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NMR Spectroscopy of Sodium Cations in Intact Cells

RAJ GUPTA* & LINDA A JELICKS
Departments of Physiology & Biophysics, and Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Introduction

The unequal distribution of Na^+ and K^+ aquo cations across cell membranes is essential for sustaining chemistry in all living cells. The concentrations, electrochemical gradients, and transmembrane fluxes of Na^+ are of fundamental importance in cell physiology and play an important role in a variety of vital cell functions, such as nerve transmission and generation of action potentials. The energy stored in the Na^+ gradient across the plasma membrane is utilized for the transport of other cations and essential nutrients into the cell. Intracellular Na^+ concentrations are regulated by the coordinate action of a number of membrane pumps and exchange systems. These include the Na^+/K^+-ATPase (Na^+-pump), Na^+:Ca^{2+} exchange, Na^+:H^+ exchange, Na^+/K^+-co-transport and Na^+ selective channels in the plasma membrane. Each tissue is characterized by a well-defined intracellular Na^+ level, which is often dependent on its physiological state and the maintenance of which appears crucial for its viability. Changes in the intracellular concentration of this cation are often an indication of tissue disease or malfunction. Thus, alterations in intracellular Na^+ concentration have been variously associated with cancer, hypertension, and diabetic states, as well as with sickle cell disease[1-9].

From the point of view of magnetic properties, the ^{23}Na nucleus is especially attractive, with an isotopic abundance of 100% and a resonance frequency close to that of the widely studied ^{13}C nucleus. The ^{23}Na cation can be observed directly through its own NMR absorption. The magnetic properties of the ^{23}Na nucleus and other alkali metal cations are compared in Table 1. While the signal produced by the ^{23}Na nucleus following a single NMR pulse is an order of magnitude smaller than that generated by protons, its relaxation times are often two orders of magnitude shorter than those of protons in a similar environment. Therefore, in time-averaging experiments, the ^{23}Na NMR signal acquired per unit time is comparable to that resulting from a similar population of protons. Because of its 3/2 nuclear spin, the ^{23}Na nucleus exhibits three single-quantum NMR transitions: a central $I_z = -1/2 \rightarrow +1/2$ transition; and two equivalent outer transitions, $-3/2 \rightarrow -1/2$ and $+1/2 \rightarrow +3/2$. The relaxation behaviour of quadrupolar nuclei with $I > 1$ is simple (i.e, a single exponential curve) only within a certain range of correlation times called the "region of extreme narrowing" ($\tau_c \ll 1/\omega_0$). When molecular tumbling becomes relatively slow as is often the case with ions interacting with biomolecules, τ_c will be greater than $1/\omega_0$ and these nuclei will exhibit significantly nonexponential relaxation. The situation is complicated by the fact that these ions are usually exchanging between several sites which may vary markedly in their correlation times (i.e., free ions and ions bound to macromolecules). The equations are quite complex for nuclei possessing spin greater than 3/2 (e.g., ^{25}Mg and ^{43}Ca). However, for the ^{23}Na nucleus under typical biological conditions, the transverse relaxation reduces to a sum of two exponentials which can be expressed by the relation (1),

$$M_Z(t) = M_0\{0.6\ e^{-t/T_{2f}} + 0.4\ e^{-t/T_{2s}} \quad \ldots (1)$$

Table 1—Magnetic Properties of ^{23}Na Nucleus and Other Alkali Metal Cations

Nucleus	I	Larmor Freq. at 4.7 Tesla (MHz)	Natural isotopic abundance (%)	Rel sensitivity for equal number of nuclei	Electric quadrupole moment
^{6}Li	1	29.4	7.4	0.092	0.0007
^{7}Li	3/2	77.7	92.6	3.17	−0.03
^{23}Na	3/2	52.9	100	1.00	0.145
^{39}K	3/2	9.3	93.1	0.0055	0.11
^{87}Rb	3/2	65.4	27.8	1.89	0.13
^{133}Cs	7/2	26.2	100	0.51	−0.003

†Dedicated to Prof P.T. Narasimhan, IIT, Kanpur on his sixtieth birthday

where $1/T_{2f}$ and $1/T_{2s}$ are determined by the relative amounts, the exchange rates, and the relaxation rates of free and bound ions. This effect becomes apparent in the shape of the resonance lines of these nuclei which are "non-lorentzian" and appear to have relative areas of 6:4, 60% of the intensity corresponding to the two outer transitions and 40% to the inner transition. The widths of these two types of transitions may be very different and under certain conditions it is possible to have outer transitions broadened beyond detectability while the central transition stays narrow, leaving only 40% of the signal observable. This presents a complication in that observed NMR intensities may not be proportional to concentrations and the possibility of invisible transitions affecting the NMR intensities should therefore be kept in mind in calculating concentrations.

Localization of Sodium Cations in Intra- and Extracellular Compartments by NMR

It is of considerable interest to study sodium ions in the intracellular compartment and their transport across membranes in intact cells and tissues. Measurement of intracellular Na^+ is generally hampered by the large difference in its concentration across the plasma membrane. Even a slight contamination by occluded extracellular fluid, because of its high Na^+ content, significantly distorts the measurement of the intracellular Na^+ levels by techniques which are not specific to the intracellular compartments. Several methods are available for the determination of intracellular sodium cation content. These include straightforward chemical analysis, electron microprobe X-ray analysis, and ion-selective microelectrodes. Chemical analysis involves rigorous separation of cells from the extracellular fluid, tissue homogenization and measurement of metal cation content using flame emission photometry or atomic absorption techniques. The electron microprobe technique is based on an analysis of element-specific X-rays emitted when a high energy electron beam impinges on a very small region of a specimen in an electron microscope. The advantages of this technique are its high sensitivity, its ability to study subcellular organelles and its detection of virtually any element. However, the technique is destructive as it requires very rapid freezing of the sample followed by dehydration and ultrathin sectioning. Cation-selective microelectrodes have also been used to study intracellular sodium activity but require impalement of cells and tissues, and suffer, even when successful impalement can be achieved, from electrode calibration problems. The potential measured is affected by transmembrane electrical potential. In addition, the electrodes often produce cell injury, are limited in use to large cells, and sample only that region of the cell at the electrode tip. A noninvasive technique for measurement of intracellular Na^+ concentration is most advantageous and NMR spectroscopy offers such a technique. NMR can be used to detect cations within intact cells and because it is nondestructive and allows the observation of ionic changes in the cellular environment as they take place within an essentially unperturbed living system it is preferred over other methods, such as atomic absorption or electron X-ray microanalysis. In addition to providing a measurement of concentrations, NMR can provide information about the physical state of the cations.

The noninvasive spectroscopic technique of ^{23}Na NMR has enjoyed a growing enthusiasm within the scientific community since the first study of the continuous wave NMR spectra of $^{23}Na^+$ in muscle, kidney and brain by Cope[10-12]. Earlier ^{23}Na NMR studies of cells and tissues were carried out in a number of laboratories[10-16].

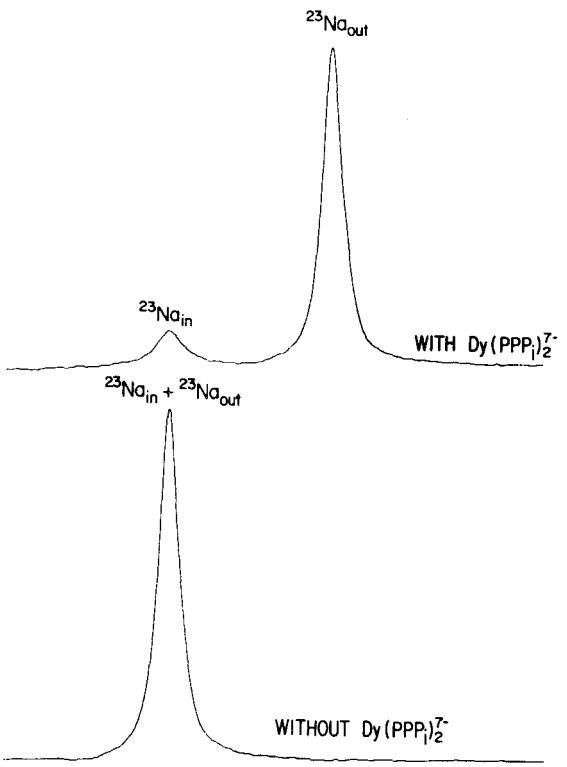

Fig. 1 — ^{23}Na NMR spectra of red cells in heparinized whole human blood with (top) and without (bottom) 3 m*M* $Dy(PPP_i)_2^{7-}$ showing spectral resolution of intra- and extracellular Na^+ ions by the paramagnetic shift reagent [The resonances of intra- and extracellular Na^+ ions are labeled as $^{23}Na_{in}$ and $^{23}Na_{out}$, respectively]

It became clear from these studies that the ^{23}Na signals from the extra- and intracellular compartments occur precisely at the same chemical shift. This is presumably because Na^+ is present mostly as hydrated cation and therefore the chemical environment or chemical shift of the ^{23}Na nucleus which is dependent on its immediate electronic environment remains unaltered in different compartments. The interesting small ^{23}Na resonance of intracellular ions is masked by the uninteresting but much larger resonance of extracellular ions. Until 1982, this lack of spectral discrimination between intra- and extracellular ^{23}Na resonances precluded the use of NMR in the study of intracellular Na^+ ions. The discovery in 1982 of a highly anionic paramagnetic shift reagent dysprosium bis(tripolyphosphate), $Dy(PPP_i)_2^{7-}$, effectively circumvented this problem[17,18] and for the first time allowed direct observation of well-resolved ^{23}Na resonances from intra- and extracellular Na^+ ions in intact cells at non-perturbing, low reagent concentrations (Fig. 1). This permitted a study of the intracellular Na^+ without interference from the extracellular ions and paved the way for future noninvasive NMR studies of monovalent cations in cells, tissues, organs and organisms[3,4,6,7,18–49]. The detection of resolved resonances from intra- and extracellular Na^+ ions exploits the fact that the anionic paramagnetic reagents cause a hyperfine shift in the frequency, without significant broadening, of the resonance of Na^+ ions in their environment. $Dy(PPP_i)_2^{7-}$, because of its highly anionic character, can not permeate through the cell membrane over the time scale of NMR measurements, and remains localized only in the extracellular compartments so that the NMR resonance of extracellular Na^+ is shifted away from the resonance of intracellular Na^+. Because the technique is noninvasive, consecutive steps in a protocol can be carried out on the same cell sample.

Other anionic reagents were independently introduced by Pike and Springer[50] and Bryden *et al.*[51], but required the use of over an order of magnitude higher reagent concentration to achieve the same spectral discrimination[23,52]. Balschi *et al.*[53] used such a reagent to obtain intracellular Na^+ in yeast cells at very low (15 mM) extracellular Na^+ concentrations. Two years later Chu, *et al.*[52] suggested $Dy(TTHA)^{3-}$ which, although again causing much smaller hyperfine shifts of extracellular Na^+ than those by $Dy(PPP_i)_2^{7-}$, possesses certain other advantages[42].

$Dy(PPP_i)_2^{7-}$ causes especially large hyperfine shifts in the NMR absorption of ^{23}Na. This is ascribed to its highly anionic nature and its ability to bind Na^+ in close spatial proximity of dysprosium. In order to obtain information on the structure of the complex of Na^+ with $Dy(PPP_i)_2^{7-}$, we studied the interaction of Na^+ with the corresponding relaxation reagent $Gd(PPP_i)_2^{7-}$. The magnitude of the paramagnetic effect of Gd^{3+} on the longitudinal nuclear relaxation rate of ^{23}Na in the complex of Na^+ with $Gd(PPP_i)_2^{7-}$ was measured. From the magnitude of this paramagnetic effect (10,000/s) and the rotational correlation time derived from Stokes' law, and assuming that only one ^{23}Na cation interacts with a Gd^{3+} to ^{23}Na distance of 4 Å was obtained using Eq. (2) for distance-dependent paramagnetic dipolar interactions[54,55].

$$\frac{1}{T_{1p}} = \frac{2fqS(S+1)g^2\beta^2\gamma_I^2}{15r^6}\left(\frac{3\tau_{c_1}}{1+\omega_I^2\tau_{c_1}^2}+\frac{7\tau_{c2}}{1+\omega_S^2\tau_{c_2}^2}\right) \quad \ldots (2)$$

where f is the ratio of concentrations of the paramagnet and the relaxing ligand, q is the relative stoichiometry of the bound ligand and bound paramagnet (i.e., the coordination number), S is the electron spin, γ_I is the nuclear gyromagnetic ratio, g is the electron's g-factor, β is the Bohr magneton, r is the metal-nucleus distance, ω_I and ω_S are the nuclear and electron angular precession frequencies, and τ_{c1} and τ_{c2} are the correlation times for the dipolar interaction, which in our case are equal to τ_R, the rotational correlation time. Interaction of 7 equidistant Na^+ cations at a distance of 5.5 Å would also be consistent with the measured paramagnetic relaxation effect.

$Dy(PPP_i)_2^{7-}$ causes an upfield paramagnetic shift in the frequency of the extracellular ^{23}Na resonance and only minimal line-broadening. In contrast, a complex of the same ligand with thulium, $Tm(PPP_i)_2^{7-}$, causes a downfield paramagnetic shift but is only half as effective as $Dy(PPP_i)_2^{7-}$, as judged by the magnitudes of the observed shifts[23,52]. The opposite directions of the observed paramagnetic shifts with Tm^{3+} and Dy^{3+} complexes indicate their dipolar origin and presumably reflect differences in orientation of the principal axes of the electron's g-tensor in the two cases.

Quantitation of Intracellular Sodium Concentration

Once the separation of intra- and extracellular ^{23}Na resonances by the shift reagent has been achieved, a comparison of the intensity of the resonance of extracellular ions (A_{out}) in the cell sam-

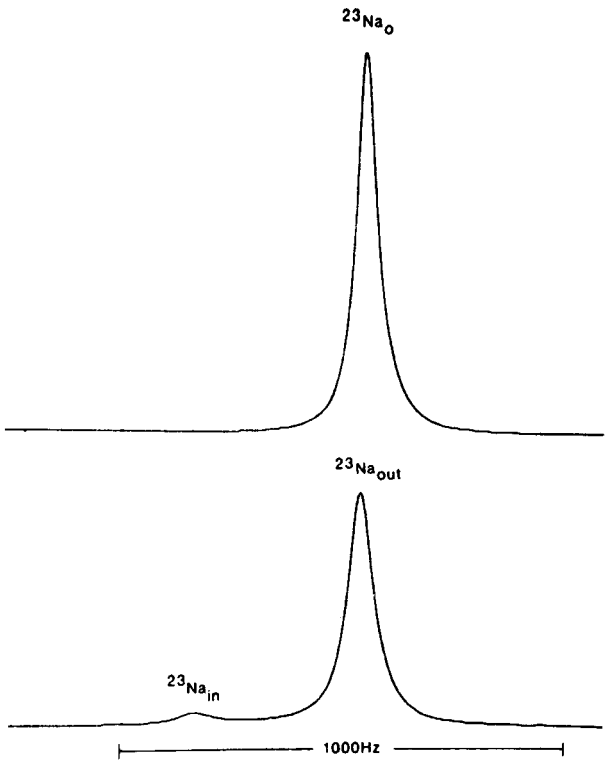

Fig. 2—Comparison of ^{23}Na NMR spectrum of red cells suspended in serum containing 3 m*M* $Dy(PPP_i)_2^-$ (lower trace) with that of the suspension medium alone (upper trace) [The ratio of intensities of the $^{23}Na_{out}$ and $^{23}Na_o$ signals directly gives the fractional extracellular space]

ple with the intensity of ^{23}Na resonance from a cell-free control (A_0) containing only the suspension medium [Na_{out}] (Fig. 2) directly yields the fractional space in the NMR-sensitive volume that is extracellular (S_{out}). It should be noted that the extracellular space defined in this way is the space seen by the ^{23}Na ions themselves and includes the space occupied by the medium as well as any vascular and interstitial spaces. The ratio of intensities of the ^{23}Na resonances of intracellular (A_{in}) and extracellular ions (A_{out}) together with a knowledge of the fractional space that is extracellular then directly yield the concentration of intracellular Na^+ ions [Na_{in}] that contribute to the observed resonance signal. Equations (3) and (4) provide the relationship between the observed resonance intensities and the "NMR-visible" intracellular Na^+ concentration[18,23].

$$S_{out} = \frac{A_{out}}{A_0} \qquad \ldots (3)$$

$$[Na_{in}] = \left\{ \frac{A_{in}}{A_{out}} \frac{S_{out}}{(1 - S_{out})} \right\} [Na_{out}] \qquad \ldots (4)$$

When [Na_{out}] is expressed in m*M*, the units of [Na_{in}] are mmols/litre cells. A knowledge of tissue water content enables calculation of [Na_{in}] on the basis of kilogram cell water.

Measurement of Intracellular Water Content

Knowledge of intracellular water volume is required for expressing the NMR-measured concentrations of intracellular ions and metabolites on the cell-water basis. Intracellular water volume can be measured gravimetrically, by comparing dry weight of cells with their wet weight, or by using ^3H_2O to determine the total water space. In both methods [^{14}C]-*inulin* is generally used as a membrane impermeant marker for the estimation of extracellular space. However, it has been shown (see e.g., ref. 22, 29) that cells can take up inulin by fluid-phase endocytosis. This results in inaccurate estimation of the amount of extracellular water, which may introduce sizeable errors in the calculation of intracellular water content. An additional source of error in the gravimetric method is partial decomposition of solid cell components to volatile compounds during drying of cells to a constant weight.

Hoffman and Gupta have described[56] an NMR method which is based on the use of ^{35}Cl and ^{2}H nuclei (Fig. 3). Measurements may be carried out without changing the NMR probe-configuration and they do not require the perturbation of the cell system by a shift reagent. The method is equally applicable to situations of slow and fast transmembrane water exchange and is based on the observation[31] that the resonance of intracellular $^{35}Cl^-$ ions in most cells is so broad as to become NMR-invisible. Therefore, the intensity of the $^{35}Cl^-$ NMR signal of a cell-suspension is proportional to the extracellular water volume within the NMR receiver coil. The intensity of the $^{35}Cl^-$ NMR signal of the cell-free medium in an identical sample-geometry is proportional to the total volume sampled by the NMR coil. The ratio of the two intensities (f_{Cl}) yields the extracellular water volume as a fraction of the total suspension volume. The ratio of the intensities of the water ^{2}H NMR signals of the cell-suspension and the cell-free medium (f_D) gives the water volume as a fraction of the total suspension volume. The intracellular water content (f_w), i.e., the volume of the intracellular water as a fraction of the total cell-volume, is given by Eq. (5)

$$f_w = \frac{f_D - f_{Cl}}{1 - f_{Cl}} \qquad \ldots (5)$$

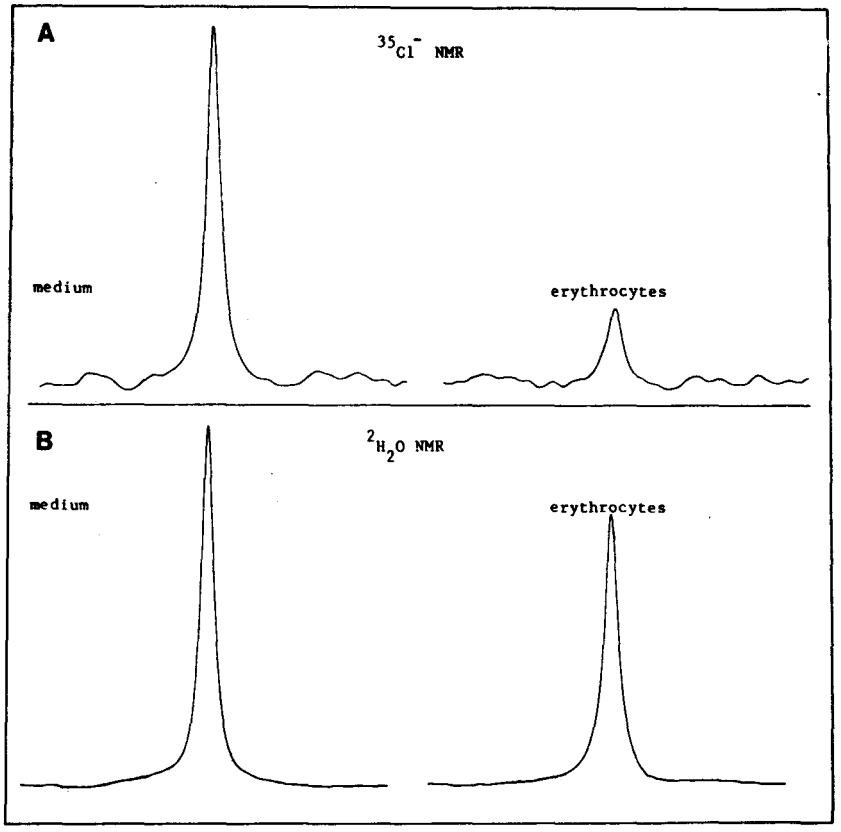

Fig. 3—Representative pairs of $^{35}Cl^-$ (A) and ^{2}H (B) NMR spectra [The left traces correspond to the cell-free medium and the right traces to the red cell suspension. Measurements may be carried out without changing the NMR probe configuration and they do not require perturbation of the cell system by addition of membrane impermeable reagents. The method is equally applicable to situation of slow and fast water exchange]

The water content values obtained by this method are expressed in units of intracellular water volume/total cell volume. This has the advantage that the values can be used directly to calculate the concentrations of intracellular constituents. No further assumptions concerning cell density are needed. For red blood cells, this method yields a value of 73.4 ± 2.4 ml water/100 ml cells[56], in good agreement with the gravimetric method, which yields 71.1 ml of water/100 ml cells. Ovarian oocytes from the frog *Rana pipiens* are much more anhydrous and yield an average intracellular water content of only $42.6 \pm 0.8\%$ (ref. 57). Previous gravimetric measurements indicated a significantly higher water content of $49.8 \pm 0.4\%$ (vol/weight). The gravimetric values may therefore include contributions from interstitial spaces and reflect difficulty in correcting for the density of the non-aqueous phase of the oocyte. Thus, NMR may well provide a useful nondestructive approach to the measurement of intracellular water content.

Applications of ^{23}Na NMR to Cells and Tissues

(a) Sodium Ions In Amphibian Oocytes And Eggs

Sodium ions appear to play an important role in the regulation of early embryonic development. *Rana* frog oocytes constitute a particularly favourable cell system for ^{23}Na NMR studies. A *Rana* female contains up to 2000-3000 large (1.8 mm diameter) oocytes arrested in first meiotic prophase. They can be readily superfused in the NMR tube and induced to undergo nearly synchronous meiotic and early mitotic divisions. Changes in ion-transport and electrical properties of the plasma membrane occur following hormonal stimulation and the effects on intracellular Na^+ have been investigated by NMR[22,28,29,33,34]. In particular, progesterone is thought to provide the physiological stimulus for reinitiation of meiotic division, and the mechanism of action, which is not well understood, is accompanied by changes in intracellular Na^+.

$Dy(PPP_i)_2$ was used to separate the resonances of intra- and extracellular Na^+ ions in *Rana* oo-

cytes, ovulated eggs, and early embryos[33]. A 4 m*M* concentration of $Dy(PPP_i)_2$ had no adverse effects on oocyte membrane potential, membrane conductance, or on the phosphocreatine level as observed by ^{31}P NMR for periods of at least 2 hr[29,33] and the reagent did not inhibit insulin or progesterone-induced nuclear breakdown. Thus, the shift reagent appears to be non-toxic to *Rana* oocytes and enabled a noninvasive analysis of intracellular Na^+ *in situ* and the detection of small changes in cell Na^+. An accurate analysis of Na^+ levels in the amphibian egg in meiotic prophase arrest, second meiotic metaphase arrest, and during early cleavage was possible[33].

The NMR data indicated that only $17 \pm 3\%$ of the total intact follicle Na^+ is detectable[22,23]. In contrast, about 40% of the total Na^+ of "denuded" oocytes (obtained by removing surrounding follicular epithelial cell layers) was found to be NMR-visible. The higher Na^+ content of denuded oocytes compared to that of follicles reflects an increase in intracellular Na^+ (about 10 mmols/kg cells) caused by exposure (30 min) to Ca^{2+}-free Ringer's solution during the denuding process. In progesterone-treated denuded oocytes NMR visible Na^+ decreased to about one-half prior to onset of nuclear breakdown (7-9 hr) and increased about 2-fold in progesterone-treated follicles following nuclear breakdown without a change in total oocyte Na^+. By ovulation (second metaphase arrest) the total intracellular Na^+ also increased and NMR-visible Na^+ now accounted for about 25% of the total egg Na^+. During early cleavage in hypotonic ($< 0.5 \times$ normal ionic strength) Ringer's solution, there was a small (6%) decrease in total Na^+, but a further increase in the NMR-visible fraction. By the 2-16 cell stage, 66-70% of the total intracellular Na^+ became NMR-visible. Thus, NMR-visible Na^+ increases markedly during early development[33].

It is quite apparent that the NMR-visible Na^+ concentration in *Rana* oocytes is significantly lower than the total concentration estimated by atomic absorption. This suggests either that all of the intracellular Na^+ is not contributing to the observed resonance, or that the observed resonance does not represent the full absorption associated with all of the magnetic transitions of the ^{23}Na nucleus. The loss of signal intensity may be due to a broadening beyond detectability of the two outer nuclear transitions by first order nuclear quadrupolar interactions involving the entire pool of cell Na^+ and is expected to be either 60% or none[14-16]. Since only about 17% of the total Na^+ is NMR-visible in prophase-arrested, follicle-enclosed oocytes and as much as 70% is visible in early cleavage stages, it does not appear possible to explain the ^{23}Na NMR data in terms of this simple picture. The observation of an intensity loss of only 30% or as much as 83%, depending upon the physiological state, in comparison to the expected full signal, must be interpreted in terms of compartmentation and selective quadrupolar broadening of the NMR-invisible Na^+. Quadrupolar interactions could cause sufficient broadening of all nuclear transitions resulting in total disappearance of the ^{23}Na resonance of a given compartment. The broadening effect may be enhanced by increases in asymmetric electric field gradients and/or immobilization of Na^+ ions. However, the compartmentalized Na^+ pool undergoing quadrupolar broadening must exchange with the NMR-visible Na^+ slowly in comparison to the magnitude of quadrupolar interaction; otherwise the observed ^{23}Na resonance would be expected to reflect the entire concentration of intracellular Na^+ ions. In the *in situ* prophase-arrested oocyte, invisible Na^+ may be only 57% if the first order quadrupolar interactions were actually causing a 60% decrease in the amplitude of NMR-visible Na^+, but as high as 83% if such interactions were not affecting the "free" Na^+ pool.

The evidence for the existence of some NMR-invisible Na^+ in the follicular oocyte and its release during early development are consistent with other studies. For example, Morrill *et al.*[58] found that a sizeable fraction of the intracellular Na^+ in prophase-arrested, follicle-enclosed oocytes could be recovered with the yolk platelet fraction and was released inpart into the cytosol by ovulation. Palmer *et al.*[59] reported that only 8% of the total Na^+ in the *Rana* follicle could be detected with Na^+-sensitive microelectrodes, although the small tip of an impaling microelectrode can sense the properties of only a small volume of the intracellular fluid and the composition of this fluid may not be representative of the total intracellular fluid when subcellular compartmentalization is present. The identity of intracellular organelles responsible for compartmentation of ions within mature oocytes is unknown. The yolk platelets, occupying a sizeable part of the oocyte, appear to be a possible site of Na^+ compartmentalization although the nucleus itself may also play a significant role. Yolk platelets, isolated in isotonic, Na^+-free, sucrose medium at room temperature did not yield a detectable NMR signal, although atomic absorption studies indicated appreciable Na^+-content (about 30% of the total oocyte Na^+). Thus, while yolk

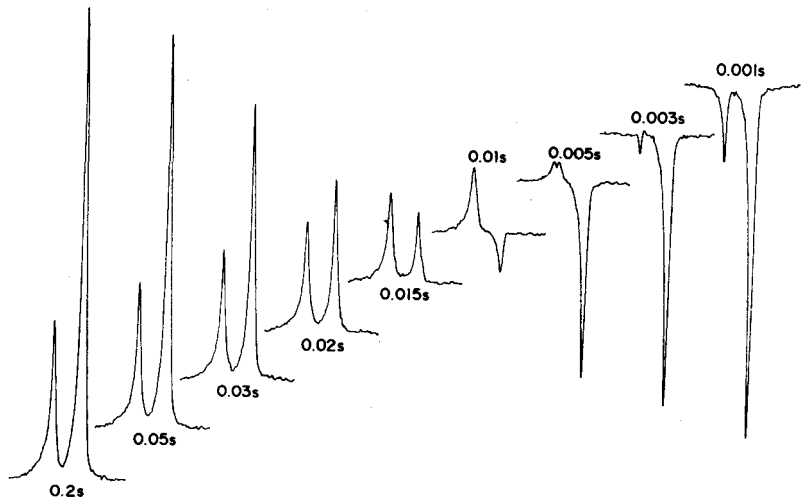

Fig. 4—Partially relaxed inversion-recovery ^{23}Na NMR spectra of *Rana* oocytes in a Ca, Mg-free medium showing evidence for compartmentation of intracellular Na^+ [The larger peak to the right in each trace arises from extracellular $^{23}Na^+$, while the smaller one to the left represents intracellular Na^+. The intracellular $^{23}Na^+$ peak appears composed of at least two components of widely differing widths and spin-lattice relaxation times; e.g. the partially relaxed spectrum at 0.005 sec clearly shows a small inverted negative intracellular $^{23}Na^+$ signal from a compartment superimposed on a predominantly positive peak of other compartments of intracellular Na^+ (ref. 33)]

platelets are rich in Na^+ this NMR-invisible Na^+ does not contribute to the oocyte NMR signal.

Partially relaxed inversion-recovery ^{23}Na FT NMR spectra were obtained to investigate the existence of intracellular compartments with differing magnetic environments and relaxation behaviour within an intact oocyte. The experiments failed to detect the existence of sizeable compartmentation of Na^+ in intact follicles in a normal Ca, Mg-containing Ringer's solution but revealed the existence of two intracellular pools with different longitudinal relaxation behavior in follicles loaded with Na^+ in a Ca, Mg-free medium (Fig. 4). (The possibility that the differently relaxing, superimposed, intracellular Na^+ resonances merely represent the central and outer transitions of the same Na^+ population appears unlikely since the use of 90° sampling pulses in the inversion-recovery sequence should have served to scramble the recovery rates of the narrow and broad transition components). Since yolk platelet Na^+ appears to be NMR-invisible, one of the two observed compartments may be the nucleus which is 400 μm in diameter. However, since this compartment is not detectable in follicles in a normal Ringer's solution, an implication would be that the Na^+ that accumulates in follicle cells during exposure to a Ca, Mg-free Ringer's solution must at least, in part, end-up in the nucleus. In any case, the NMR studies provide evidence for the existence of significant levels of NMR-invisible Na^+ in the amphibian egg and for its release during early development[33].

The role of Na^+ in the mechanism of insulin action in reinitiation of meiotic divisions in follicles and denuded oocytes was also studied[34]. In a normal Ca, Mg-containing Ringer's solution, both NMR-observable and total Na^+ were increased only slightly in insulin-treated denuded oocytes in comparison to controls. However, when follicles were allowed to accumulate Na^+ in a Ca, Mg-free medium containing 1 m*M* EDTA for 1.5 hr, the NMR-visible intracellular Na^+ level increased from 12 to 74 mmols/litre cell water. The follicles lost preloaded Na^+ when returned to Ca, Mg-containing Ringer's solution but the decrease in Na^+ occurred at a slower rate in the presence of 10 μ*M* insulin. About 3 hr after being transferred to a Ca, Mg-containing medium, insulin-treated follicles retained 46 mmols of NMR-visible intracellular Na^+/litre cell water whereas untreated follicles exhibited a significantly lower level of NMR visible Na^+ (33 mmols/litre cell water). Thus, Na^+-loaded oocytes retained a markedly (about 40%) higher level of NMR-visible intracellular Na^+ in insulin-containing medium than in insulin-free controls. In oocytes with low free Na^+ levels, however, only a slight (7%) increase in NMR-visible Na^+ relative to controls was apparent. These observations[34], together with insulin-induced hyperpolarization and decrease in membrane Na^+-conductance[22,29], suggest that insulin stimulates a nonconducting pathway for Na^+ influx in the oocyte plasma membrane. As in the case of rat cardiac myocytes (discussed later)[7], in a Ca, Mg-containing medium, the cytoplasmic level of intracel-

lular free Na^+ in *Rana* oocytes is low and the Na^+-pump may not be saturated with this cation. Any transient increase in internal Na^+ due to increased influx would shift the operating point and would result in enhanced activation of the Na^+-pump. This would tend to return the intracellular Na^+ towards the level found in the absence of insulin. After pre-incubation in a Ca, Mg-free medium, however, the level of intracellular Na^+ is increased and the Na^+ pump is likely to be operating close to its maximal efficiency. Therefore, an insulin-induced increase in influx would result in a markedly higher intracellular Na^+ level.

Part of the increased uptake of Na^+ is apparently due to an increase in fluid phase turnover via endocytosis. About 2% of the total oocyte fluid volume exchanges per hour via endocytosis[22,29]. Since the extracellular medium contains about 110 m*M* Na^+, fluid uptake via endocytosis could account for about 2 mmols/kg/hr Na^+ uptake by the prophase-arrested oocyte, i.e. about one-third of the Na^+ uptake by the prophase oocyte may be via endocytotic vesicles. Insulin increases fluid uptake and turnover by stimulating the endocytotic pathway 2 to 3-fold during the first hour after treatment. This increased endocytosis could be contributing significantly to the insulin-induced increase in NMR-visible Na^+. Insulin also causes an increase in intracellular *p*H as measured by ^{31}P NMR. In *Rana* oocytes, insulin-induced elevation of pH_i was found to be Na^+-dependent indicating that intracellular *p*H may be regulated by Na^+/H^+ exchange[29]. Thus, insulin may act, at least in part, by stimulating the Na^+/H^+ exchange system in the oocyte plasma membrane. The observed increase in NMR-visible Na^+ may be due in part to increased Na^+ uptake in exchange for H^+ as well as increased fluid phase turnover. Insulin stimulation of Na^+/H^+ exchange in the oocyte plasma membrane most likely occurs via a decrease in the activation energy of this system. This would allow Na^+, moving down its free energy gradient into the cell, to provide the energy required to move protons outward, against their free energy gradient, with a resulting increase in intracellular *p*H[60].

(b) Sodium Ions In Blood Cells

(1) *Human erythrocytes*

The concentrations of intra- and extracellular Na^+ of human red blood cells are amenable to study by ^{23}Na NMR via $Dy(PPP_i)_2$. Exposure to the shift reagent does not cause hemolysis, and does not affect the ATP, 2,3-DPG, or free Mg^{2+} levels of human red blood cells and thus has no adverse effect on the energetics of the cell or on the maintenance of ionic gradients[23]. The intensity of the intracellular ^{23}Na resonance is independent of the extracellular concentration of $Dy(PPP_i)_2$, up to at least 5 m*M*, showing that the presence of the reagent does not significantly perturb the concentration or environment of intracellular Na^+ ions. From the spectrum of fresh erythrocytes, an intracellular Na^+ ion concentration of 4.1 mmols/litre cells was estimated[3,23]. This value is significantly (20%) lower than the total concentration of Na^+ in the same red cells as estimated by atomic absorption. The simplest explanation for these results is that the entire pool of intracellular ^{23}Na ions does not contribute to the observed resonance and that the cell makes a part of the intracellular Na^+ somehow invisible to the NMR technique. The resonance of this small, yet measurable, Na^+ ion pool must be experiencing quadrupolar broadening large enough to cause its disappearance.

In the absence of a paramagnetic shift reagent, human red blood cells exhibit only a single ^{23}Na resonance due to overlap of the signals from intra- and extracellular ions. Hemolysis of packed, ATP-rich, human red cells by repeated freeze-thawing caused a $17 \pm 2\%$ increase in the overall ^{23}Na NMR signal[3,36]. This observation indicates that a small NMR-invisible pool of intracellular ^{23}Na ions exists in the human red cell and that ions in this pool become observable upon cell lysis. Using $Dy(PPP_i)_2$ to distinguish between intra- and extracellular ions, the contribution of extracellular Na^+ to the observed signal from well-packed erythrocytes has been estimated to be about 24%. Since the contribution of extracellular Na^+ to the observed signal remains unchanged upon cell lysis, the NMR-invisible pool must represent 0.17/0.76 or about 22% of the total intracellular Na^+ concentration. Essentially all of the Na^+ becomes observable by NMR in the lysed state. A similar (about 20%) increase in the ^{23}Na NMR signal was observed when well-packed fresh red cells were lysed by detergent solubilization of the cellular membranes. In contrast, no NMR-invisible Na^+ was apparent in red blood cells depolarized by treatment with 2.5 or 10 μM gramicidin, as indicated by the absence of any significant increase in the Na^+ signal upon freeze-thawing[36]. These results indicate the existence of a red cell compartment dependent upon the presence of an intact membrane potential that contains a small pool of Na^+ ions experiencing a large quadrupolar interaction. The inability of this Na^+ pool to exchange

rapidly with the free cytosolic Na^+ makes it NMR-invisible. It may be that this pool represents several layers of Na^+ ions associated with negative charges on the cell membrane. The exchange of these ions with free cytosolic Na^+ may be slow (<100/s) in the presence of a negative membrane potential, but is rapid in its absence.

(2.) *Amphiuma red blood cells*

The ^{23}Na NMR technique has been employed to measure Na^+ fluxes during volume regulation in *Amphiuma* red blood cells, which possess a particularly robust electroneutral Na^+:H^+ exchange[42]. The cytotoxicity of the shift reagents was evaluated using the dynamic cell volume regulatory response as a bioassay. The Na^+ shift reagents are known to complex Ca^{2+} [$Dy(PPP_i)_2$ much more so than DyTTHA] and the medium containing these reagents must be titrated to the appropriate [Ca^{2+}] needed to maintain normal cell function. Doing so extends the concentration range beyond which the reagents have cytotoxic effects. At low concentration, the Na^+ shift reagents $Dy(PPP_i)_2$ (<4 m*M*) or DyTTHA (<6 m*M*) do not enter the cells or alter intracellular Na^+, K^+ and Cl^- concentrations, and have no effect on ion transport in *Amphiuma* red blood cells over a 5 hr period when extracellular [Ca^{2+}] is maintained at >0.5 m*M* (ref. 42). At higher reagent concentrations or lower Ca^{2+} concentrations, these cells tend to loose K^+ and gain Na^+ in manner suggestive of membrane damage. It is however clear from this study[42] that despite Ca^{2+} chelation by $Dy(PPP_i)_2$, it is possible to adjust free Ca^{2+} by titrating the reagent with Ca^{2+}. In a solution containing 0.5 m*M* free Ca^{2+}, the shift of Na^+ resonance by 4 m*M* $Dy(PPP_i)_2$ is about 70% as large as that obtained[42] in the absence of any Ca^{2+}. It is interesting that even at free Ca^{2+} levels of 2 mM, which should be sufficient for studies of most Ca^{2+}-dependent processes, $Dy(PPP_i)_2$ provides a shift greater than that provided by other reagents under similar conditions, without any evidence of alterations in complex cellular regulation of membrane ion-transport.

Under conditions where *Amphiuma* red blood cells were allowed to loose Na^+ via a physiological transport pathway, the transported Na^+ appeared to be NMR-visible to the same extent inside or outside the cell. However, in agreement with human erythrocyte studies described above, lysing the cells altered the Na^+ environment so as to increase the total NMR-visible Na^+ by 19%, consistent with the presence of some NMR-invisible Na^+ even in these red blood cells[42].

(3) *Human lymphocytes*

The ^{23}Na NMR technique is also appropriate for the study of living lymphocytes and acceptable spectral signal to noise ratio is easily attainable[3,23]. Because several considerations have led to the hypothesis that an abnormality in the control of intracellular monovalent cations may play a role in the development of some leukemic cells, we measured the NMR-visible Na^+ contents of human normal and leukemic lymphocytes using $Dy(PPP_i)_2$ (ref. 3). Lymphocytes obtained from patients with chronic lymphocytic leukemia (CLL) and normal controls were suspended in their own serum in order to reflect the *in vivo* environment as closely as possible. The ^{23}Na NMR spectra from normal and abnormal (CLL) lymphocytes yielded significantly different NMR-visible intracellular Na^+ ion concentrations (17.5 ± 1.2 mmols/litre cells for normal and 8.7 ± 0.8 mmols/litre for abnormal cells)[3]. These results appear to indicate that NMR-visible Na^+, which is presumed to be predominantly free, is only half the normal level in leukemic lymphocytes. Accordingly, the Na^+ electrochemical gradient across the plasma membrane is two-fold larger in leukemic lymphocytes in comparison to normal lymphocytes. The ability of the ^{23}Na NMR technique to noninvasively monitor the state of intracellular ions in abnormal lymphocytes may be useful as a simple *in vitro* assay for determining the effectiveness of action of cancer chemotherapeutic agents[3].

(c) Intracellular Sodium Ions In Cardiac Myocytes

The maintenance of electrochemical gradients in heart muscle is crucial to the maintenance of resting potential, the development of action potential, and initiation of contraction. Using ^{23}Na NMR via $Dy(PPP_i)_2$ the concentration of Na^+ in cardiac myocytes at rest, as well as the magnitude and reversibility of change in intracellular Na^+ level induced by insulin-deficiency and by removal of extracellular Ca^{2+} have been studied[7]. When Ca^{2+} and insulin are present in the extracellular medium, the intracellular Na^+ level has been measured to be 8.8 ± 1.2 mmols/litre cells[7]. Upon transfer of the myocytes to a Ca^{2+}-free medium a 3-fold increase in the NMR-visible Na^+ to a level of 22.8 ± 2.6 mmols/litre myocytes has been observed. An increase of this magnitude was seen even though the calcium concentration in the extracellular medium was changed only from 300 μM to 10 μM. This increase in intracellular Na^+ was reversed by the addition of 0.3 to 1 m*M*

$CaCl_2$ to the medium. The myocytes thus remained functional in a Ca^{2+}-free medium in the NMR tube[7] and were able to pump their Na^+ down to the characteristic low level when they were transferred again to a Ca^{2+}-containing medium. Insulin-deficiency in the extracellular medium caused only a small (21%) decrease in the NMR-visible intracellular Na^+ to a level of 6.9 ± 0.5 mmols/litre cells in a normal Ca^{2+}-containing physiological medium. In a Ca^{2+}-free, insulin-deficient medium, the NMR-visible Na^+ was increased but to a level (14.6 ± 2.0 mmols/litre cells) markedly (64%) lower than that found in insulin-containing cells (22.8 ± 0.6 mmols/litre cells) under similar conditions. Thus, in a Ca^{2+}-free medium, insulin induced a substantial increase in the NMR-visible cell Na^+. The isolated cardiac myocytes used in this study were functionally intact rectangular cells which contracted repeatedly, synchronously and specifically in response to electrical stimulation in 1 m*M* Ca^{2+} and possessed nearly normal high energy phosphate content. The NMR-measured value (8.8 ± 1.2 mmols/litre cells) of intracellular Na^+ in cardiac myocytes is comparable to a recent measurement of about 8.1 mmols/kg rat myocytes by flame photometry and is similar to values reported by Fossell and Hoefeler for intact perfused hearts[40]; however, it is significantly higher than the <3 m*M* estimated by Pike *et al.*[41].

The NMR technique allowed measurement of Na^+ loading of isolated adult cardiac myocytes when extracellular Ca^{2+} was removed and demonstrated Na^+ extrusion when extracellular Ca^{2+} was restored. Extrusion of intracellular Na^+ was accompanied by uptake of Ca^{2+} from the extracellular medium and this might occur, in part, via the Na^+/Ca^{2+} exchange system in the sarcolemma. While these effects can also be measured by the usual chemical techniques, the NMR method has the advantage that sequential Na^+ loading and unloading may be demonstrated in the same sample of cells without interference from extracellular sodium. Insulin-deficiency caused only a small decrease (21%) in intracellular Na^+ in Ca^{2+}-containing medium but a marked decrease (36%) in intracellular Na^+ in Ca^{2+}-free medium. These results are consistent with insulin stimulation of Na^+ influx in mammalian cardiac myocytes. In the presence of Ca^{2+}, the level of intracellular Na^+ is low and the Na^+-pump may not be saturated with this cation. Any increase in internal Na^+ by insulin-stimulation of Na^+ influx would shift the operating point resulting in enhanced activity of the Na^+-pump. This would tend to reduce the steady state intracellular Na^+ level towards the value measured in the absence of insulin. In a Ca^{2+}-free medium, however, the increased intracellular Na^+ may saturate the Na^+ site of the Na^+-pump so that it is operating close to V_{max}. Therefore, an increased Na^+ influx causes a more marked increase in the steady state level of this cation. It is interesting to note that increased stimulation of Na^+/K^+ exchange across cell membranes due to increased internal Na^+ would cause depletion of extracellular K^+ and may explain the well known hypokalemic effect of insulin in man.

(d) Sodium Ions In Mammalian Renal Cells

(1) *Rat outer medullary kidney tubules*

The distal nephron in the human kidney is responsible for the fine control of the total body fluid volume and the electrolyte balance, a function regulated partly by adrenal steroids. In defining the regulatory mechanisms operative in the distal tubule, the measurement of the intracellular Na^+ level is of paramount importance, since this level regulates Na^+/K^+-ATPase activity, the final determinant of the amount of Na^+ reabsorbed by the kidney.

Measurements of intracellular Na^+ levels in suspensions of separated tubules from the outer medullary segment of the thick ascending limb of the loop of Henle obtained from rat kidney have been carried[31] out using $Dy(PPP_i)_2$. The intracellular Na^+ level was 37 ± 1 mmols/litre cells water at 22°C and there was a significant decrease in the intracellular Na^+ level measured after 30 min equilibration of the tubular preparation at 37°C. The level measured at 37°C was 23 ± 2 mmols/litre cell water, consistent with a sizeable increase in Na^+/K^+-ATPase activity and a concomitant decrease in intracellular Na^+ level at the higher temperature[31].

The intracellular Na^+ measured at 22°C in the suspension of outer medullary kidney tubules was responsive to defined physiological stimuli. Furosemide, an inhibitor of the $Na^+/K^+/Cl^-$ cotransport system, the major Na^+ transporting system in the thick ascending limb of the loop of Henle, reduced the intracellular Na^+ level from a control value of 35 ± 4 to one of 24 ± 6 mmols/litre cell water. In addition, amiloride (1 m*M*), an inhibitor of the Na^+ channels in medullary collecting tubules (a minor fraction in the tubular preparation used) and the Na^+/H^+ exchange process in segments of the rat thick ascending limb reduced the intracellular level of Na^+ from 37 ± 2 to

23 ± 6 mmols/litre cell water. The Na^+/H^+ exchange mechanism may therefore be an important regulator of intracellular Na^+ levels in the cells of thick ascending limb of the loop of Henle. In contrast, ouabain, a specific Na^+/K^+-ATPase inhibitor, caused a marked rise in the measured intracellular Na^+ to 58 ± 5 from a basal level of 37 ± 2 mmols/litre cell water[31].

In validation of the measurement of the intracellular Na^+ levels, the accessibility of the entire extracellular space to the shift reagent was tested by comparing the extracellular space containing $^{35}Cl^-$ ions with the extracellular space defined by the shift reagent in the same sample of the tubular suspension. The measurement of extracellular space using $^{35}Cl^-$ NMR was based on the expectation that the resonance of intracellular $^{35}Cl^-$ ions may not be detectable due to interactions with intracellular proteins and other components which would be expected to cause a large quadrupolar broadening. Indeed when the extracellular ^{35}Cl signal was broadened beyond detection, by adding the $Zn-HbO_2$ complex, there was no observable ^{35}Cl NMR signal in the tubular suspension. Since $Zn-HbO_2$ would not be expected to penetrate the cell membrane, it was concluded that the intracellular ^{35}Cl signal was NMR-invisible. This invisibility of the intracellular $^{35}Cl^-$ ions meant that the ^{35}Cl NMR signal provided a measure of the extracellular space available to this ion. The extracellular space measured using the $^{35}Cl^-$ ion resonance was, within experimental error, the same as that defined by $Dy(PPP_i)_2$ (ref. 31).

Incubation of outer medullary kidney tubules at room temperature in a medium containing ouabain to inhibit Na^+ efflux and amphotericin B to simultaneously increase Na^+ permeability of the plasma membrane raised the NMR-visible intracellular Na^+ level to 110 ± 10 mmols/litre cell water, a level equal to $82 \pm 7\%$ of that in the incubation medium (135 m*M*). These observations exclude the possibility that nuclear quadrupolar interactions reduce the NMR signal of the free intracellular Na^+ pool by 60%. The somewhat lower estimate of intracellular free Na^+ in comparison to the extracellular Na^+, under conditions which would tend to equilibrate Na^+ across the plasma membrane, could arise from incomplete equilibration of ions among intracellular compartments[31].

(2) *Rat proximal tubules*

Transport of sodium across the proximal tubule cells, as in other transporting epithelia, is regulated by the intracellular Na^+ activity. This in turn facilitates transport of a variety of substances, including HCO_3^-, Cl^-, P_i, carbohydrates, lactate and aminoacids. Thus, precise information regarding the intracellular concentration of Na^+ is essential to the understanding of transport processes across the proximal tubule. Although NMR is noninvasive and permits serial measurements of nonbound intracellular Na^+, its limitations include the requirement for a dense cell suspension and the inability to ascertain the compartmental localization of Na^+. The noninvasiveness and specificity of NMR, however, are decisive advantages over the alternative methods in the study of Na^+ in proximal tubules.

NMR spectroscopy has been used to measure intracellular Na^+ in a suspension of proximal tubules of rat kidney subjected to various maneuvers known to stimulate or inhibit the transport of sodium[35] and thus would appear to offer a useful quantitative approach to the analysis of Na^+ handling by renal epithelial cells. Oxygenation and gentle stirring were required for maintaining tissue viability and no toxic effects of $Dy(PPP_i)_2$ up to concentrations of 5 m*M* were noticeable in a Ca^{2+}-enriched medium. Intracellular Na^+ concentration in these proximal tubules was 34.1 ± 1.8 m*M* at 22°C. Raising the temperature to 37° resulted in a fall in the intracellular Na^+ concentration to 16.3 ± 0.6 m*M*, presumably due to temperature dependence of the Na^+/K^+-ATPase activity. This would increase the extrusion of Na^+ through the basolateral membrane and thus decrease the intracellular Na^+ concentration. Addition of ouabain resulted in a 2-fold increase in Na^+ concentration to 30.9 ± 2.9 m*M*, while in the presence of nystatin, a membrane channel former, there was a 5-fold increase in internal Na^+ (ref. 35).

The basal Na^+ levels in proximal and outer medullary kidney tubules should serve as a basis of reference for further inquiries.

(3) *Sodium in tubules of diabetic rats: Role of glucose*

Renal hypertrophy is a common consequence of diabetes mellitus that precedes and possibly accounts for the increased glomerular filtration rate. Despite the wealth of information regarding the descriptive aspects of the renal hypertrophy of diabetes mellitus, little is known about the cellular mechanisms that result in the enlargement of the kidneys. It has been postulated that the glucose-mediated increase in the intracellular concentration of Na^+ initiates the chain of events leading to the increase in cell size and eventually cell number. At 96 hr after the induction of the diabetes,

there was a 60% increase in the intracellular Na^+ measured by ^{23}Na NMR using $Dy(PPP_i)_2$ compared to control ($P<0.01$) (ref. 6). No further increase in intracellular Na^+ was observed during the subsequent 11 weeks of observation. Ouabain-inhibitable Na^+/K^+-ATPase activity was substantially higher in the renal tubules of diabetic rats, the increase being proportional to that of intracellular Na^+. The rise in glucose concentration from 5 to 25 m*M* resulted in a 52% increase ($P<0.001$) in intracellular Na^+ in proximal tubules of non-diabetic animals comparable to that seen in diabetic animals[6]. Based on these findings, it has been proposed that the rise in intracellular Na^+ observed in the proximal tubules of diabetic rats is due to increased filtered load of glucose that stimulates the entry of glucose and Na^+ into renal cells resulting in an increase in intracellular Na^+. The increase in intracellular Na^+ may stimulate the discharge of Ca^{2+} from intracellular stores into the cytosol and may inhibit the exit of Ca^{2+} through the Na^+/Ca^{2+} antiport. The resulting rise in the concentration of Ca^{2+} has been shown to contribute to the activation of protein kinase C. Experiments with cell cultures have demonstrated that these events result in the rapid expression of a set of proto-oncogenes that code for DNA binding proteins. These proteins by additional mechanisms may prepare the cell for DNA replication[6].

(4) *Na/K—ATPase regulation by steroids*

A possible role of intracellular Na^+ ions in the mechanism of mineralocorticoid and/or glucocorticoid regulation of Na^+/K^+-ATPase activity has been examined[32] by ^{23}Na NMR. Such a role is suggested by the observation that amiloride, a substance known to block Na^+ channels and Na^+/H^+ exchange, markedly reduces the intracellular Na^+ level (by 38%) in segments of the thick ascending limb of the loop of Henle, and inhibited both mineralocorticoid and glucocorticoid Na^+/K^+-ATPase activation[32]. Addition of dexamethasone, a glucocorticoid, to a suspension of rat outer medullary kidney tubule segments results in a significant elevation of the intracellular Na^+ level. The dexamethasone-stimulated Na^+ level was measured to be 46.1 ± 1.2 m*M* (25°C) after 2 hr incubation, while that in the control suspension of tubules, under identical conditions, was 36.9 ± 0.9 m*M*. This effect appeared to be glucocorticoid-specific since aldosterone, a mineralocorticoid, did not change the Na^+ level significantly (38.0 ± 0.4 m*M*). These data implicate glucocorticoids in the regulation of Na^+ ion-transport in the thick ascending limb of the loop of Henle[32]. Elevation of intracellular Na^+ concentration after aldosterone treatment has been observed in mineralocorticoid-sensitive tissue. The observation that glucocorticoids, and mineralocorticoids, appear to elevate Na^+, together with the observation that amiloride both reduces intracellular Na^+ levels and inhibits steroidal Na^+/K^+-ATPase activation, would appear to implicate the intracellular Na^+ level, as a possible mediator, in the steroidal regulation of the Na^+/K^+-ATPase activity. To investigate the possibility that the Na^+ ion was the primary and direct mediator of the steroidal activation of Na^+/K^+-ATPase, the intracellular Na^+ level was elevated artificially to a level of 52 ± 3 m*M* from 26 ± 3 m*M* by superfusing tubule segments in medium containing ouabain at 37°C. Over the time interval required to elicit a maximal steroidal response in the outer medullary tubular preparation (2 hr), no change in Na^+/K^+-ATPase activity was detectable[32]. These results suggested that a change in intracellular Na^+ level was not the primary mediator of either mineralocorticoid or glucocorticoid regulation of Na^+/K^+-ATPase activity in the rat kidney outer medulla. Rather, the data were consistent with some permissive role of Na^+ ions. Superfusion in ouabain-containing medium over longer perods (18 hr), however, did result in an elevation of Na^+/K^+-ATPase activity, an increase markedly larger than that observed after steroidal stimulation[32]. However, intracellular Na^+ levels, which were elevated after 2-4 hr incubations in ouabain-containing medium, were restored to a level not significantly different from control values, after 18-20 hr of treatment. Thus, a chronic elevation in intracellular Na^+ caused an increase in the number of Na^+/K^+-ATPase enzyme sites. The experiments demonstrate a homeostatic mechanism potentially important in any chronic aberration of cellular Na^+ homeostasis[32]. The demonstration of such a response in the suspension of tubular segments responsible for the fine control of total body Na^+ balance, exposes this homeostatic mechanism as one of significant physiological and pathological importance. In essential hypertension smooth muscle cells loose their ability to regulate cellular Na^+ homeostatsis, thus allowing elevation of Na^+, that would normally be restored to basal levels by this homeostatic mechanism.

(c) ^{23}Na NMR AND CANCER

Transmembrane ion fluxes and associated changes in intracellular ion concentrations have long been hypothesized as mediators in cell growth. Studies of intracellular concentrations of Na^+ in both neoplastic and nonneoplastic tissue

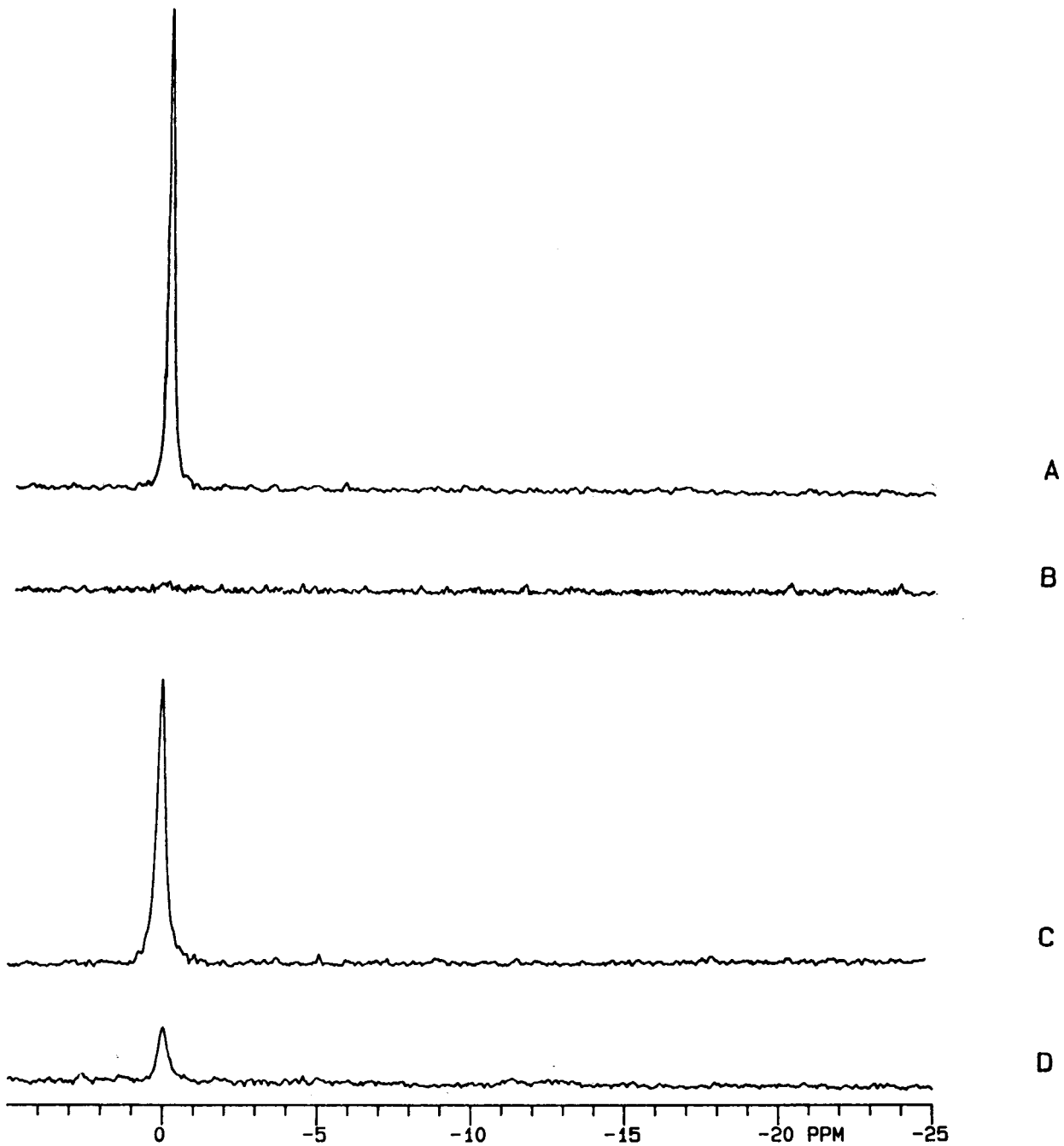

Fig. 5—^{23}Na double quantum NMR spectra of human plasma (A), plasma containing 5 m*M* $Dy(PPP_i)_2^{7-}$ (B), intact blood (C) and intact blood with 5 m*M* $Dy(PPP_i)_2^{7-}$ (D) [The spectra were recorded in the absolute value mode. Each spectrum is the fourier transform of 2048 free induction signals. Other NMR parameters were: acquisition time, 0.1 s; 90° pulse width, 30 μs; sequence recycle time, 0.17 s; double quantum preparation time, 15 ms; evolution time, 20 μs, and Larmor frequency, 132 MHz]

have strongly implicated the Na^+ cation as a mediator in cell growth and proliferation. We have explored the possible involvement in malignancy of intracellular Na^+ in a study of uterine leiomyoma and leiomyosarcoma (the benign and malignant tumors of the smooth muscle) and their nonneoplastic counterpart, myometrium, as well as colonic adenocarcinoma, a malignant tumor, and its nonneoplastic counterpart colonic mucosa[4].

The intracellular Na^+ measured using $Dy(PPP_i)_2$ in benign uterine leiomyomas was 5.1 ± 1.2 mmols/litre tissue. This value is about 3-fold less than the intracellular Na^+ concentration of 14.1 ± 1.2 mmols/litre tissue measured in myomet-

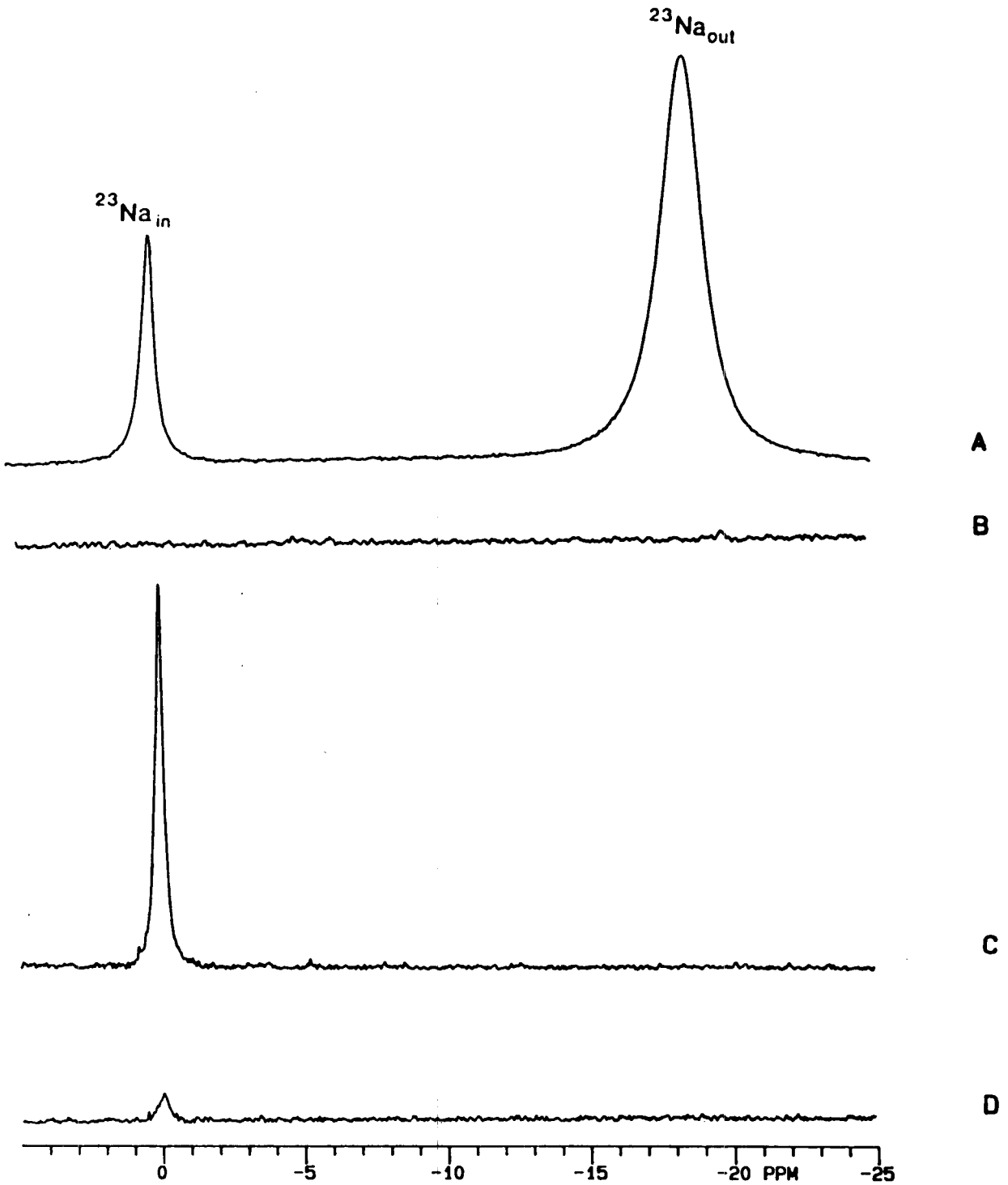

Fig. 6—^{23}Na NMR spectra of human red blood cells washed with Hank's balanced salt solution [Conventional spectrum (A) representing a time-average of 256 transients displayed with a vertical scale reduced by a factor of 10 relative to other spectra, double quantum spectra of Hank's medium (B), red cell suspension (C), and red cell suspension treated with 5 m*M* $Dy(PPP_i)_2$ (D). Double quantum acquisition parameters were the same as in Fig. 5]

rium, the normal smooth muscle layer of the uterus from which the tumor originates. In marked contrast to the sharply lower concentration of intracellular Na^+ observed in leiomyoma, uterine leiomyosarcoma, a rare malignant tumor of smooth muscle, yielded an intracellular Na^+ concentration of 34.6 ± 4.5 mmoles/litre tissue which is an almost 2.5-fold increase over the nonneoplastic counterpart[4].

The intracellulr Na^+ in well-differentiated adenocarcinoma of the colon, a malignant tumor that retains a morphological and functional resemblance to its tissue of origin, was 13.9 ± 1.5 mmoles/litre tissue, about 2-fold higher than the 8.0 ± 1.1 mmoles/litre tissue measured in colonic mucosa, the nonneoplastic counterpart. Poorly differentiated adenocarcinoma, a more highly invasive malignant tumor that deviates further in structure and function from its tissue of origin, displayed an even higher intracellular Na^+ concentration of 21.7 ± 0.6 mmoles/litre tissue, an almost 3-fold increase over the nonneoplastic counterpart and a 1.5-fold increase over the well-differentiated malignancy.

These measurements demonstrated alterations in the intracellular Na^+ in neoplastic human tissue relative to its tissue of origin. In addition, the degree of cellular proliferative activity and/or tumor invasiveness seemed to have a direct correlation with the concentration of intracellular Na^+. As has been observed with colonic and uterine tissue, the progression from normalcy to neoplasia, from benignity to malignancy, and from well-differentiation to poor differentiation were, with one exception, accompanied by increase in the intracellular Na^+. The one exception was the leiomyomas and most of these specimens were taken from peri- or postmenopausal women. Leiomyomas in postmenopausal women tend to regress and, therefore, the lower intracellular Na^+ is consistent with a regressing tissue possessing little or no proliferative activity. The colonic adenomatous polyp, the benign correlate of colonic adenocarcinoma, would provide the proliferative intermediate between normalcy and malignancy. However, such tissue was unobtainable for this study. The data suggest that benignity and malignancy may be distinguishable on the basis of intracellular Na^+ and that ^{23}Na NMR spectroscopy has the potential of becoming a powerful diagnostic tool. That is, once obtained, a measurement of intracellular Na^+ may represent as good a diagnostic and/or prognostic indicator as histopathologic viewing[4].

(f) Double Quantum NMR Of Intra- And Extracellular ^{23}Na In Cells And Tissues

The use of shift reagents to separate intra- and extracellular Na^+ has had much success and is applicable to many biological systems. The one disadvantage to the use of shift reagents lies in the fact that they can be toxic to certain cells and tissues. Because of this possible toxicity, a totally noninvasive NMR approach is preferable in some instances. It has been suggested that double quantum NMR may allow the noninvasive discrimination of different pools of Na^+ *in vivo*[61-63]. For quadrupolar nuclei ($I \geqslant 3/2$) double quantum coherence can be excited by biexponential relaxation[64] which is observed for ^{23}Na when the extreme narrowing condition ($\tau_c \ll 1/\omega_0$) does not hold. When $\tau_c > 1/\omega_0$, the outer transitions ($-3/2 \rightarrow -1/2$ and $1/2 \rightarrow 3/2$) decay more quickly than the inner transition ($-1/2 \rightarrow 1/2$) and the resulting biexponential relaxation can induce violations of the coherence transfer selection rules. The evolution of the spin system under such conditions can be described by representing coherence in terms of irreducible spherical tensor operators[64,65]. Double quantum coherence can be detected using the phase cycled INADEQUATE double quantum filter:

$$90_x - \tau/2 - 180_y - \tau/2 - 90_x - \Delta - 90_x - t\,(\text{acquire})$$

After the first pulse the spin system contains single quantum tensor components of rank 1 which are transformed to single quantum components of rank 3 during τ, the preparation time, due to the effects of biexponential transverse relaxation. The 180_y pulse at the midpoint of the interval τ merely refocuses the chemical shift. The third pulse generates double quantum tensor components of rank 3 which are transformed into single quantum components of rank 3 by the last pulse of the sequence. These components become detectable during the acquisition time t as they evolve into single quantum components of rank 1 as a result of biexponential transverse relaxation. The detected signal is given by Eq. (6)[63]:

$$M_0(3/4)(e^{-\tau/T}2f - e^{-\tau/T}2s)e^{-\Delta/T}dq\cos(2\omega\Delta)$$
$$(e^{-t/T}2f - e^{-t/T}2s)e^{i\omega t} \qquad \ldots (6)$$

where T_{2f} is the T_2 of the outer transitions, T_{2s} is the T_2 of the inner transition, T_{dq} is the relaxation rate of the double quantum transition, ω is the resonance offset, and M_0 is the equilibrium longitudinal magnetization.

It has been suggested that the intracellular Na^+ may be selectively detected with double quantum methods obviating the need for using the shift reagents, such as $Dy(PPP_i)_2$ or DyTTHA to discriminate between intra-

and extracellular Na^+ pools[61]. We have attempted to use the double quantum experiment to detect intracellular Na^+ in human blood in order to test the validity of such an approach[66]. Double quantum spectra of plasma and intact blood are shown in Fig. 5. These were obtained using a 256-step phase cycled INADEQUATE pulse sequence. Preparation (τ) and evolution (Δ) times were optimized to obtain maximum double quantum-filtered signal from the intact blood sample and set at 15 ms and 20 μs, respectively. The double quantum spectrum of plasma alone is shown in Fig. 5A and it is clear that biexponentially relaxing Na^+ present in plasma would contribute significant intensity to the total observed ^{23}Na double quantum signal of the intact blood. The addition of 5 m*M* $Dy(PPP_i)_2$, however, dramatically reduced the plasma double quantum signal as shown in Fig. 5B. We have interpreted this to be due to quenching of the plasma double quantum coherence by paramagnetic interaction of Na^+ with $Dy(PPP_i)_2$ which results in rapid transverse relaxation of ^{23}Na via a non-quadrupolar mechanism. The ^{23}Na double quantum signals of intact blood with and without $Dy(PPP_i)_2$ (5 m*M*) are shown in Figs 5C and 5D, respectively. The dramatic difference in the amplitudes of the filtered signal indicates a dominant contribution from extracellular Na^+ to the observed signal in Fig. 5C (ref. 66).

NMR spectra were also obtained from red blood cells washed with Hank's balanced salt solution with and without $Dy(PPP_i)_2$ (5 m*M*). The chemical shifts of the unshifted and shifted ^{23}Na resonances in a conventional single pulse NMR spectrum are shown in Fig. 6A. Na^+ in the saline medium alone did not give rise to a detectable double quantum signal (Fig. 6B). Figure 6C shows the double quantum spectrum of the washed cell suspension (80% hematocrit). The magnitude of the observed signal is comparable to that from intact blood in Fig. 5C. To determine whether the detected double quantum signal was arising solely from intracellular Na^+, 5 m*M* $Dy(PPP_i)_2$ was added to separate the intra- and extracellular Na^+ double quantum signals. The resulting spectrum is shown in Fig. 6D. A dramatic reduction in the double quantum signal at the unshifted frequency and no signal at the frequency of the shifted extracellular Na^+ were observed, presumably again due to quenching of the signal from extracellular Na^+ by the paramagnetic reagent. The intensity difference between the signals in Figs 6C and 6D suggests a dominant contribution to the double quantum signal from extracellular Na^+ even though the medium alone (without cells) exhibited no double quantum signal. Double quantum coherence from extracellular Na^+ in this case presumably arises from Na^+ interaction with membrane sites on the cell surface which would cause biexponential relaxation. When a paramagnetic reagent is added, the transverse relaxation of extracellular Na^+ is enhanced to the extent that the extracellular double quantum signal is not detectable under our experimental conditions. Double quantum coherence of extracellular Na^+ of a magnitude similar to that observed with 80% red cells was also observed in a suspension containing 40% cells, although in the latter case the interval corresponding to maximal signal was longer[66].

These results demonstrate that the double quantum NMR of cells and tissues allows detection of biexponentially relaxed Na^+. However, it is readily apparent that the detected signal is not solely due to intracellular Na^+ but contains a dominant contribution from extracellular Na^+ which can be quenched by interaction with a paramagnetic relaxation reagent permitting discrimination of contributions from intra- and extracellular Na^+. While the double quantum technique alone cannot be used to quantitate intracellular Na^+ due to significant contributions from the extracellular Na^+, it may be useful in qualitative studies of compartmentation and NMR-invisibility.

(g) ^{23} Na NMR Imaging

Sodium is the second most abundant NMR nucleus in tissue and is primarily present in the extracellular fluid at a concentration of about 145 m*M*. The intracellular Na^+ concentration of 10 to 20 m*M* is maintained by the Na^+/K^+-ATPase pump. Because changes in the physiological state of the cell can alter these relative concentrations ^{23}Na NMR imaging may be useful in studying diseased states. In ^{23}Na imaging studies, image contrast can arise from two general mechanisms: (i) any increase in the extracellular space, i.e., regions of edema, results in an increase in ^{23}Na concentration; and (ii) in the event of cell death the intracellular Na^+ concentration increases dramatically causing the average tissue concentration to increase by as much as an order of magnitude. The biexponential T_2 relaxation behavior of Na^+ provides an additional contrast mechanism. In biological samples the intracellular Na^+ relaxes much more rapidly (T_2 values of about 2 ms) than extracellular Na^+ (T_2 values of about 50 ms).

In vivo imaging of tissue sodium was first performed using animal models and demonstrated the dramatic image contrast expected following stroke and myocardial infarction[67-69]. For these experiments a field strength of 2.7 T was used and data acquisition took up to 4 hr. Since no chemical shift information is available, a conventional three-dimensional fourier imaging sequence was used to typically obtain 32 planes with approximately 2.5 mm resolution. Images of sodium distributions in humans have also been carried out. Hilal *et al.*[70] have obtained an image of the Na^+ distribution

within the head at 1.5 T. Particularly noticeable in these images is the intense signal (observed as a bright area) arising from the high Na^+ concentration within the cerebrospinal fluid, which serves to outline the brain and brainstem. An abnormal Na^+ distribution was observed, appearing as a bright region, in the presence of an arteriovenous malformation of the brain.

Preliminary investigation into the application of shift reagents to separate intra- and extracellular Na^+ in imaging studies have been carried out[71]; however, practical application requires the development of higher field systems as well as a nontoxic shift reagent. The use of ^{23}Na NMR for *in vivo* diagnostic imaging and spectroscopy is thus still limited by the inability to distinguish the intra- from the extracellular Na^+. Although ^{23}Na has a sensitivity of over one order of magnitude less than ^1H, as pointed out elsewhere in the paper, its relaxation times are over two orders of magnitude shorter than those of ^1H. Therefore, with time-averaging, ^{23}Na produces a signal per unit time comparable to that of ^1H. This imaging characteristic, the 100% natural abundance of the isotope, as well as the finding that NMR-visible intracellular $[Na^+]$ is indeed altered in neoplasia and other disease states should provide impetus to refine the technique of ^{23}Na NMR to the point where the intracellular compartment can be observed *in vivo*. The development of a nontoxic, membrane impermeable, paramagnetic shift reagent would permit *in vivo* spectroscopy and chemical shift imaging of the intracellular space. Alternatively, it may be possible to use differences in the relaxation behaviour of the intra- and extracellular Na^+ to obtain an image representing the intracellular Na^+ compartment.

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