMR relaxation times in unsealed membrane. A 200 mM phloretin stock solution in DMSO solvent was added to 2.5 ml unsealed membrane sample which has 3 mM NaCl. The data with the unit of ms. The protein concentrations of the membrane samples were 8.0 and 8.9 mg/ml. For the control experiment with phloretin, a stock solution of 200 mM phloretin was added to 2.5 ml 5H8 with 3 mM NaCl.

0 mM	0.3 mM	0.6 mM	1.2 mM	2.0 mM
A: with membrane				
44±3	42±3	40±2	39±2	42±2
44±2	44±3	45±2	45±2	45±3
B: without membrane				
71±3				70±2
69±1				70±1

In order to test the phloretin effect at the same concentration as amiloride, I added phloretin to the membrane sample up to a concentration of 6 mM. The membrane sample had a protein concentration of 8.7 mg/ml.

0 mM	1 mM	2 mM	4 mM	6 mM
A: with membrane				
45±2	41±2	41±1	44±1	43±1
B: without membrane				
69±1				70±1

Na^+-H^+ transport rate that I obtained at 2% hematocrit was 34 mmol/L cell/hr without amiloride and 8.8 mmol/L cell/hr with amiloride. Thus 74% Na^+ influx was inhibited by amiloride, in agreement with Canessa's values of 10-40 mmol/L cell/hr for Na^+-H^+ transport rate and 60-80 % of this transport being sensitive to amiloride (39).

The shift reagent $Dy(TTHA)^{3-}$ was used to distinguish the pools of Na^+ inside and outside the RBC membrane (Figure 11). The extracellular Na^+ which surrounded $Dy(TTHA)^{3-}$ yielded the downfield peak. The transport rate was obtained by measuring the peak area changes of intra- and extracellular Na^+ resonances (see Methods). The spectra show that as time increased the intracellular Na^+ peak increased, increasing faster in the pH 8.0 medium than in the pH 6.0 medium (Figure 12). The rate obtained from NMR spectra at 20% hematocrit for Na^+ depleted cells at 1 hour observation time was 0.40 mmol/L cell/hr which varied among individual blood samples (Table 12). The rates from NMR were smaller than the rates from AA. This is because the NMR measurements were done at a higher hematocrit and over a longer observation time. The hematocrit and the extracellular [Na^+] effects were also studied by ^{23}Na NMR (Tables 13-15). At 70% Ht and 150 mM NaCl in suspension, the transport rate was 9.3 mmol/L cell/hr. By decreasing the Ht to 35%, the rate increased to 39 mmol/L cell/hr. At 35% Ht, decreasing [Na^+] in the suspension from 150 mM to 65 mM, the rate decreased to 33 mmol/L cell/hr. As the observation time of these experiments was 10 minutes, these rates were higher than the rate at 1 hour observation time. The percentage of cell lysis is below 5% for all Na^+ influx experiments using NMR. The calculated rates for duplicate samples at 70% Ht, 150 mM NaCl in suspension were 9.3 and 3.2 mmol/L cell/hr, with a coefficient of variation of 69%.

Figure 11. ^{23}Na NMR spectrum of inner and outer tube combination. The inner tube contains 25 mM NaCl and the outer tube contains 119 mM NaCl, 7 mM $Na_3Dy(TTHA)$, 10 mM glucose, 10 mM HEPES pH=7.4.

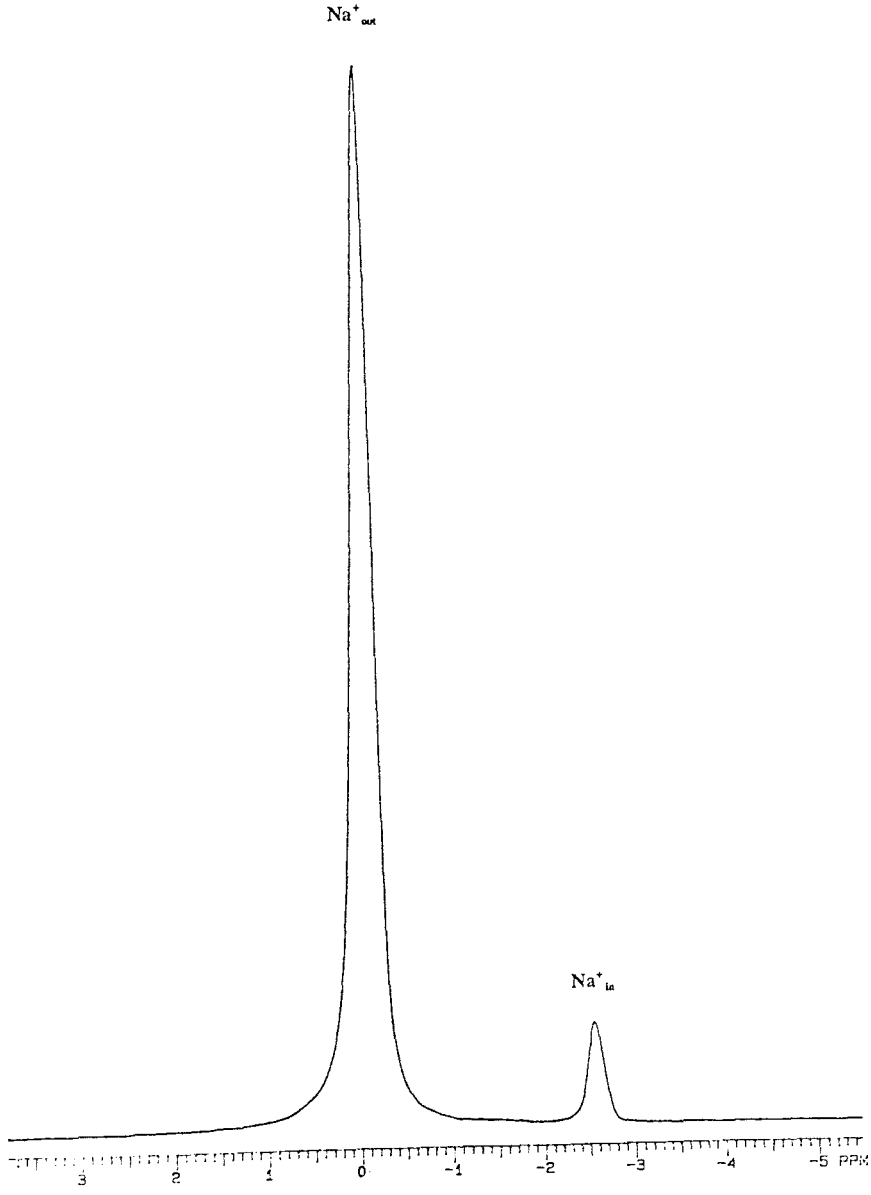

Na^+_{out}
Na^+_{in}
3
2
1
0
-1
-2
-3
-4
-5 PPM

Figure 12. Na^+ influx rate measurements by ^{23}Na NMR. The acid loaded RBCs (see Methods) were put into two Na^+ influx media (129 mM NaCl, 7 mM $Na_3Dy(TTHA)$, 10 mM glucose, 10 mM buffer, Tris-MES for pH=6.0, Tris-MOPS for pH=8.0, 0.01 mM bumetanide, 0.1 mM ouabain). The osmolarity of both media was 370 ± 10 mosm to prevent swelling in acid loaded RBCs by increased Cl^- content. The hematocrit of both suspensions was 20%.

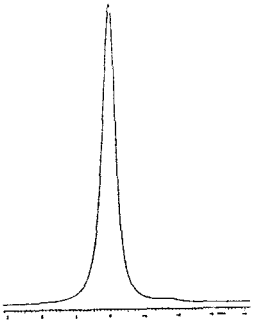
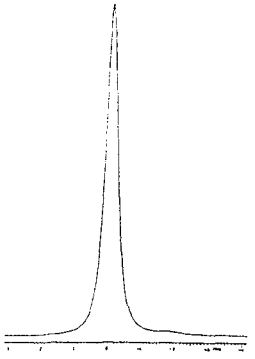
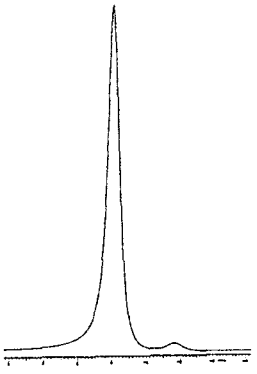
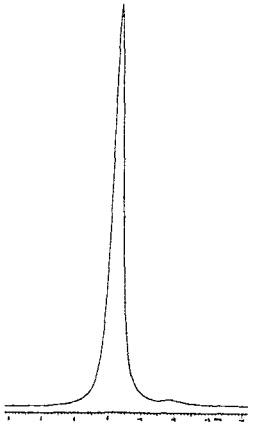
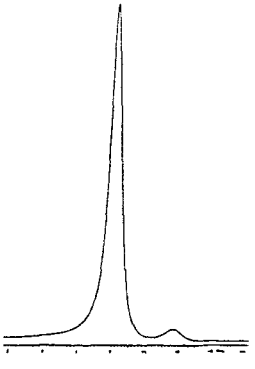
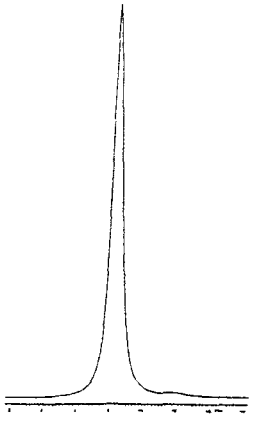
Time/min	pH=8.0	pH=6.0
2.5		
22.5		
42.5		

Table 12. Na^+ influx rate measurements by ^{23}Na NMR at 20% Ht for 1 hour. The RBCs used in this experiment were pretreated with nystatin to deplete intracellular sodium. The Na^+-H^+ transport rate from this sample is 0.40 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 6.0 medium: slope=0.0106 mM/min, r^2=0.8100.				
2.5	0.3801	0.0080	0.021	0.80
12.5	0.4002	0.0076	0.019	0.72
22.5	0.4028	0.0105	0.026	0.98
32.5	0.4128	0.0108	0.026	0.98
42.5	0.4141	0.0133	0.032	1.20
pH=8.0 medium: slope=0.0172 mM/min, r^2=0.9078.				
2.5	0.3705	0.0076	0.021	0.78
12.5	0.3824	0.0098	0.026	0.97
22.5	0.3897	0.0110	0.028	1.06
32.5	0.3924	0.0120	0.031	1.15
42.5	0.3869	0.0161	0.042	1.55

Table 13. Na^+ influx measurements by ^{23}Na NMR at 70% Ht for 10 minutes. Same suspension conditions as for Figure 12. The transport rate of this sample was 9.3 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 8.0 medium: slope=0.3025 mM/min, r^2=0.9626.				
2	75.804	16.385	0.216	14.07
6	76.220	19.364	0.254	16.13
10	76.968	20.056	0.261	16.49
pH 6.0 medium: slope=0.2250 mM/min, r^2=0.7500.				
2	92.595	13.667	0.148	12.36
6	91.438	14.988	0.164	13.54
10	91.927	15.055	0.164	13.54

Table 14. Na^+ influx rate measurements by ^{23}Na NMR at 35% Ht for 10 min. Same suspension conditions as for Figure 12. The transport rate was 39 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 8.0 medium: slope=0.660 mM/min, r^2=0.8957.				
2	163.48	7.28	0.0445	10.90
6	164.85	10.30	0.0625	15.10
10	166.70	11.20	0.0672	16.18
pH 6.0 medium: slope=0.011 mM/min, r^2=0.7674.				
2	196.74	10.90	0.0554	16.63
6	201.98	11.01	0.0545	16.37
10	203.53	11.33	0.0557	16.72

Table 15. Na^+ influx rate measurements by ^{23}Na NMR at 35% Ht for 10 min at low extracellular [Na^+]. Same suspension conditions as for Figure 12, expect that [Na^+] in suspension is 65 mM. The trasnport rate of this sample was 33 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 8.0 medium: slope=0.595 mM/min, r^2=0.8576.				
2	100.448	10.778	0.107	20.86
6	101.570	13.190	0.130	24.92
10	102.370	13.710	0.134	25.62
pH 6.0 medium: slope=0.046, r^2=0.9998.				
2	93.046	8.360	0.0898	12.69
6	95.182	8.677	0.0912	12.87
10	95.762	8.957	0.0926	13.06

3.2. Na^+ efflux measurement by ^{23}Na NMR

As we can see from the ^{23}Na NMR spectra, shown in Figure 12, because of large concentration differences, the intracellular Na^+ peak areas were very small compared with the extracellular Na^+ peak areas. It was therefore hard to determine accurately the intracellular Na^+ concentration changes by integrating the small intracellular peaks. A Na^+ efflux measurement method was developed by placing Na^+-loaded cells in two media at different pH values to better observe changes in intracellular Na^+ concentrations and more accurately measure the rate of RBC Na^+-H^+ exchange using ^{23}Na NMR (Figure 13 and Tables 16,17,18). As time increased, the intracellular Na^+ peak decreased, decreasing faster in the pH 6.0 medium than in the pH 8.0 medium (Figure 13, Table 16). The rate of Na^+ efflux varied among individual blood samples and was in the range 3 to 13 mmol/L cell/hr (Table 16, 17, 18). The data in Tables 17 and 18 are duplicates of the same blood sample. The rates were 4.7 and 3.3 mmol/L cell/hr for the same blood sample indicating that the intraindividual reproducibility (coefficient of variation 25%) of Na^+ efflux measurements was better than that of Na^+ influx measurements. The percentage of cell lysis in Na^+ efflux measurements was less than 2%.

3.3. Measurement of intracellular pH in RBC by ^{31}P NMR

When the intracellular Na^+ concentration changed in acid-loaded cells, the intracellular pH also changed due to Na^+-H^+ transport. The indicator methylphosphonic acid (MeP) was used to obtain intracellular pH measurements by ^{31}P NMR spectroscopy (44,45). From pH 6.0 to pH 8.0, the pH dependence of MeP-inorganic phosphate chemical shift has a sensitivity of 0.39 pH unit/ppm, independent of the hemoglobin oxygenation state (45). The acid-loaded RBCs with an intracellular pH 6.29 were put into two different pH suspension media, pH 6.0

Figure 13. Na^+ efflux rate measurements by ^{23}Na NMR. Na^+ loaded RBCs were put into two pH suspension media (150 mM KCl, 7 mM $Dy(TTHA)^{3-}$ in Tris form, 0.15 mM $MgCl_2$, 10 mM glucose, 10 mM buffer, Tris-MES for pH=6.0 and Tris-MOPS for pH=8.0, 0.1 mM ouabain, 0.01 mM bumetanide.) The hematocrits of both solutions were 20%.

pH=6.0

pH=8.0

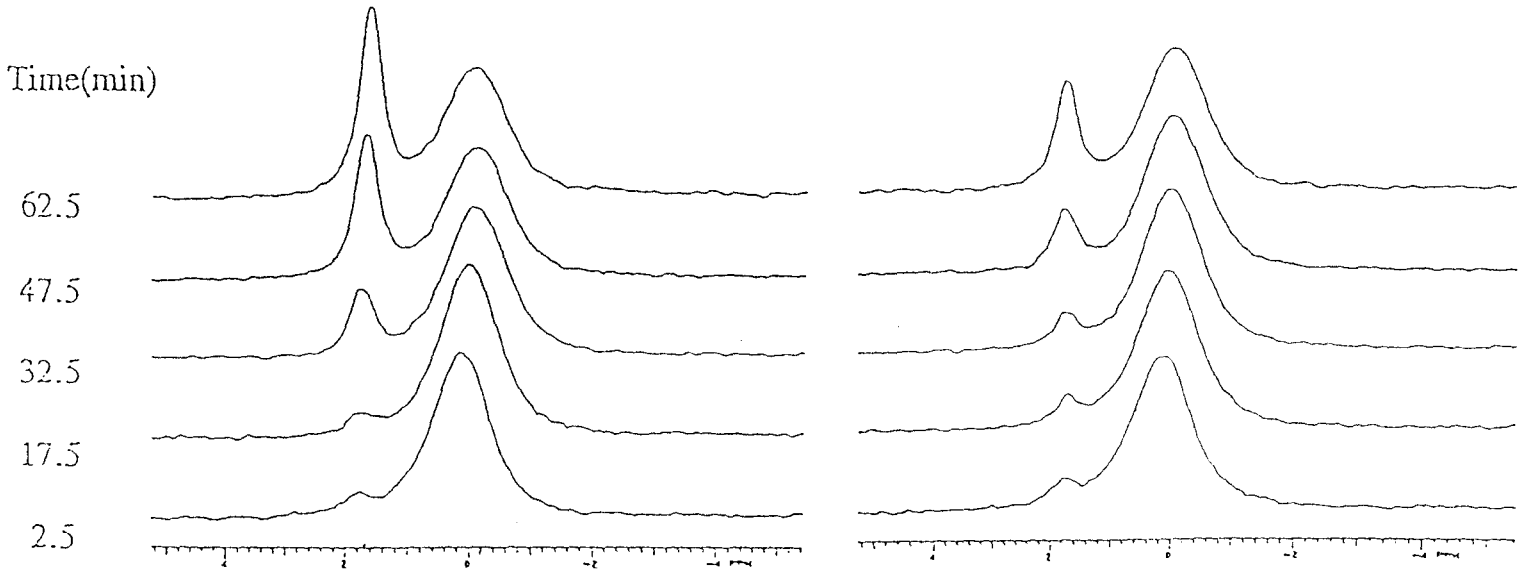


Table 16. Na^+ efflux rate measurements by ^{23}Na NMR. Same conditions as for Figure 13. The starting intracellular sodium concentration was 63 mM. The sodium efflux rate was 13 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 8.0 medium: slope=-0.1932 mM/min, r^2=0.7783.				
2.5	0.0179	0.1818	10.1564	56.10
17.5	0.0211	0.1874	8.8815	55.23
32.5	0.0213	0.1818	8.5352	54.95
47.5	0.0315	0.1746	5.5429	51.41
62.5	0.0582	0.1625	2.7921	43.52
pH 6.0 medium: slope=-0.4118 mM/min, r^2=0.9216.				
2.5	0.0167	0.1771	10.60	56.36
17.5	0.0181	0.1880	10.39	56.23
32.5	0.0364	0.1730	4.75	49.88
47.5	0.0762	0.1488	1.95	38.41
62.5	0.0972	0.1460	1.50	34.39

Table 17. Na^+ efflux rate measurements by ^{23}Na NMR. Same conditions as for Figure 13. The sodium efflux rate was 4.7 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 8.0 medium: slope=-0.1150 mM/min, r^2=0.8708.				
2.5	0.0182	0.3223	17.7088	37.36
17.5	0.0198	0.3201	16.1667	37.13
32.5	0.0258	0.3210	12.4419	36.35
47.5	0.0473	0.3118	6.5920	33.62
62.5	0.0722	0.2893	4.0069	30.49
pH 6.0 medium: slope=-0.1931 mM/min, r^2=0.9111.				
2.5	0.0164	0.3518	21.4512	37.80
17.5	0.0199	0.3604	18.1106	37.42
32.5	0.0351	0.3491	9.9459	35.53
47.5	0.0812	0.3229	3.9766	30.43
62.5	0.113	0.2828	2.5409	26.81

Table 18. Na^+ efflux rate measurements by ^{23}Na NMR. Same conditions as for Figure 13. The sodium efflux rate was 3.3 mmol/L cell/hr (n=1).

Time/min	$Peak_{out}$	$Peak_{in}$	$Ratio_{in/out}$	$[Na^+]_{in}$
pH 8.0 medium: slope=-0.1333 mM/min, r^2=0.9004.				
2.5	0.0203	0.3172	15.6256	37.04
17.5	0.0219	0.3063	13.9909	36.72
32.5	0.0320	0.3119	9.7469	35.45
47.5	0.0548	0.2982	5.4416	32.53
62.5	0.0827	0.2771	3.3507	29.13
pH 6.0 medium: slope=-0.1875 mM/min, r^2=0.9084.				
2.5	0.0279	0.3531	12.6559	36.40
17.5	0.0283	0.3566	12.6007	36.39
32.5	0.0478	0.3485	7.2908	34.15
47.5	0.0916	0.3031	3.3090	29.03
62.5	0.1246	0.2899	2.3266	26.02

and pH 8.0. After one hour incubation at 37 °C, the RBCs in pH 6.0 suspension had $pH_i=6.17\pm0.05$ (n=2), the RBCs in pH 8.0 suspension had $pH_i=6.52\pm0.03$ (n=2) (Table 19). These intracellular pH change are a result of Na^+-H^+ exchange. In the pH 8.0 suspension there was a larger pH gradient than in the pH 6.0 suspension. Na^+ influx will predominate in the pH 8.0 sample replacing intracellular H^+ and increasing intracellular pH. In the pH 6.0 suspension the intracellular pH was similar to that of the extracellular pH, so there were no significant changes in the cell pH. The initial intracellular pH varied among individual samples. The sample showed in Figure 14 had a starting intracellular pH 6.7 and after 30 minutes incubation in pH 5.7 and pH 8.0 media, the final cell pH values were 6.4 and 6.9.

4. Transport Rates and Relaxation Time Measurements in RBCs from Hypertensive Patients

All hypertensive patients were white and had diastolic blood pressure higher than 90 mmHg. The Na^+-Li^+ and Na^+-H^+ transport rates were measured by AA in intact RBCs from five hypertensive patients (see Methods). The ^7Li and ^{23}Na T_1 relaxation times were measured on the membrane prepared from patient's blood (Table 20). I only studied blood samples from five hypertensive patients. The sample size was too small to do statistical analysis and draw any conclusions concerning the involvement of these two ion transport pathways in hypertension.

Table 19. The intracellular pH of RBCs obtained from MeP and P_i chemical shift. RBCs were placed in acid- and MeP loading suspension medium (130 mM KCl, 20 mM MeP, 10 mM glucose, 20 mM Tris-Mes buffer, pH=5.7, 0.15 mM $MgCl_2$, 0.1 mM ouabain, 0.01 mM bumetanide) for 10 minutes at 37 °C and 10% Ht, then methazolamide and DIDS were added and incubated for another 30 minutes. The cells were then washed four times with AWS. The acid and MeP loaded RBCs were finally placed into two different pH 6.0 and 8.0 suspension media for 1 hour. The ^{31}P NMR spectra were taken from packed RBCs before and after 1 hour incubation at 37 °C. The blood sample used for this experiment is different form that used for Figure 15.

	Chemical Shift			
	P_i	MeP	MeP-P_i	pH_{in}
Time 0	0.66	23.97	23.21	6.29
pH 8.0	0.82	23.44	22.62	6.54
	0.76	23.44	22.68	6.50
pH 6.0	0.41	23.74	23.33	6.20
	0.65	23.03	23.38	6.13

Figure 14. Intracellular pH changes during Na^+-H^+ exchange measured by ^{31}P NMR. MeP was loaded into the RBCs and intracellular pH was obtained from the chemical shift separation between the MeP and the P_i peaks. The MeP loaded RBCs was placed into two pH suspensions of 5.7 and 8.0. After 30 minutes, the cells were centrifuged and packed to take ^{31}P NMR spectra. The pH was obtained from the standard curve of Labotka et al. (44). (A) Time 0 minute. Cell pH=6.7. (B) 30 minutes in pH 5.7 suspension. Cell pH=6.4. (C) 30 minutes in pH 8.0 suspension. Cell pH=6.9.

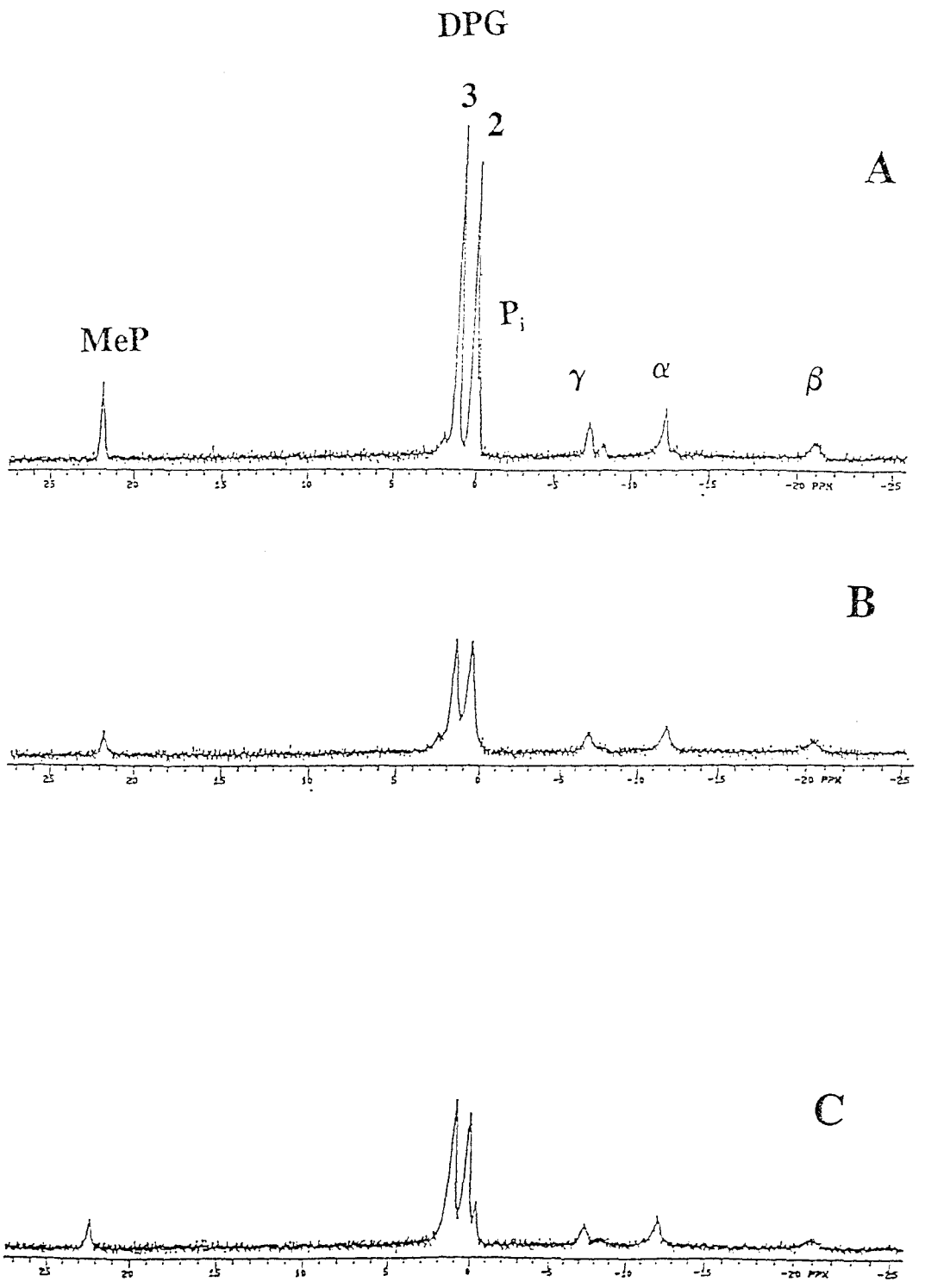

DPG
3
2
A
MeP
P_i
γ
α
β
B
C

Table 20. Transport rates and NMR relaxation time measurements in RBCs from hypertensive patients. The ^{23}Na T_1 was measured for 29 mM NaCl in membrane, and the ^{7}Li T_1 was measured for 11 mM LiCl in membrane.

Patient	Na^+-Li^+ rate	Na^+-H^+ rate	^{23}Na T_1	^{7}Li T_1	K_{Na}	K_{Li}	[Protein]
	mmol/L cell/hr	mmol/L cell/hr	ms	s	M^{-1}	M^{-1}	mg/ml
1	0.25	28.9	58.2	12.0	1012	2179	6.78
2	0.17	20.8	56.9	9.85	119	121	6.68
3	0.45	3.75	58.2	7.86	3548	123	5.00
4	0.20	2.60	60.7	13.0	96	1195	5.34
5	0.13	4.90	61.0	7.02	280	131	5.20

CHAPTER V

DISCUSSION

1. ^{23}Na and ^{7}Li NMR Relaxation Time Study of Na^+-H^+ Exchange Protein in the Human RBC Membrane

Canessa, M. (11) reported that the binding of alkaline metal ions for the human RBC membrane exchange protein had the relative affinity $H^+ > > Li^+ > Na^+$. We anticipated that the intracellular pH will change the distribution of free and bound intracellular Na^+ and ^{23}Na NMR relaxation times. As expected, when the intracellular pH decreased, the T_1 and T_2 values of ^{23}Na in the acid-loaded RBCs increased (Table 1, Figure 2). Because a small difference in relaxation times between two blood samples occurred, one may conclude that this is due to variations in intracellular sodium concentration. The ^{23}Na relaxation values were 30.6 ± 0.8 ms for T_1 and 10.6 ± 0.6 ms for T_2 in pH 7.4, and 39.4 ± 2.7 ms for T_1 and 15.9 ± 2.3 ms for T_2 in pH 5.8 cells in the intracellular sodium concentration range 1.0 to 22 mM. Therefore by studying the effect of intracellular sodium concentration on relaxation times (Table 2, Figure 3), in a large range of sodium concentrations, we found, however, that the ^{23}Na NMR relaxation times were not significantly different. There was, however, a significant difference in ^{23}Na relaxation values between cells at two different intracellular pH values. An increase in intracellular pH caused a decrease in ^{23}Na NMR relaxation times, indicating that protons compete with Na^+ binding sites in human RBCs. As the pH could also change the oxygenation state of RBCs, the Bohr effect was studied in N_2 and CO RBCs (Table 3). The ^{23}Na NMR

relaxation times showed no significant difference in the three different oxygenation states ruling out this possibility.

Studies on unsealed membrane were conducted for both Na^+ and Li^+ ions. The pH effect on ^{23}Na and ^7Li relaxation times found with intact RBCs was also observed with membrane. In the low intracellular sodium concentration range (2.0 to 8.0 mM), the ^{23}Na T_1 values were lower in the pH 8.0 samples than those in the pH 6.5 samples (Table 5, Figure 5), which indicated that H^+ competed with Na^+ ions. In the high sodium concentration range, however, the binding sites start being saturated and the relaxation times in two different pH samples were similar. The pH effect was also shown with Li^+ ions on membrane samples. By decreasing the pH of the membrane samples, the T_1 values of ^7Li NMR increased, indicating a competition between H^+ and Li^+ (Table 6, Figure 6). When decreasing the pH of membrane samples, the ^{23}Na NMR T_1 values increased only slightly (Table 7, Figure 7). This is because the binding of Na^+ ions was weaker than the binding of Li^+ ions to the Na^+-H^+ exchange protein (11), and therefore the competition between H^+ and Na^+ was not as pronounced as the competition between H^+ and Li^+. Adding Li^+ ions to the membrane samples already containing Na^+, resulted in an increase in the ^{23}Na NMR relaxation times in the pH range from 6.8 to 7.8 (Table 7, Figure 7). This indicates again that Li^+ competes with Na^+ binding sites in the human RBC membrane.

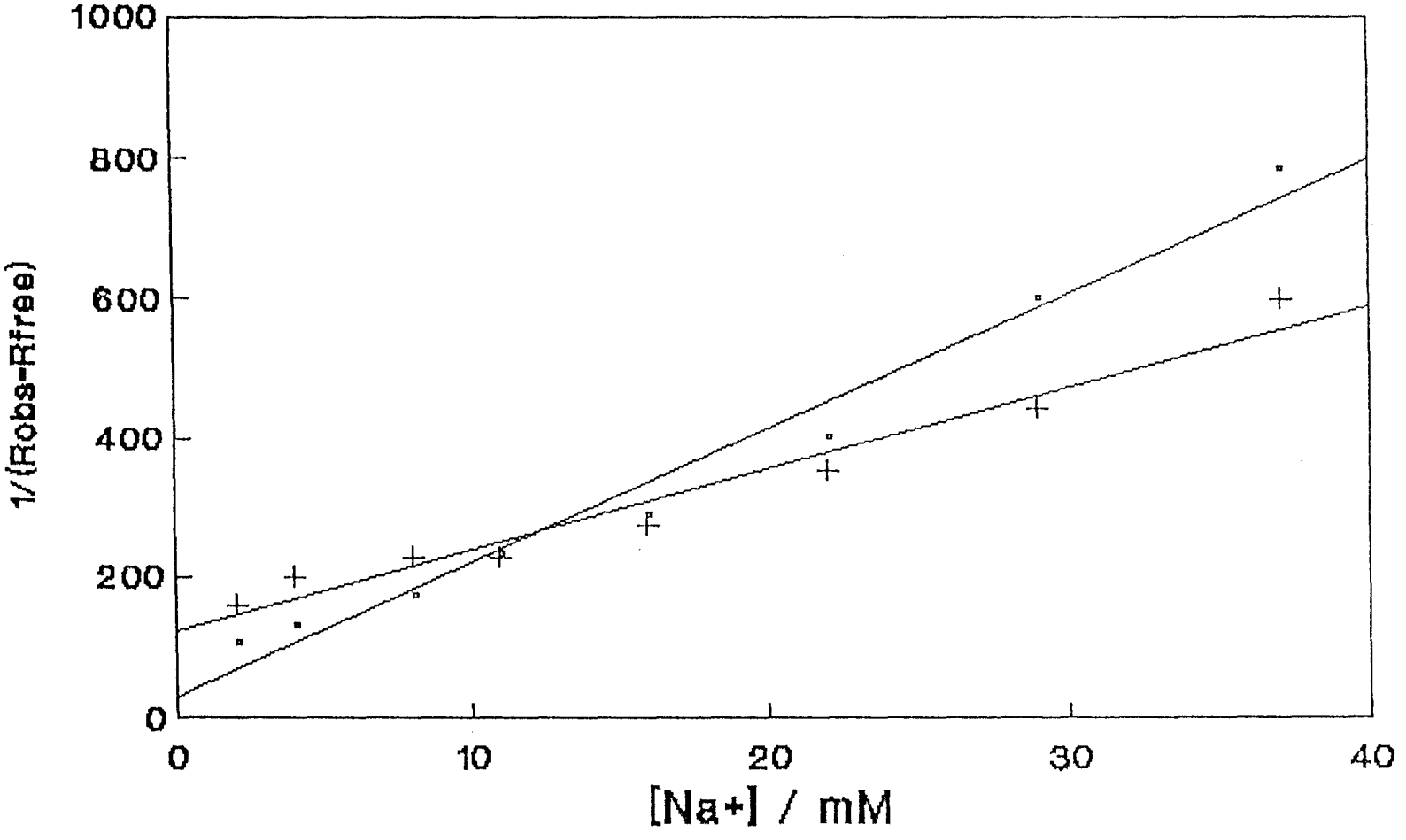
Using James-Noggle plots (53), we generated the binding constants for Na^+ to the RBC membrane. The binding constant, K_b, to the RBC membrane, is calculated by using T_1 values and assuming a two-state (free and bound Na^+ ions) model undergoing fast exchange:

$$R_{obs} = 1/T_{1(obs)} \qquad R_{free} = 1/T_{1(free)}$$

$$\Delta R^{-1} = (R_{obs}-R_{free})^{-1} = K_b^{-1}\{[B](R_{bound}-R_{free})\}^{-1} + [M^+]\{[B](R_{bound}-R_{free})\}^{-1},$$

where $[M^+]$ and $[B]$ are the total concentrations of Na^+ ions and membrane binding sites. The

Figure 15. James-Noggle plot for the Na^+ binding constants to the RBC membrane at two different pH values. Using the average data from Table 5, the binding constants for membrane samples at pH 8.0 and pH 6.5 were generated by taking the slope of the line and dividing it by the Y axis intercept. The binding constants were 96 M^{-1} at pH 6.5 and 493 M^{-1} at pH 8.0. The squares denote data for the pH 8.0 sample and the pluses are for the pH 6.5 sample.


1000
800
600
400
200
0
1/(Robs-Rfree)
0
10
20
30
40
[Na+] / mM

Na^+ binding constant in unsealed human RBC membrane was 96 ± 33 M^{-1} ($n=3$; $r^2=0.96$) at pH_{in} 6.5 and 493 ± 106 M^{-1} ($n=3$; $r^2=0.98$) for pH_{in} 8.0 (Figure 15) which is consistent with weaker Na^+ binding at acidic intracellular pH.

Amiloride and phloretin show competition effects on both Na^+ and Li^+ NMR relaxation times. By adding 6 mM amiloride to membrane, the ^7Li T_1 increased by 70% (Table 9) and the ^{23}Na T_1 increased by 35% (Table 8). As Li^+ binds more strongly than Na^+ (11), amiloride showed a stronger effect on $^7Li^+$ T_1 values. Phloretin (at 6 mM) increased the ^7Li T_1 values by 32% (Table 10), but showed no effect on the ^{23}Na T_1 values (Table 11). Because phloretin binds more weakly to the membrane than amiloride, it can not show any appreciable effect on the weakly bound Na^+.

2. NMR Measurements of Na^+-H^+ Exchange in human RBC suspensions

Using ^{23}Na NMR spectroscopy, we showed that the intra- and extracellular Na^+ peaks changed with time for acid-loaded and Na^+-depleted RBCs suspended at two different pH values (Table 12). We studied the hematocrit effect on the rate of Na^+-H^+ exchange for acid-loaded RBCs (Table 13, 14). As the hematocrit decreased, the Na^+-H^+ exchange rate increased as expected. At low hematocrit, fewer cells are surrounded by more Na^+ in the suspension, which causes fast Na^+ influx. By decreasing the Na^+ concentration in the suspension at the same hematocrit, the exchange rate decreased as expected (Table 15).

For Na^+ efflux measurements, we loaded RBCs with Na^+ using nystatin treatment. The final intracellular sodium concentration after loading was hard to control. For the same starting extracellular sodium concentration (150 mM) and 10% Ht, the final $[Na^+]_{in}$ varied from 35 to 60 mM, and the sodium efflux rate varied from sample to sample. The intraindividual reproducability was, however, excellent with an error of approximately 1 mmol/L cell/hr

(Tables 17 and 18). The coefficient of variation for Na^+ efflux was 25% as opposed to 69% for Na^+ influx measurements, which indicated that the ^{23}Na NMR method has a higher reproducibility under Na^+ efflux conditions. As the binding affinity of the transport membrane protein for Na^+ is not the same on both sides of the cell membrane (11), the NMR determined rates for Na^+-H^+ exchange measured under Na^+ efflux conditions are not comparable with the AA determined rates of Na^+-H^+ exchange measured under Na^+ influx conditions. It is therefore possible to use one dimensional ^{23}Na NMR spectroscopy to monitor the Na^+ concentration changes in RBCs undergoing Na^+-H^+ exchange.

^{31}P NMR spectroscopy was used successfully to detect intracellular pH changes during Na^+-H^+ transport (Table 19). By placing acid-loaded RBCs in two Na^+ influx media at different pH values, the intracellular pH increased in the pH 8.0 medium due to the large pH gradient but did not change appreciably in the pH 6.0 medium where a smaller pH gradient was present (Table 19).

The question concerning Na^+-H^+ exchange and Na^+-Li^+ countertransport being mediated by the same or a different transport protein in human RBC membranes has been studied by many investigators (3, 9-12, 21-26). The effect of inhibitors is controversial. Amiloride-sensitive Na^+-H^+ transport is activated by cytoplasmic lithium (9) but amiloride showed no inhibition effect on Na^+-Li^+ countertransport (22, 23). This could due to the improper experimental conditions used in the previous studies or due to the insensitivity of the assay transport methods. We worked directly with the human RBC membrane where the transport protein is located. Our study showed that amiloride, which was previously known as only an inhibitor for Na^+-H^+ exchange, competed with both Na^+ and Li^+ ions in human RBC membrane. We were unable to observe phloretin competition with Na^+ ions for the RBC membrane. This was because phloretin showed weaker binding than amiloride for the RBC

membrane as manifested by the smaller changes in the ^7Li NMR relaxation times for the same inhibitor concentrations (Tables 9 and 10). We also expected, however, that phloretin competed more weakly with Na^+ than did amiloride. It is therefore not surprising that we were unable to detect competition between phloretin and Na^+ for the human RBC membrane. Our relaxation data in the presence of amiloride are therefore consistent with the idea that Na^+-H^+ exchange and Na^+-Li^+ countertransport are mediated by the same membrane transport protein in human RBCs.

Our ion transport results show that alkaline metal NMR spectroscopy is a sensitive method to study Na^+-H^+ and Na^+-Li^+ transport in human RBCs. Future studies should focus on the application of the NMR methods to blood samples from hypertensive and manic depressive patients. It would be exciting to know whether the rates of Na^+-H^+ and Na^+-Li^+ exchange are correlated to the binding of metal ions to the human RBC membrane in these disease states. NMR relaxation time studies of RBCs from hypertensive and manic-depressive patients could provide novel binding information which may lead to a molecular understanding of the etiology of essential hypertension and/or manic-depression.

APPENDIX I

DERIVATION OF EQUATION 3

The total number of mmol $Na^+ = [Na^+]_{out0} \times (1\text{-}Ht) \times V_{total} + [Na^+]_{in0} \times Ht \times V_{total}$ (1)

The total number of mmol Na^+ = number of mmol Na^+_{in} + number of mmol Na^+_{out} (2)

R = Ratio of mmol Na^+_{in}/(mmol $Na^+_{out} \times 0.8$) (3)

From (3), the number of mmol Na^+_{out} = mmol $Na^+_{in}/(0.8 \times R)$ (4)

Combining (2) and (4),

The total number of mmol $Na^+ = \{1 + 1/(0.8 \times R)\} \times$ number of mmol Na^+_{in} (5)

$[Na^+]_{in}$ = mmol $Na^+_{in}/(Ht \times V_{total})$ (6)

Combining (5) and (6),

$Ht \times V_{total}[Na^+]_{in}$ = total number of mmol of $Na^+/\{1 + 1/(0.8 \times R)\}$ (7)

Combining (1) and (7),

$\{1 + 1/(0.8 \times R)\} \times (Ht \times V_{total} \times [Na^+]_{in}) = \{[Na^+]_{out0} \times (1\text{-}Ht) + [Na^+]_{in0} \times Ht\} \times V_{total}$ (8)

Canceling the V_{total} term from both sides, equation (8) becomes equation (9) for the calculation of intracellular Na^+ concentration:

$[Na^+]_{in} = \{[Na^+]_{out0} \times (1\text{-}Ht) + [Na^+]_{in0} \times Ht\}/\{Ht \times (1 + 1/0.8R)\}$ (9)

The meaning of the symbols is as follows:

$[Na^+]_{out0}$ is the $[Na^+]$ in suspension at time 0 min, Ht is the hematocrit of cell suspension, V_{total} is the volume of sample in NMR tube, and $[Na^+]_{in0}$ is intracellular $[Na^+]$ at time 0 min.

APPENDIX II

DERIVATION OF EQUATION 4

The total number of mmol $Na^+ = [Na^+]_{in0} \times Ht \times V_{total}$ (1)

The total number of mmol Na^+ = mmol Na^+_{in} + mmol Na^+_{out} (2)

R = Ratio of mmol Na^+_{in}/(mmol $Na^+_{out} \times 0.8$) (3)

From (3), mmol Na^+_{out} = mmol $Na^+_{in}/(0.8 \times R)$ (4)

Combining (2) and (4),

The total number of mmol $Na^+ = \{1 + 1/(0.8 \times R)\} \times$ number of mmol Na^+_{in} (5)

Combing (1) and (5),

mmol $Na^+_{in} \times (1 + 1/0.8R) = [Na^+]_{in0} \times Ht \times V_{total}$ (6)

$[Na^+]_{in}$ = mmol $Na^+_{in}/(Ht \times V_{total})$ (7)

Combining (6) and (7),

$Ht \times V_{total} \times [Na^+]_{in} = [Na^+]_{in0} \times Ht \times V_{toatl}/(1 + 1/0.8R)$ (8)

Canceling the V_{total} and Ht term from both sides, we have

$[Na^+]_{in} = [Na^+]_{in0}/(1 + 1/0.8R)$ (9)

The meaning of the symbols used above is the same as for the derivation of equation (3) shown in the previous page.

BIBLIOGRAPHY

1. Nakhoul, N.L., Boron, W.F.: Physiological roles of Na^+-H^+ exchange in epithelial cells. In Grinstein, S. (ed.) Na^+-H^+ Exchange. Boca Raton, FL: CRC Press, Inc., 1988, pp 167-178.

2. Mahnensmith, R.L., Aronson, P.S.: The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Res. 6: 773-788, 1985.

3. Semplicini, A., Mozzato, M.G., Sama, B., Nosadini, R., Fioretto, P., Trevisan, R., Pessina, A.C., Crepaldi, G., Palu, C.D.: Na^+-H^+ and Li^+-Na^+ exchange in red blood cells of normotensive and hypertensive patients with insulin dependent diabetes mellitus (IDDM). Am. J. Hypertension 2: 174-177, 1989.

4. Lifton, R.P., Sardet, C., Pouyssegur, J., Lalouel, J.M.: Cloning of human genomic amiloride-sensitive Na^+-H^+ antiporter gene, identification of genetic polymorphisms and localization on the genetic map of chromosome 1p. Genomics 7: 131-135, 1990.

5. Escobales, N., Canessa, M.: Amiloride-sensitive Na^+ transport in human red cells: Evidence for a Na^+-H^+ exchange system. J. Membr. Biol. 90: 21-28, 1986.

6. Escobales, N., Canessa, M.: Ca^{2+}-activated Na^+ fluxes in human red cells. J. Biol. Chem. 260: 11914-11923, 1985.

7. Dascalu, A., Nevo, Z., Korenstein, R.: Regulation of the Na^+-H^+ exchanger under conditions of abolished proton gradient: isosmotic and hyperosmotic stimulation. FEBS Lett. 282: 305-309, 1991.

8. Aronson, P.S., Nee, J., Suhm, M.A.: Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. Nature (London) 299: 161, 1982.

9. Parker, J.C.: Interactions of lithium and protons with sodium-proton exchanger of dog red blood cells. J. Gen. Physiol. 87: 189, 1986.

10. Schmalzing, G., Schlosser, T., Kutschers, P.: Li^+ as a substrate of the synaptosomal Na^+-H^+ antiporter. J. Biol. Chem. 261: 2759, 1986.

11. Canessa, M., Morgan, K., Semplicini, A.: Genetic differences in lithium-sodium exchange and regulation of the sodium-hydrogen exchanger in essential hypertension. J. Cardiovasc. Pharmacol. 12: S92-S98, 1988.

12. Canessa, M., Brugnara, C., Escobales, N.: The Li^+-Na^+ exchange and Na^+-K^+-Cl^- cotrasport systems in essential hypertension. Hypertension 10: I4-10, 1987.

13. Williams, R.R., Hunt, S.C., Wu, L.L., Hasstedt, S.J., Hopkins, P.N., Ash, K.O.: Genetic and epidemiological studies on electrolyte transport systems in hypertension. Clin. Physiol. Biochem. 6: 136-149, 1988.

14. Dorus, E., Cox, N.J., Gibbons, R.D., Shaughnessy, R., Pandey, G.N., Cloninger, C.R.: Lithium ion transport and affective disorders within families of bipolar patient: Identification of a major gene locus. Arch. Gen. Psychiatry 40: 545-552, 1983.

15. Hannaert, P.A., Garay, R.P.: A kinetic analysis of Na^+-Li^+ countertransport in human red blood cells. J. Gen. Physiol. 87: 353-368, 1986.

16. Sarkadi, B., Alifimoff, J.K., Gunn, R.B., Tosteson, D.C.: Kinetics and stoichiometry of Na^+-dependent Li^+ transport in human red blood cells. J. Gen. Physiol. 72: 249-265, 1978.

17. Mota de Freitas, D.E., Espanol, M.T., Dorus, E.: Lithium transport in red blood cells of bipolar patients. Lithium Therapy Monographs 4: 96-120, 1991.

18. Canessa, M., Adragna, N., Solomon, H.S., Connolly, T.M., Tosteson, D.C.: Increased sodium-lithium countertransport in red cells of patients with essential hypertension. N. Engl. J. Med. 302: 772, 1980.

19. Weder, A.B., Schork, N.J.: Mixture analysis of erythrocyte lithium-sodium countertransport and blood pressure. Hypertension 13: 145-150, 1989.

20. Weinberger, M.H., Smith, J.B., Fineberg, N.S., Luft, F.C.: Red-cell sodium-lithium countertransport and fractional excretion of lithium in normal and hypertensive human. Hypertension 13: 206-212, 1989.

21. Semplicini, A., Canessa, M., Mozatto, M.G., Ceolotto, G., Marzola, M., Buzzaccarini, F., Casolino, P., Pessina, A.C.: Red blood cell Na^+-H^+ and Li^+-Na^+ exchange in patients with essential hypertension. Am. J. Hypertension 2: 903-908, 1989.

22. Pandey, G.N., Sarkadi, B., Haas, M., Gunn, R.B., Davis, J.M., Tosteson, D.C.: Lithium transport pathways in human red blood cells. J. Gen. Physiol. 72: 233-247, 1978.

23. Carr, S., Thomas, T.H., Wilkinson, R.: Sodium-lithium countertransport activity and its sensitivity to inhibitors with erythrocyte ageing in man. Clin. Chim. Acta 178: 51-58, 1988.

24. Jennings, M.L., Adams-Lackey, M., Cook, K.W.: Absence of significant sodium-hydrogen exchange by rabbit erythrocyte sodium-lithium countertransporter. Am. J. Physiol. 249: C63-C68, 1985.

25. Kahn, A.M.: Difference between human red blood cell Na^+-Li^+ countertransport and renal Na^+-H^+ exchange. Hypertension 9: 7-12, 1987.

26. Kahn, A.M., Allen, J.C., Cragoe Jr., E.J., Shelat, H.: Sodium-lithium exchange and sodium-proton exchange are mediated by the same transport system in sarcolemmal vesicles from bovine superior mesenteric artery. Circ. Res. 65: 818-828, 1989.

27. Pandey, G.N., Ostrow, D.G., Hass, M., Dorus, E., Casper, R.C., Davis, J.M., Tosteson, D.C.: Abnormal lithium and sodium transport in erythrocytes of a manic patient and some members of his family. Proc. Natl. Acad. Sci. USA 74: 3607-3611, 1977.

28. Mota de Freitas, D.E., Silberg, J., Espanol, M.T., Dorus, E., Abraha, A., Dorus, W., Elenz, E., Whang, W.: Measurement of lithium transport in RBC from psychiatric patients receiving lithium carbonate and normal individuals by ^{7}Li NMR spectroscopy. Biol. Psychiatry 28: 415-424, 1990.

29. Ramasamy, R., Mota de Freitas, D.E., Bansal, V.K., Dorus, E., Laboka, R.: Nuclear magnetic resonance studies of lithium transport in erythrocyte suspensions of hypertensives. Clin. Chim. Acta 188: 169-176, 1990.

30. Springer, C.S. Jr.: Transmembrane ion pumping: High resolution cation NMR spectroscopy. Ann. N.Y. Acad. Sci. 508: 130-148, 1987.

31. Ramasamy, R., Mota de Freitas, D.E., Geraldes, C.F.G.C., Peters, J.A.: Multinuclear NMR study of the interaction of the shift reagent lanthanide(III) bis(triphosphate) with alkali-metal ions in aqueous solution and in the solid state. Inorg. Chem. 30: 3188-3191, 1991.

32. Mota de Freitas, D.E., Espanol, M.T., Ramasamy, R., Labotka, R.J.: Comparison of Li^+ transport and distribution in human red blood cells in the presence and absence of Dysprosium(III) complexes of triphosphate and triethylenetetraminehexaacetate. Inorg. Chem. 29: 3972-3979, 1990.

33. Ramasamy, R., Mota de Freitas, D.E., Jones, W., Wezeman, F., Labotka, R., Geralders, C.F.G.C.: Effects of negatively charged shift reagents on red blood cell morphology, Li^+ transport, and membrane potential. Inorg. Chem. 29: 3980-3985, 1990.

34. Espanol, M.T., Mota de Freitas, D.E.: ^{7}Li NMR studies of lithium trasnport in human erythrocyte. Inorg. Chem. 26: 4356, 1987.

35. Ramasamy, R., Espanol, M.C., Long, K.M., Mota de Freitas, D.E., Geraldes, C.F.G.C.: Aqueous shift reagents for ^{7}Li NMR trasnport studies in cells. Inorg. Chim. Acta. 163: 41-52, 1989.

36. Pettegrew, M.W., Post, J.F.M., Panchaligam, K., Withers, G., Woessner, D.E.: ^{7}Li NMR study of normal human erythrocytes. J. Magn. Reson. 71: 504-519, 1987.

37. Mota de Freitas, D.E., Espanol, M.T., Rong, Q.: ^{7}Li NMR relaxation studies of Li^{+} ion interactions with human erythrocyte membrane components. In preparation.

38. Gadian, D.G.: Nuclear Magnetic Resonance and its Application to Living Systems. Oxford: Clarendon Press, p 113, 1982.

39. Canessa, M.: Kinetic properties of Na^{+}-H^{+} exchange and Li^{+}-Na^{+}, Na^{+}-Na^{+}, and Na^{+}-Li^{+} exchanges of human red cells. Methods Enzymol. 173: 176-191, 1989.

40. Magnuson, J.A., Shelton, D.S., Magnuson, N.S.: A nuclear magnetic resonance study of sodium ion interaction with erythrocyte membranes. Biochem. Biophys. Res. Commun. 39: 279-283, 1970.

41. Ong, R.L., Cheung, H.C.: ^{23}Na-NMR studies of Na^{+} interaction with human red cell membranes from normotensives and hypertensives. Biophys. Chem. 23: 237-244, 1986.

42. Urry, D.W., Trapane, T.L., Andrews, S.K., Long, M.M., Overbeck, H.W., Oparil, S.: NMR observation of altered sodium interaction with human erythrocyte membrane of essential hypertensives. Biochem. Biophys. Res. Commun. 96: 514-521, 1980.

43. Nissen, H., Jacobsen, J.P., Horder, M.: Assessment of the NMR visibility of intraerythrocytic sodium by flame photometric and ion-competitive studies. Magn. Reson. Med. 10: 388-398, 1989.

44. Labotka, R.J., Kleps, R.A.: A phosphate-analogue probe of red cell pH using phosphorus-31 nuclear magnetic resonance. Biochemistry 22: 6089-6095, 1983.

45. Labotka, R.J.: Measurement of intracellular pH and deoxyhemoglobin concentration in deoxygenated erythrocytes by phophorus-31 nuclear magnetic resonance. Biochemistry 23: 5549-5555, 1984.

46. Petersen, A., Jacobsen, J.P., Horder, M.: ^{31}P NMR measurements of intracellular pH in erythrocytes: Direct comparison with measurements using freeze-thaw and investigation into the influence of ionic strength and Mg^{2+}. Magn. Reson. Med. 4: 341-350, 1987.

47. Steck, T.L., Kant, J.A.: Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol. 31: 172-177,1974.

48. Pike, M.M., Yarmuch, D.M., Balschi, J.A., Lenkinski, R.E., Springer, C.S.: Aqueous shift reagents for high resolution cationic nuclear magnetic resonance. II. Mg-25, K-39, and Na-23 resonances shifted by chelidamate complexes of dysprosium (III) and thulium (III). Inorg. Chem. 22: 2388, 1983.

49. Bollag, D.M., Edelstein, S.J.: Protein Methods Wiley-Liss: pp 50-55, 1991.

50. Dozy, A.M., Kleihauer, E.F., Huisman, T.H.: Studies on the heterogeneity of

hemoglobin. J. Chromatography 32: 723, 1968.

51. Agre, P., Parker, J.C.: Red Blood Cell Membranes, Structure, Function, Clinical Implications. Marcel Dekker, Inc., pp 538, 1989.

52. Cantor, C., Schimmel, P.: Biophysical Chemistry. Freeman, pp 963-964, 1980.

53. James, T.L., Noggle, J.H.: ^{23}Na nuclear magnetic resonance relaxation studies of sodium ion interaction with soluble RNA. Proc. Natl. Acad. Sci. USA 62: 644-649, 1969.

VITA

The author, Suilan Mo, was born May 6, 1965, in Chongqing, China.

In September, 1982, she entered China's University of Science and Technology and received the Bachelor Degree of Science with a major in Chemistry in June, 1987.

In January 1990, she joined Loyola University of Chicago to pursue graduate studies in Chemistry. She was awarded a teaching assistantship from January 1990 to May 1991. She was supported by a research assistantship as part of a research grant awarded to Dr. Duarte Mota de Freitas by the National Institute of Mental Health from June 1991 to February, 1992.

APPROVAL SHEET

The thesis submitted by **Suilan Mo** has been read and approved by the following committee:

Dr. Duarte Mota de Freitas, Director
Associate Professor of Chemistry, Loyola

Dr. Kenneth Olsen
Professor of Chemistry, Loyola

Dr. Daniel Graham
Assistant Professor of Chemistry, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

The thesis is, therefore, accepted in partial fulfillment of the requirements for the degree of **Master of Science**.

5/6/92
Date

Director's Signature