NUCLEAR MAGNETIC RESONANCE STUDIES ON INTRACELLULAR SODIUM IN HUMAN ERYTHROCYTES AND FROG MUSCLE

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ABSTRACT The nuclear magnetic resonance (NMR) spectrum of sodium was determined in muscle and erythrocytes using conventional continuous wave techniques. NMR spectra of fresh intact muscle revealed a single line with a width of about 38 Hz equivalent in intensity to about 53% of the total muscle sodium, in general agreement with previous work. Prolonged washing with sodium-free solutions led to a marked loss of both total and NMR-detectable sodium. The NMR-visible sodium remaining in the muscle was somewhat larger than the fraction calculated to remain extracellular and, presumably, was intracellular. The original sodium signal is thus interpreted as arising from both extracellular sodium and the narrow line portion of the signal from intracellular sodium. NMR spectra of sodium were also obtained for human erythrocytes under conditions preserving the sodium transport system. The intensity of the sodium signal in fresh cells was 98% of that present in the same samples after complete hemolysis of the cells. The NMR sodium present in intact cells was 92% of the sodium recovered by flame photometric determination of sodium from ashed samples. It is concluded that no NMR-"invisible" sodium occurs in human erythrocytes and that the presence of such sodium is not necessary for the normal functioning of the sodium transport system in erythrocytes.

INTRODUCTION

In recent years a number of papers have appeared dealing with the physical state of water and ions, particularly sodium, in a variety of tissues as revealed by changes in the NMR spectra (1–10). Although the main intent of this past work has been to describe the physical state of the intracellular water and ions, in most of the multicellular tissues studied an appreciable fraction of the total molecules is extracellular.

In view of the numerous references in the physiological literature which attest to the difficulty of distinguishing the intracellular and extracellular compartments of any tissue by any analysis, whether experimental or philosophical, one might expect to encounter considerable difficulty in interpreting NMR spectra from a tissue which has multiple intracellular and extracellular compartments. Our preliminary attempts to interpret the NMR spectrum of intracellular sodium, utilizing frog

muscle, amply confirmed this expectation. Nonetheless we report the results briefly because they confirm previous work and demonstrate that our technical procedures give results similar to those of other workers.

Human erythrocytes were used in the main experiments to be discussed, partly because it is possible, by utilizing standard procedures of washing the cells, to obtain a compact preparation of cells virtually free of extracellular sodium ions (at least for a short period), and partly because erythrocytes possess an intracellular system of much simpler structure than muscle.

After the completion of our work several papers appeared which have important ramifications for the interpretation of our data and the earlier published results on sodium NMR in muscle. Shporer and Civan (11) showed that the ^{23}Na signal in a liquid crystal (sodium linoleate in water) is split into a relatively narrow central component and broad components symmetrically disposed to higher and lower frequency. Chen and Reeves (12) made similar observations in another lyotropic nematic phase and in addition found a model system in which the high- and low-frequency satellite peaks are broadened beyond recognition. Lindblom (13) found similar results for ^{23}Na NMR in the liquid crystal lecithin-sodium cholate-water, while Edzes et al. (14) observed such splittings and line broadening for ^{23}Na signals in oriented DNA. Recently Berendsen and Edzes (15) presented a detailed analysis of previously published ^{23}Na NMR results in muscle, together with new pulse ^{23}Na NMR studies. They concluded that the commonly reported failure to observe a large fraction of the expected ^{23}Na signal in muscle and other biological tissues is the result of the occurrence of "medium range" order (over distances of about 100 Å or more) among charged macromolecules in the cell. The ^{23}Na line is thus split into a triplet, with the broad satellite peaks, which account for 60% of the overall intensity, often unobserved. The presence of NMR-invisible sodium cannot, according to this picture, provide a measure of sodium that is tightly bound, as Cope had originally proposed (1). Berendsen and Edzes concluded therefore that sodium in biological tissue behaves nearly the same as in the ordinary liquid state.

Our experiments were directed at two biological systems with typical sodium transport behavior: frog muscle, a system for which there is, of course, abundant evidence for medium range order in the contractile apparatus, and human erythrocytes, in which no such order has been demonstrated. On the basis of the analysis of Berendsen and Edzes, one would expect, therefore, little or no invisible intracellular sodium in erythrocytes in strong contrast to the situation in frog muscle.

METHODS

NMR Spectra

^{23}Na NMR spectra were obtained using a Varian HR-60 spectrometer (Varian Associates, Palo Alto, Calif.) operating at 15.8 MHz. The spectrometer was modified in a manner similar to that described previously (16) to include a proton field/frequency lock. The trans-

mitter coil was double tuned to 15.8 and 60 MHz, while a separate 60 MHz receiver coil was wound above and below the 15.8 MHz receiver coil and coaxial with it. Water in the sample usually served as the locking signal. Both 15.8 and 60 MHz frequencies were derived from a single frequency synthesizer. The ^{23}Na spectrum was scanned by sweeping an audio side band frequency, and the start of each frequency sweep automatically triggered the address advance of a Varian C-1024 time-averaging computer. Each recorded spectrum was the summation of 400 5-s sweeps, each over a range of about 300 Hz.

Signal areas were measured with a planimeter from chart records. Preliminary experiments with NaCl solutions of different concentration showed an excellent linear relation between peak area and Na^+ concentration. In all experiments care was taken to avoid saturation.

For a few studies of broad lines the spectrometer was used without proton lock and with a low-frequency (25 Hz) modulation to produce derivative curves. In these experiments the V-4352 linear sweep unit was used to sweep the field and to trigger the C-1024 computer. Because there was no field/frequency lock some field drift occurred during repetitive scans, but in the 34 min period used for these particular measurements this drift results in only slight line broadening (about 5 Hz).

Solutions

The solutions used are listed in Table I.

Preparation of Muscle Samples

Large adult specimens of *Rana pipiens* were sacrificed by decapitation. The semitendinosus muscles were carefully dissected from the animal, blotted on filter paper to remove surface fluid, and trimmed at the ends to minimize the amount of adherent tendon. After blotting, the muscles were gently packed into an NMR tube to a premarked height to give a total sample volume of 3.5 ml. Particular care was taken to minimize any void spaces in the vicinity of the receiver coil. The total amount of muscle in the sample was 3.4–3.8 g.

After the NMR signal had been recorded (recording time about 33 min), the muscles were transferred to a platinum crucible, dried over a vacuum, and then ashed. The ashed residue was then dissolved in 3.5 ml of 0.1 N HCl and transferred back to the original NMR tube. The ^{23}Na signal of this solution was recorded at identical instrument control settings. The signal area was taken as a measure of the total muscle sodium. Although small air pockets may have existed in the sample tube containing the intact muscles, we assume that any such voids were randomly distributed through the 3.5 ml volume. Thus the total sodium per unit volume should be the same before and after ashing.

TABLE I

COMPOSITION OF SOLUTIONS USED (mM)

	Na	K	Li	Mg	Ca	Cl	Tris	PO_4	pH
Ringer's*	115	2.5	—	1.5	1.8	124	—	3.1	7.1
Lithium-Ringer's*	—	2.5	115	1.5	1.8	124	—	3.1	7.1
Erythrocyte-saline‡	140	5	—	2	1	152	2	—	7.3
Lithium-erythrocyte-saline‡	—	5	140	2	1	152	2	—	7.3

* Solutions also contained 3 mg tubocurarine per liter of solution

‡ Solutions also contained 2 mM each of glucose and adenosine

In some experiments, after recording the first ^{23}Na signal, the individual muscles were removed from the tube and bathed in lithium-Ringer's or potassium-Ringer's solution. The muscles were then blotted and repacked into the same tube for a second measurement of the ^{23}Na spectrum. In a few cases this procedure was repeated for a subsequent soak. After the final soaking period, the muscle samples were dried, ashed, and a final spectrum obtained to determine the total residual sodium in the muscles.

We estimate the experimental error in the measurement of signal areas to be about 10–15% for the intact and ashed muscles. For the weaker signals found after extensive soaking the error may be as large as 40%.

Preparation of Erythrocytes

Fresh human blood was obtained from two sources: laboratory volunteers, in which case the blood was drawn into heparin-containing syringes, or from a commercial blood bank, in which case the blood was delivered in plastic bags containing 450 ml whole blood and 50 ml ACD[1] solution as anticoagulant. Subsequent treatment of the blood was the same for both batches.

The blood was first spun at 5000 *g* for 5 min to separate the cells from the plasma proteins. The pellet was then suspended to a cytocrit of about 30% and washed three times with erythrocyte-Ringer's solution. After the final wash the cells were left suspended in the storing medium at a relative cytocrit of 30–50% and refrigerated at a temperature 4–10°C. Ampicillin trihydrate was added to retard bacterial growth.

Samples were prepared for NMR spectroscopy as follows: a pellet was formed at the bottom of a standard test tube by centrifuging a cell suspension containing 10 μM strophanthidin or ouabain for 5 min at 5000 *g*. Approximately 5 ml of these packed cells were gently transferred with a syringe with a large bore (16 gauge) needle to the special NMR tubes. The cells were then recentrifuged for 10 min at 5000 *g* to pack the pellet in the bottom of the NMR tube. The excess pellet was then carefully removed with a syringe until the sample contained exactly 4.0 ml of packed cells. The sample was then placed in the spectrometer and the ^{23}Na signal obtained (running time 15–45 min) at 20°C.

Hemolysis of Sample

The total sodium in the pellet was determined by hemolyzing the pellet to disrupt the cells without transferring it from the NMR tube. Adequate hemolysis was achieved simply by freezing the sample quickly by immersing the tube in a mixture of acetone and dry ice and then thawing the pellet at 37°C. Microscopic examination of the hemolysate indicated about 1% of the cells appeared grossly intact after a single freeze-thaw treatment. None survived a second freeze-thaw cycle. Consequently, the standard procedure involved two cycles of freeze-thaw followed by a second determination of the ^{23}Na spectrum under identical conditions as for the first. Total sodium was also determined on a sample of the hemolyzed pellet by flame photometric techniques previously described (17).

Because our standard procedures for handling erythrocytes did not control the relative oxygenation of the hemoglobin, some variability in the sodium signal might be introduced because of differences in the tertiary structure of reduced and oxygenated hemoglobin. Therefore, in a few experiments, before the final centrifugation, the cell suspension was equilibrated with carbon monoxide to convert the hemoglobin to carboxyhemoglobin. No differences were found in the spectra of sodium so treated and the procedure was not adopted routinely.

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[1] ACD solution contains (grams per liter) citric acid: 8; sodium citrate: 22; dextrose: 24.5.

Sodium Efflux from Erythrocytes

Erythrocytes to be used for isotope experiments were suspended overnight at 10°C in the usual saline containing ^{22}Na. They were then processed exactly as described above for the NMR experiments including the final spin in the NMR tube. Instead of being placed in the spectrometer, however, the pellet was allowed to sit for a period of time comparable with that required for a spectrum determination and then diluted 1000 times by discharge into a flask of nonradioactive saline. Aliquots of this suspension were removed at intervals and centrifuged to form a pellet. The supernatant was carefully removed and the pellet counted to determine the contained radioactivity. Since the aqueous solution was very much less radioactive than the cells, it was not necessary either to wash the pellet or to correct the counts for extracellular radioactivity.

Preparation of Hemoglobin Solution

For certain experiments to be described in Results, it was necessary to have relatively concentrated solutions of undenatured hemoglobin. Such were prepared by the technique of vacuum dialysis.

Fresh erythrocytes were hemolyzed in 4 vol of distilled water. The suspension was sedimented at 48,000 *g* for 20 min. Because of the high specific gravity of the hemoglobin solution, a high speed centrifugation was necessary to sediment the fragmented cells. The supernatant was separated from the pellet and poured into a dialysis tube suspended in a vacuum jar. The jar was then evacuated to a pressure of about 10 mm of Hg, sealed, and placed in the refrigerator for about 24 h during which time the hemoglobin solution became about eightfold concentrated. The hemoglobin concentration, relative to the initial hemolysate, was determined by comparing the light absorbancy at 430 nM.

RESULTS

Muscle

Typical ^{23}Na spectra of a frog muscle, before and after the ashing, are shown in Fig. 1. The "visible" ^{23}Na signal observed from the intact frog muscle has a line width

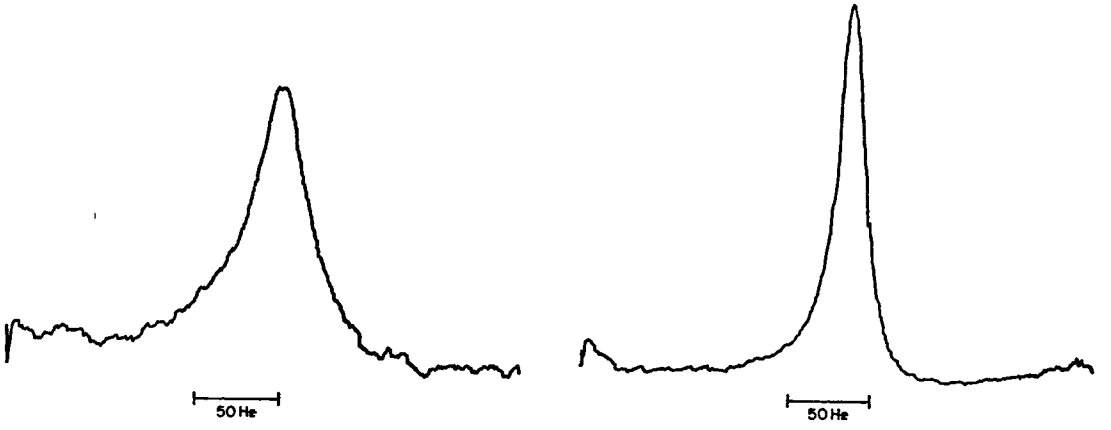

FIGURE 1 NMR sodium signal from whole frog muscle. Left: intact, fresh muscle. Right: signal from same muscle after it had been ashed and the residue dissolved in 0.1 N HCl. The gain sensitivity was one-half that for the intact muscle spectrum.

of about 38 Hz. The increase in the width over that of an aqueous NaCl solution could be due to an increase in ^{23}Na quadrupolar relaxation rate, which may arise from asymmetry in its local environment and/or from a motional restriction of the sodium ion (18). The results of 10 such experiments are listed in Table II. Just over half of the total muscle sodium present in the ashed sample can be detected in the intact muscles leaving an invisible fraction of 47%. In these experiments no effort was made to control the amount of sodium in the extracellular space and hence the values for total muscle sodium vary rather widely. Our results, however, do confirm the previous work which indicates that a significant fraction of the sodium in whole frog muscle is not detected by NMR spectroscopy. Our estimate of the invisible fraction is intermediate between those data reported earlier by other workers: Martinez et al. (6), 36%; Cope (1, 2), 65%; Czeisler et al. (7), 80%.

In view of the large and variable contribution of the extracellular sodium to total muscle sodium, a second series of experiments was undertaken in an attempt to remove the extracellular sodium. In these experiments, after the initial spectra had been obtained, the muscles were bathed in lithium-Ringer's solution for various periods, and then repeat spectra were obtained. The results are shown in Table III.

The most rapid loss of NMR signal occurred in the first 10–25 min of the lithium wash. Since this is the interval of time during which loss of muscle sodium occurs largely from the extracellular space (19), one might conclude that the fractions of visible and invisible NMR sodium correspond to the extra and intracellular sodium,

TABLE II

SODIUM CONCENTRATION OF INTACT AND ASHED FROG MUSCLE AS DETERMINED BY ^{23}Na NMR-VISIBLE SIGNAL*

Intact	Ashed	Percent visible Na^+
mM	*mM*	
18.9	28.7	65.9
16.8	26.8	62.7
19.0	31.4	60.5
16.5	30.9	53.4
10.7	22.8	46.8
10.6	22.9	46.1
13.7	27.5	50.0
12.2	25.1	48.7
14.0	30.3	46.0
12.3	23.6	52.0
		Average = 53.2

* All Na^+ concentrations corrected to volume of intact muscle, were determined from standard NMR signal at room temperature.

TABLE III

SODIUM CONCENTRATION IN FROG MUSCLE AT VARIOUS BATHING TIMES AS DETERMINED BY ^{23}Na NMR-VISIBLE SIGNAL

Intact	Li-Ringer's solution			Ashed
	First bathing 10 min	Second bathing 25 min	Third bathing 40 min	
mM	*mM*	*mM*	*mM*	*mM*
17.3	7.7	2.6	—	7.9
14.9	12.1	4.4	1.1	5.5
21.9	—	4.9	1.9	7.2
15.2	—	—	2.4	8.5
14.7	—	—	2.3	9.3
10.4	—	—	4.0	5.8
13.3	7.8	3.4	1.4	4.7
19.7	8.2	3.4	1.3	3.8

respectively. The last two columns of Table III indicate, however, that this interpretation is oversimplified. As might be expected, after 40 min of washing, the total muscle sodium has declined significantly; however, the NMR-visible sodium still amounts to about 30% of the total residual sodium. (In the Discussion, a correction factor for residual extracellular sodium is considered and shown to be inadequate quantitatively to account for the visible sodium.) This result is in agreement within experimental error with the predictions of the Berendsen-Edzes model, which requires a narrow (visible) component of the signal of 40% of the total sodium.

In order to have some estimate of the minimum line width which the invisible sodium could have and still escape detection by our techniques, we investigated a model system, sodium bound to an ion exchange resin (Amberlite IR-120, from Rohm and Haas Co., Philadelphia, Pa., 8% cross-linking in divinyl benzene [DVB]). This resin, suspended in NaCl solution, displays a ^{23}Na signal that is a composite of a sharp line (10–15 Hz width) and a broad line (about 210 Hz width). On washing with water, the sharp signal is removed, but the broad signal remains, as shown in Fig. 2. We mixed a small quantity of washed, sodium-containing resin with an inert material of comparable particle size ($ZrSO_4$) to produce an overall concentration of Na^+ of 17 mM, about twice that of the sodium remaining in muscle after 30 min bathing in lithium-Ringer's solution. Using low-frequency modulation to enhance the signal from the broad line, we observed the 210 Hz-wide line from this resin sample with a signal-noise ratio of about 3. Under comparable conditions we were unable to detect any signal from a muscle sample bathed for 30 min. This observation suggests that we could not have detected a line of width greater than about 200 Hz. This estimate of minimum line width is fully compatible with the observations by Cope (5) and Berendsen and Edzes (15) of one component with $T_2 \approx 1$ ms (equivalent to a line width of about 310 Hz).

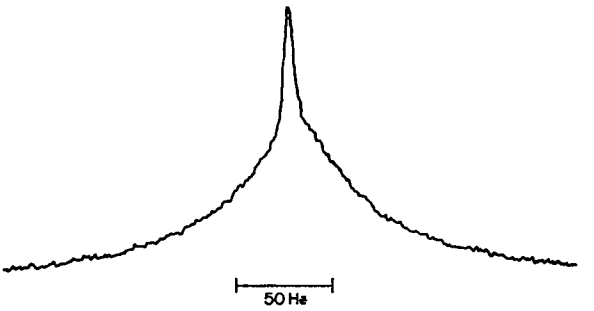

FIGURE 2 NMR sodium signal of sodium bound to an ion exchange resin. (Amberlite IR-120, 8% cross-linking in DVB)

Erythrocytes

Sodium Efflux from Packed Erythrocytes. In order to demonstrate that the sodium transport system was functioning normally under the conditions of our experiments, a single determination of the tracer efflux of sodium from intact and strophanthidin-poisoned cells, was made as described in Methods. The results of this experiment are shown in Fig. 3, which illustrates the decline in cell radioactivity with time after dispersing the pellet in nonradioactive solution. At the arrow sufficient ouabain was added to make the resulting solution 10 μM. The subsequent efflux was reduced by a factor of nearly 7, although it was not completely abolished. The time constant for the efflux from the cells was 5.2 h in the absence of external ouabain and about 37 h in the presence of the glycoside. These time constants accord well with those previously published by several authors (e.g., Garrahan and Glynn [20]).

Effect of Hemoglobin upon ^{23}Na Spectrum In Vitro. Since a variety of proteins and ion exchange resins bind sodium ions sufficiently strongly to broaden the NMR signal beyond recognition, it seemed prudent to investigate the extent to which hemoglobin, present in an aqueous medium, might alter the sodium spectrum.

Two types of experiments were done. In both cases the starting material was a low sodium (1.4 mM) solution of hemoglobin prepared by the vacuum dialysis technique described in Methods.

In the first experiment, the effect of a constant hemoglobin concentration on the sodium signal obtained from solutions of progressively increasing concentration was determined. Such solutions were obtained by adding serially small volumes of 2.8 M sodium chloride solution to the same concentrated hemoglobin solutions (approximately 1.6 times more concentrated than erythrocyte hemoglobin which is about 5 mM). The results are shown in Fig. 4. The figure shows clearly that the area of the sodium signal is a linear function of the sodium concentration, except possibly at the lowest sodium concentrations where the low signal-to-noise ratio makes an accurate determination of signal area difficult. Fig. 4 also shows that the line

width is little affected by increasing sodium concentrations, there being only a 10% decrease in line width in the range 15–45 mM sodium.

The second experiment was concerned with the effect of varying the hemoglobin concentration at constant sodium concentration. A sample aliquot of concentrated hemoglobin solution was prepared with a sodium concentration of 17 mM. The hemoglobin concentration was varied by progressively diluting the sample with 17

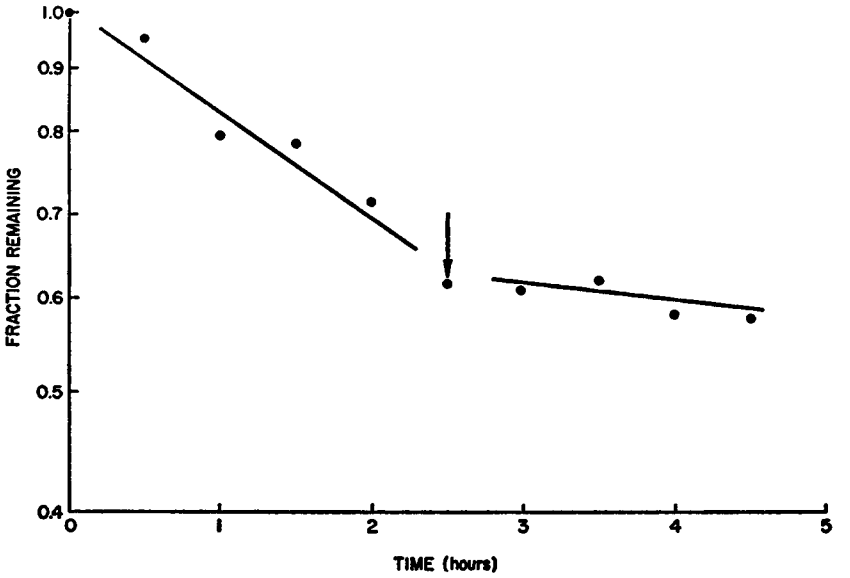

FIGURE 3 Semilogarithmic plot of time-course of sodium efflux from human erythrocytes suspended in standard saline solution (see Table 1). At the arrow, sufficient 400 μM ouabain solution was added to the incubation flask to make the final concentration 10 μM. Temperature was 37°C.

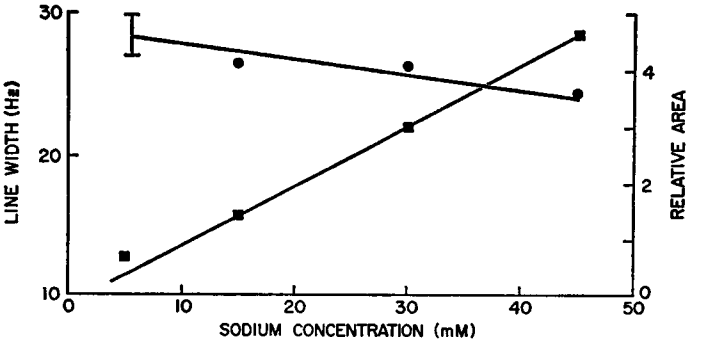

FIGURE 4 Effect of sodium concentration on line width and signal area in the presence of concentrated hemoglobin solutions (approximately 8 mM). Signal half-width: solid circles (at the lowest sodium concentration a range of values is given indicated by the vertical bar). Signal area: solid squares. Data plotted relative to mean signal area.

mM sodium chloride solutions. At each dilution the sodium signal was measured. The effect of hemoglobin on line width is shown in Fig. 5, over the range 0–8 mM. The line width is almost doubled at the highest hemoglobin concentration, and the concentration dependence is nonlinear above a hemoglobin concentration of about 4 mM. Fig. 5 also illustrates clearly that the signal area was essentially unaffected even by highly concentrated hemoglobin solutions, since the signal area is reduced only 4% in the presence of $\approx$ 8 mM hemoglobin.

These experiments indicate that the area of the sodium signal in aqueous solution is quantitatively unaffected by even larger-than-normal concentrations of hemoglobin and form the basis for our use of the strength of the sodium signal in hemolyzed erythrocyte solutions as a measure of the total sodium in the sample.

Spectrum of ^{23}Na in Intact Cells. Cells to be used for NMR spectra determinations were washed at least twice with lithium saline containing 10 μM ouabain or strophanthidin, then formed into a pellet as described in Methods and placed in the spectrometer. After the initial run, the cells were hemolyzed as described in Methods and a repeat spectrum obtained to give the NMR-visible sodium concentration in the presence of hemoglobin and stroma proteins but in the absence of intact cell membranes. A result typical of 31 determinations on bloods from 10 different apparently healthy subjects is shown in Fig. 6. The spectrum for both intact and hemolyzed cells is broadened symmetrically about twofold as compared with the standard. The signal area, however, is only trivially increased by hemolysis, indicating that no appreciable sodium exists in the intact cell that is not detected by

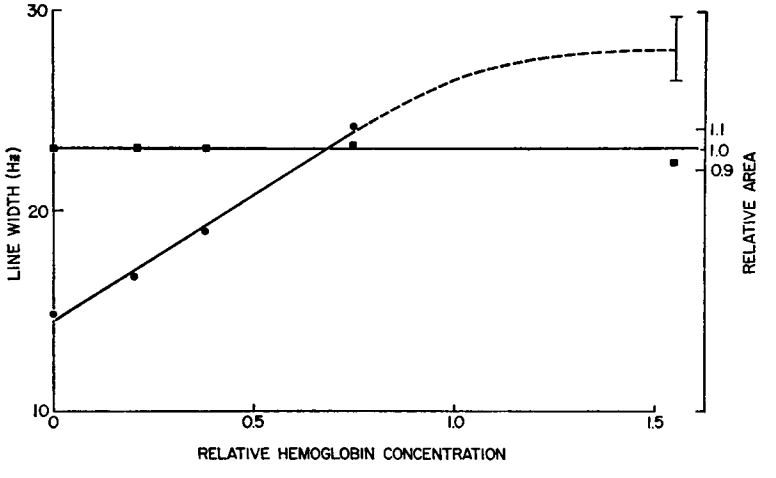

FIGURE 5 Effect of hemoglobin upon sodium signal area and line width, at constant sodium concentration (17 mM). Signal half-width: solid circle. Signal area: solid squares. The hemoglobin concentration is plotted relative to that occurring in the intact cell (about 5 mM). At the highest hemoglobin concentration, the extreme values are indicated by the vertical bar.

the NMR probe. The collected data are tabulated in Table IV. The ratio of the sodium signal in intact cells to the signal in the hemolyzed pellet is 0.98. For reference, the total sodium as determined on each sample by flame photometric analysis is included. The mean sodium content, measured by flame analysis, was about 6% higher than the value obtained by NMR. This discrepancy is considered further in the Discussion.

TABLE IV

INTERNAL SODIUM CONCENTRATION IN HUMAN ERYTHROCYTES

Sample	Donor	Na (mmol/liter packed cells)			Relative concentration		
		Intact	Hemolyzed	Flame photometer	Intact / Hemolyzed	Hemolyzed / Flame	Intact / Flame
H	3	13	13	13	1.00	1.00	1.00
I	4	9	12	12	0.75	1.00	0.75
J	1	12	14	15	0.86	0.94	0.80
K	6	—	8	8	—	1.00	—
L	2	—	8	11	—	0.73	—
M	4	6	8	9	0.75	0.89	0.66
N	4	13	12	15	1.08	0.80	0.86
O	1	18	16	18	1.13	0.89	1.00
P	3	15	15	16	1.00	0.94	0.94
Q	6	8	9	9	0.89	1.00	0.89
R	2	12	12	11	1.00	1.09	1.09
S	5	7	8	8	0.87	1.00	0.87
T	4	15	14	15	1.07	0.93	0.99
U	1	19	19	19	1.00	1.00	1.00
V	3	15	15	16	1.00	0.94	0.94
W	6	8	9	11	0.89	0.82	0.72
X	2	11	11	13	1.00	0.85	0.85
Y	5	7	8	8	0.87	1.00	0.87
AA	2	16	16	16	1.00	1.00	1.00
AB	3	18	17	19	1.06	0.90	0.95
AC	4	17	15	18	1.13	0.83	0.93
AE	5	13	15	15	0.87	1.00	0.87
AF	6	8	8	11	1.00	0.73	0.73
A	MSB	15	12	15	1.25	0.80	1.00
B	TJS	17	18	18	0.94	1.00	0.94
C	JB	17	18	18	0.94	1.00	0.94
D	MMS	18	17	18	1.06	0.94	0.99
E	JB	25	25	23	1.00	1.09	1.09
F	MMS	23	26	23	0.89	1.13	1.00
G	TJS	27	27	27	1.00	1.00	1.00
HH	MSB	23	21	22	1.10	0.96	1.05
					0.98 ±0.02	0.94 ±0.02	0.92 ±0.03

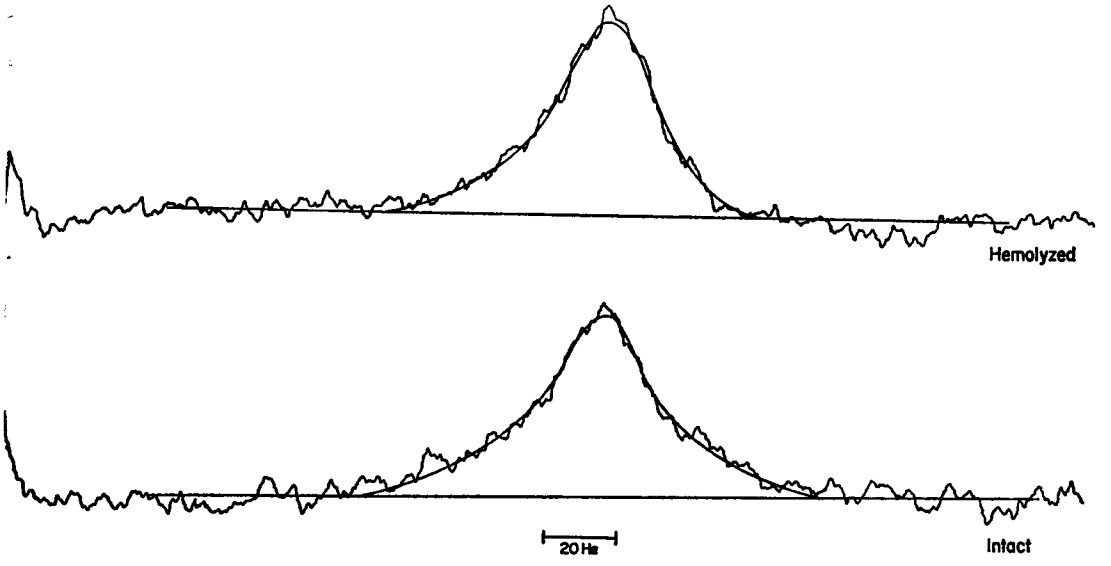

FIGURE 6 Representative spectra of the sodium signal taken obtained from erythrocytes before (below) and after (above) hemolysis by two stages of freeze-thawing. (Sample HH, Table IV). The area enclosed between the thin smooth solid lines superimposed on the spectrum was used to calculate relative signal area.

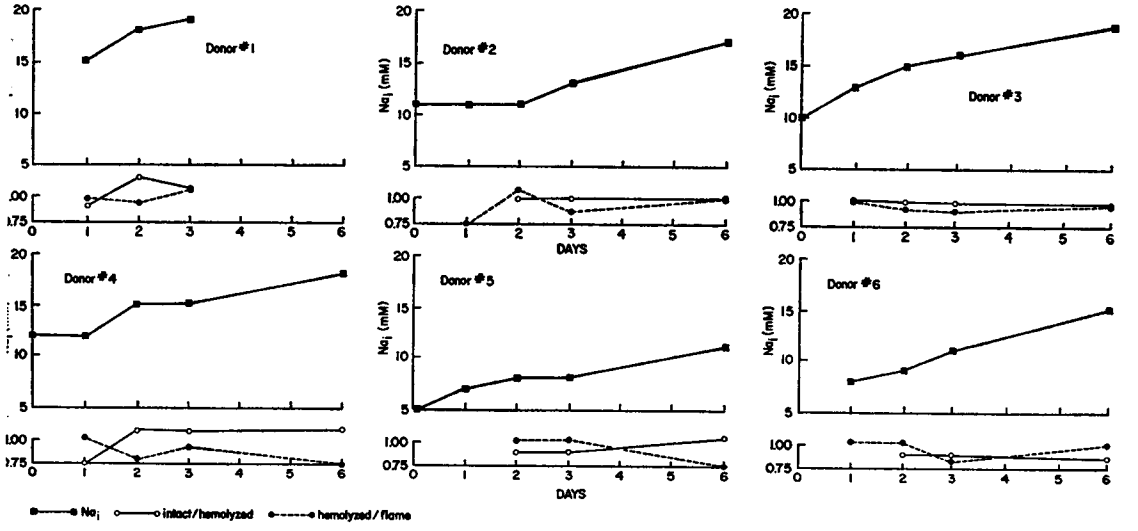

FIGURE 7 Summary of total and NMR-visible sodium in the erythrocytes of six human donors as a function of time of incubation at low temperature (4–6°C) in standard saline solution containing 10 μM ouabain (see Table I.)

In order to determine the effect of changes in internal sodium upon the amount of NMR-invisible sodium, six cell suspensions (from different donors) were treated with ouabain (10 μM) and aged in the cold for periods of up to 6 days. The mean internal sodium nearly doubled during this interval, but the fraction of sodium undetected by NMR remained unchanged and small. The six experiments are illustrated for reference in Fig. 7.

DISCUSSION

Muscle

The preliminary experiments on frog muscle agree with those of previous workers in indicating that a substantial fraction of whole muscle sodium is not detected by continuous wave NMR spectroscopy. Additional experiments to correlate the NMR components of sodium with the extracellular and intracellular components were only partially successful. The intent of the prolonged washing with lithium-Ringer's solution was to deplete the muscle of extracellular sodium, thus revealing the NMR spectrum of the intracellular fraction. A washing procedure such as used here will never completely free the extracellular space of sodium because a diffusion gradient for sodium must exist between the sarcolemma and the bulk solution phase exterior to the muscle bundle. A calculation of the amount of such residual extracellular sodium, however, indicates that only about 2–5% of the total residual muscle sodium might actually be outside of the sarcolemma after a prolonged lithium wash of the duration used (40 min). Since this estimate is considerably less than the fraction remaining visible to the NMR, one concludes that most of the NMR-visible sodium remaining after prolonged washing is intracellular and amounts to about 30% of the total residual sodium.

Although the data in Table III provide no detailed information on the time-course of loss of the NMR visible during immersion in lithium solutions, they do suggest that considerable NMR-visible sodium is lost in the first few minutes of washing, presumably largely because of sodium washout from the extracellular space. This experimental result is different from some earlier work (3, 7) which indicated that the intensity of the NMR signal remained nearly constant for at least 10 min under conditions in which the total sodium content of the muscle was being reduced. The present data are compatible with recent analyses (11, 15) of sodium NMR spectra which indicate that when a population of sodium ions experiences an asymmetrical electrical field, 40% of the signal is contained in the central peak, the remainder being concealed in two satellite peaks broadened beyond experimental recognition. Since the ratio between central and satellite peak intensity is fixed by quantum mechanical considerations, any procedure which reduces the total content of the muscle (such as a lithium wash) must reduce the intensity of the central NMR signal.

Erythrocytes

Since one of the reasons for using erythrocytes to study the NMR spectrum of sodium was to avoid the difficulties in interpretation occasioned by the presence of sodium in extracellular spaces, it is important to discuss the degree to which the intent was realized. The sodium concentration of the lithium wash solution was analyzed occasionally after the final wash and found to be much less than 1 mM. Since the extracellular space of the pellet, under our experimental conditions, was estimated to be about 10%, and the cell sodium was found to be of the order of 10–20 mM/liter packed cells, the extracellular sodium in the pellet at the start of an NMR spectrum would be less than 1% of that inside the cell. NMR spectra require on occasion, however, as much as 45 min to determine. During that period, some intracellular sodium will leave the cell and accumulate in the extracellular space of the pellet. To minimize this leakage, most of the wash solutions contained a glycoside in concentrations sufficient to produce maximal inhibition of the sodium efflux. Reference to Fig. 3 indicates that under these circumstances the residual efflux has a time constant of about 30–40 h. Therefore, even during the longest period required to obtain a spectrum, the extracellular sodium would have accumulated to no more than a few percent of the internal, and in most cases much less. We conclude therefore, that the spectra of sodium in erythrocytes reflect solely the intracellular sodium.

The physiological conditions of the cells used in these experiments can be inferred from the tracer experiment illustrated in Fig. 3. These cells which had been subjected to the same regimen of washing and centrifuging (except for lack of glycoside in the wash solutions) as the cells used for spectral determinations yielded rate constants for exchange in the presence and absence of glycoside in quantitative agreement with the published data from several sources. We conclude therefore that the sodium efflux mechanism, and probably also all of the other transport properties of the erythrocyte, were actually functioning properly in the preparations from which NMR spectra were obtained.

The data collected on erythrocytes has already been summarized in Table IV. Column 7 of that table shows that the NMR technique consistently underestimated the sodium concentration in erythrocytes by about 6%. A similar underestimate of 4% was found when the results of NMR assay of sodium in hemoglobin solutions were compared with the flame photometric analysis. We suspect that the effect is due to the significant broadening of the NMR signal produced by the hemoglobin, since the standard solution had a much narrower line width. The apparent loss of signal could also be due to unequal influences of other ions, principally potassium, upon the flame emission and NMR spectra. The flame emission analyses were all corrected for the interference due to potassium, but the NMR spectra were not. There is one report in the literature (21) that potassium reduces the peak height of the

^{23}Na NMR spectrum, although it is not clear that this affects the total area of the spectrum which was the parameter measured in the present work.

The obvious broadening of the sodium signal seen in our experiments with erythrocytes is probably due to the intracellular hemoglobin, since identical broadening was also seen in hemoglobin solutions. Part of the effect could also have been due to the membrane proteins, since Magnuson et al. (22) have shown that appropriate additions of erythrocyte ghosts to isotonic NaCl solutions can cause nearly as much broadening of the sodium line as found in our experiments.

Two conclusions to be drawn from the present work on erythrocytes are: first, there is very little difference in the spectrum of sodium whether it is intracellular or extracellular; and secondly, there is very little, if any sodium undetected by the NMR technique in either the extra- or intracellular location. These conclusions are based on the data of Table IV, column 6, which show the signal area in intact cells is 98% of that in hemolyzed cells. The experimental design essentially allows a comparison of the sodium signal in an intracellular location (intact cells) with the signal from extracellular location (hemolyzed cells) under virtually identical conditions of ionic composition, ionic strength, viscosity, etc. Such trivial augmentation of the extracellular signal as was found could easily be due to the slight increase in volume of distribution of the hemoglobin and sodium in the hemolyzed cells, since after hemolysis the intracellular material has access to the extracellular volume (about 10%) of the pellet from which it was previously excluded. Furthermore, column 6 should have revealed quantitatively significant quadrupolar interaction because of the presence of a strong electrical double layer in the immediate vicinity of the membrane if such had existed.

Although we feel that the data in column 6 are the more likely to reveal physiologically significant sodium binding, it can be argued that any interaction occurring in the presence of intracellular material and not seen in fluid of composition similar to normal extracellular space is significant for transport phenomena. If this argument is accepted, then such interactions should be seen in the data of column 8, which compares the relative concentrations of sodium as determined by NMR and flame emission analyses. The maximum undetected sodium is only 8%, and we have already indicated reasons which suggest the smaller NMR signal may in part be due to factors (i.e., viscosity, ion interference, etc.) unrelated to specific sodium interactions.

The present experiments were not designed to detect the presence of a small fraction of the total sodium that may be bound or otherwise involved in protein interaction in a special location important for the regulation of sodium transport in erythrocytes. The present data, however, provide no indication that strong interaction of the bulk of the intracellular sodium ions with intracellular constituents is necessary to account for such transport phenomena as exclusion of sodium from the cell interior or regulation of sodium entry and exit in human erythrocytes.

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