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NMR SPECTROSCOPY of CELLS and ORGANISMS

Volume II

Raj K. Gupta

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Editor

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PREFACE

Applications of NMR spectroscopy in the study of cells and organisms represent an ever-expanding area of research actively pursued in many universities and research institutions. Notable advances have been made in recent years in the use of ^{1}H, ^{19}F, ^{31}P, ^{13}C, and ^{23}Na NMR to study tissue physiology and biochemistry in vivo. These volumes are aimed at describing the noninvasive use of NMR to study electrolytes and metabolites in living cells. NMR measurements of intracellular pH, free Mg^{2+}, free Ca^{2+}, and Na^{+} ions, membrane ion-transport processes, cell water content, and steady state rates of intracellular reactions by saturation transfer, as well as mapping of metabolic pathways for the processing of organic molecules by ^{13}C NMR, are described. In vivo ^{31}P NMR studies of the metabolic state of tumor tissue, nuclear relaxation studies of tissue water, as well as multinuclear spectroscopic imaging of intact organisms, are also included. The intent of these volumes is to familiarize the physiologist/biologist/chemist with the advances and progress in cellular applications of NMR and the future potential of this technique.

THE EDITOR

Raj K. Gupta, Ph.D. is a Professor of Physiology & Biophysics and a Professor of Biochemistry at the Albert Einstein College of Medicine of Yeshiva University in New York City.

Professor Gupta obtained his graduate education leading to a Ph.D. in chemical physics from the Indian Institute of Technology, Kanpur, India. He was a postdoctoral fellow in biophysics at the IBM Watson Laboratory, Columbia University, New York and at the IBM Thomas J. Watson Research Center, Yorktown Heights, New York from 1969 to 1972. Subsequently he spent a year as a visiting scientist at the National Institutes of Health, Bethesda, Maryland. In 1973, Professor Gupta joined the Institute for Cancer Research in Philadelphia as a member of its senior research staff and later also held a joint appointment as an associate professor of biochemistry and biophysics at the University of Pennsylvania. From 1976 to 1981, he was the recipient of a research career development award from the U.S. Public Health Service pursuing research on NMR spectral studies of the structure and mechanism of heme- and metal-containing proteins. He left Philadelphia in 1982 to become a Professor at Albert Einstein, where, along with some teaching and consulting, he is actively involved in NMR research on intact cells, tissues, and organisms. Professor Gupta has played a key role in the development and use of NMR techniques for the study of intracellular $^{23}Na^{+}$, $^{39}K^{+}$, Ca^{2+}, and Mg^{2+} ions and pH.

Professor Gupta is a member of the American Chemical Society, the American Physical Society, the Biophysical Society, and the American Society of Biological Chemists. He has served on numerous scientific review panels and on the editorial board of *Biophysical Journal*. Professor Gupta has published scores of research articles and reviews on NMR spectroscopy and its biochemical and physiological applications.

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^{23}Na NMR SPECTROSCOPY OF INTACT CELLS AND TISSUES

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I. INTRODUCTION

Na^+ ions, their electrochemical gradients, and transmembrane fluxes 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. Each tissue in a given physiological state is characterized by a well-defined intracellular Na^+ level, the maintenance of which appears essential for its normal functioning. Changes in the intracellular concentration of this ion 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.[1-7] An increase in intracellular Na^+ concentration occurs during cellular proliferation, while tissue differentiation is accompanied by decreased internal Na^+.[1,8] Na^+ ions have been implicated in the mechanisms of mitogenic as well as oncogenic phenomena.[2] The association between Na^+ ions and hypertension has long been recognized and it has been postulated that an increase in intracellular Na^+ of arteriolar smooth muscle may be the primary cellular defect in hypertension.[4] Similarly, it has long been known that in contrast to normal erythrocytes, sickle red blood cells gain intracellular Na^+ in the deoxygenated state.[7] Recent studies also implicate an altered intracellular Na^+ in diabetes.[5,6]

Intracellular Na^+ levels 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^+-cotransport, and Na^+ selective channels in the plasma membrane. Changes in Na^+ ion concentration and the electrochemical gradient could affect the activities of a cell in numerous ways. The intracellular Na^+ concentration is a direct regulator of the plasma membrane Na^+-pump, which in turn influences the energetics of the cell. Na^+ electrochemical gradient is coupled to the uptake of certain amino acids, bases, and possibly other nutrients into many types of cells and may thereby exert an influence on cell growth. Further, a change in Na^+ ion concentration could profoundly alter the concentration of other intracellular ions. For example, discharge of H^+ ions from the cell with a concomitant rise in intracellular pH could accompany Na^+ influx via the Na:H exchange mechanism, or a change in intracellular Ca^{2+} or Mg^{2+} may occur during Na^+ influx via the Na^+:Ca^{2+} or a Na^+:Mg^{2+} exchange mechanism. Intracellular pH, Ca^{2+}, or Mg^{2+} ions may in turn be intimately involved in fine control of cellular functions.[1] For example, an increase in intracellular Na^+ ion in the hypertensive state would be expected to lead to a significant elevation in intracellular free Ca^{2+} resulting from decreased Na^+:Ca^{2+} exchange in arteriolar smooth muscle cells, with a consequent increase in the contractile tone and vasoconstriction.[4]

The magnetic properties of the ^{23}Na nucleus are equally encouraging, with a natural abundance of 100% and a resonance frequency close to that of ^{13}C. While the signal produced by the ^{23}Na nuclei following a single NMR pulse is an order of magnitude smaller than that generated by protons, its relaxation times are about 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 $1/2 \rightarrow +1/2$. 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 detection while the central transition stays narrow. This presents a complication in that observed NMR intensities may not be proportional to concentrations. The possibility of invisible transitions should therefore be considered in equating intensities to concentrations.

II. NMR OBSERVATION OF INTRACELLULAR SODIUM IONS

As outlined above, it is of considerable interest to study intracellular Na^+ ions and their transport across surface 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 little 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, such as flame photometry and atomic absorption spectroscopy. To avoid calculated correction factors or extensive washing to remove extracellular Na^+, a noninvasive direct measurement of intracellular Na^+ concentration is most advantageous. NMR spectroscopy offers such a technique. It can be used to detect cations within intact cells. The noninvasive nature of NMR is particularly attractive when compared with the impalement of cells and tissues that is necessary in order to measure intracellular ion concentrations with cation-sensitive microelectrodes. 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. NMR also has the advantage over other methods, such as atomic absorption or electron X-ray microanalysis, in that it is nondestructive and allows the observation of ionic changes in the cellular environment as they take place within an essentially unperturbed living system.

Early ^{23}Na NMR studies of cells and tissues were carried out in a number of laboratories.[9-15] However, two reservations precluded the use of ^{23}Na signal as a quantitative measure of intracellular Na^+. First, the exact volume of cells in the NMR window from which the observed signal was originating was difficult to estimate with reasonable accuracy. Second, a sizeable contamination of the ^{23}Na signal by extracellular ions was unavoidable. The interesting small ^{23}Na resonance of intracellular ions was masked by the uninteresting but much larger resonance of extracellular ions. Until recently, 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,[16,17] and for the first time allowed direct observation of well-resolved ^{23}Na resonances from intra- and extracellular Na^+ ions in living cells at nonperturbing, low reagent concentrations (Figure 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, and organisms.[3,6,17-35] Other anionic reagents were simultaneously and independently introduced by Pike and Springer,[36] but were not as effective and much higher concentrations were required to achieve the same resolution under physiological conditions.[22,37] The detection of resolved resonances from intra- and extracellular Na^+ 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, cannot permeate through the cell membrane over the time scale of NMR measurements, and remains localized only in the extracellular compartments so that the NMR absorption 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.

Dysprosium bis(tripolyphosphate) 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 Na^+ complex with dysprosium bis(tripolyphosphate), we studied the interaction of Na^+ with the corresponding relaxation reagent gadolinium bis(tripolyphosphate). The paramagnetic effect of Gd^{3+} on the longitudinal nuclear relaxation rate of ^{23}Na in the complex of Na^+ with gadolinium bis(tripolyphosphate) was estimated. From the magnitude of this

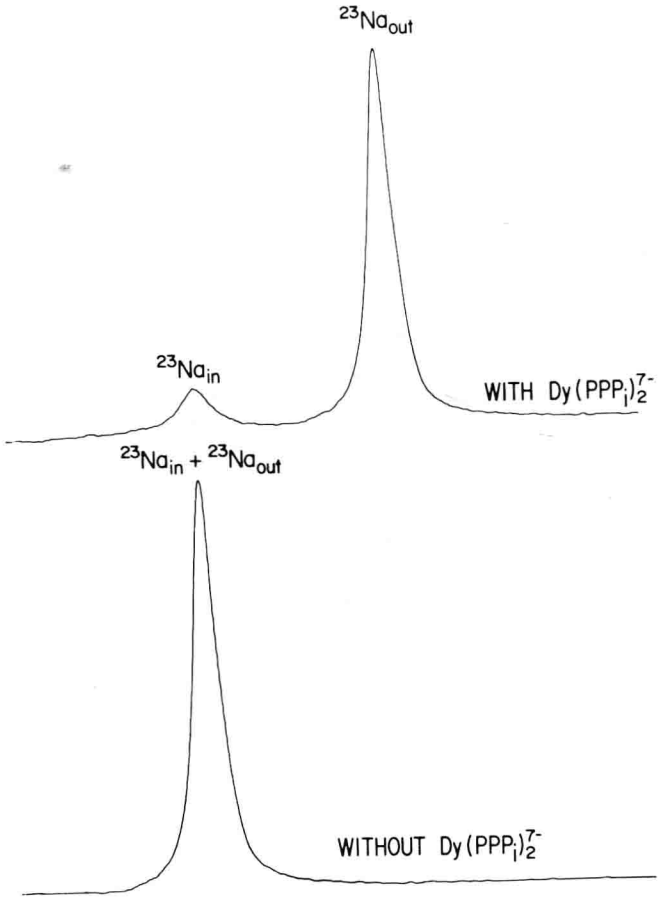

FIGURE 1. ^{23}Na NMR spectra of red cells in heparinized whole human blood with (top) and without (bottom) 3 m*M* $Dy(PPP_i)_2^{7-}$ showing spectral resolution of intra- and extracellular Na^+ ions by the paramagnetic shift reagent. The resonances of intra- and extracellular Na^+ ions are labeled as $^{23}Na_{in}$ and $^{23}Na_{out}$, respectively. (From Gupta, R. K., Gupta, P., and Moore, R. D., *Annu. Rev. Biophys. Bioeng.*, 13, 221, 1984. With permission.)

paramagnetic effect (about 10,000/sec) and the rotational correlation time derived from Stokes' law, a Gd^{3+} to ^{23}Na distance of 4 Å was obtained using the theory of distance-dependent paramagnetic dipolar interactions.[38,39] A structure of the complex consistent with this distance is shown in Figure 2.

$Dy(PPP_i)_2$ 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 $Tm(PPP_i)_2$ causes a downfield paramagnetic shift but thulium bis(tripolyphosphate) is only half as effective as dysprosium bis(tripolyphosphate), as judged by the magnitudes of the observed shifts.[22,37] The opposite directions of the observed paramagnetic shifts indicate their dipolar origin and presumably reflect differences in orientation of the principal axes of the electronic g-tensor in the two cases.

III. QUANTITATION OF INTRACELLULAR SODIUM IONS BY NMR

Once the separation of intra- and extracellular ^{23}Na resonances by the shift reagent has

$Dy(PPP)_2^{7-}$

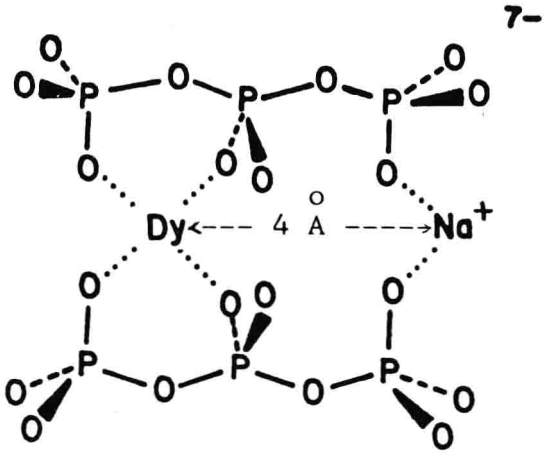

FIGURE 2. A possible structure of the complex of the anionic shift reagent $Dy(PPP_i)_2^{7-}$ with Na^+ ion, consistent with the paramagnetic effects of the relaxation reagent $Gd(PPP_i)_2^{7-}$ on the ^{23}Na nucleus.

been achieved, a comparison of the intensity of the resonance of extracellular ions (A_{out}) in the cell sample with the intensity of ^{23}Na resonance from a cell-free control (A_0) containing only the suspension medium [Na_{out}] (Figure 3) directly yields the fractional space in the NMR window 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 interstitial spaces. The ratio of intensities of the ^{23}Na resonances of intracellular (A_{in}) and extracellular ions 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. The following equations provide the relationship between the observed resonance intensities and the "NMR-visible" intracellular Na^+ concentration:[17,22]

$$S_{out} = \frac{A_{out}}{A_0} \tag{1}$$

$$[Na_{in}] = \left\{\frac{A_{in}S_{out}}{A_{out}(1 - S_{out})}\right\} [Na_{out}] \tag{2}$$

When [Na_{out}] is expressed in millimolars, the units of [Na_{in}] are millimoles per liter cells. A knowledge of tissue water content enables calculation of [Na_{in}] on the basis of kilogram cell water.

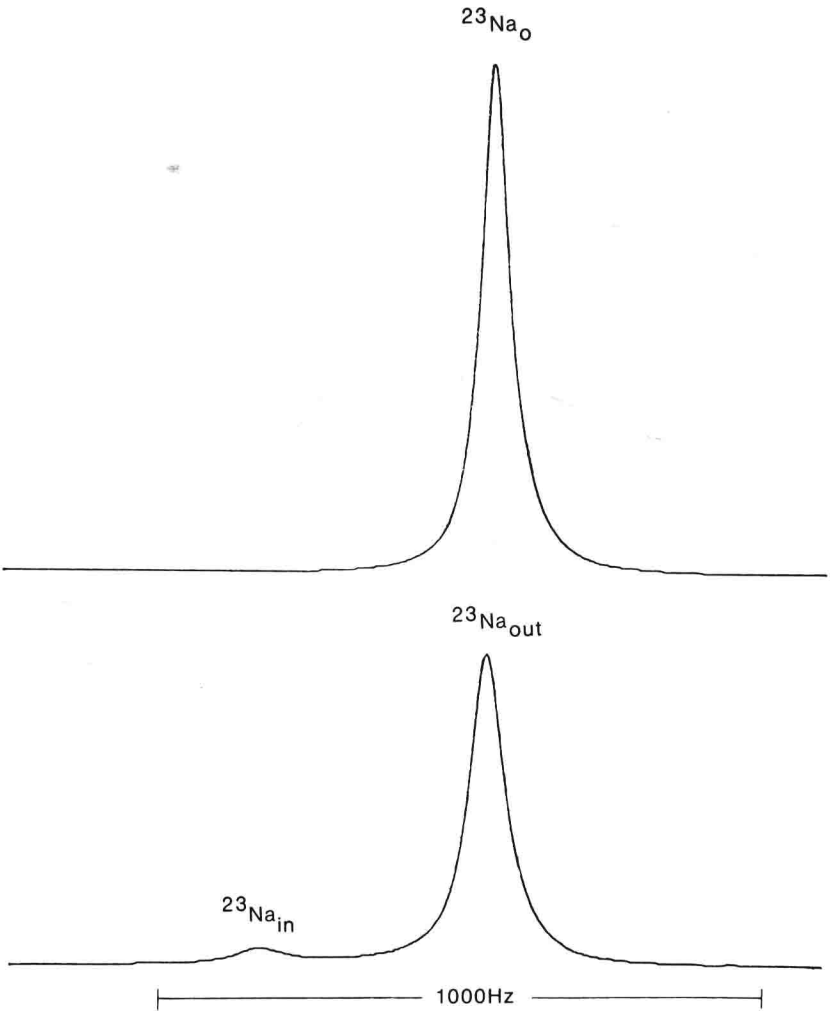

FIGURE 3. Comparison of ^{23}Na NMR spectrum of red cells suspended in serum containing 3 m*M* $Dy(PPP_i)_2$ (lower trace) with that of the suspension medium alone (upper trace). The ratio of intensities of the $^{23}Na_{out}$ and $^{23}Na_o$ signals directly gives the fractional extracellular space.

IV. MEASUREMENT OF CELL WATER CONTENT BY A COMBINATION OF ^2H AND ^{23}Na NMR

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 has previously been measured gravimetrically, by comparing dry weight of cells with their net 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 that cells can take up inulin by fluid-base endocytosis.[21] This results in inaccurate estimation of the amount of extracellular water, which may introduce sizable 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.

Recently, Cowan et al.[40] have proposed an NMR method to measure intracellular water volume by combining ^1H and ^{23}Na spectral measurements. This generally involves the use