The determination of intracellular water space by NMR

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A new method for the determination of intracellular water space using NMR spectroscopy is described. The method is based on the measurement of ^{59}Co NMR signal intensity of an inert, stable and membrane-impermeable cobalt(III) compound such as $Co(CN)_6^{3-}$ or $Co(imidazole)_6^{3+}$ and the ^2H or ^1H NMR signal intensities of the freely permeable water. As an example of the method, the variation of the intracellular water space of human erythrocytes as a function of osmolality was measured

Intracellular water space *^2H-NMR* *^{59}Co-NMR* *Erythrocyte*

1. INTRODUCTION

The determination of intracellular water space is essential in a great variety of experiments such as those investigating metabolism, transport properties and transmembrane potentials. In general, in any calculation of intracellular concentrations based on signal intensities the intracellular water space must be known. The most commonly used method is the radioactive isotope dilution method; in which the extracellular volume is determined using radioactively labeled, membrane-impermeable compounds such as [^{14}C]oligo- and polysaccharides or ^{131}I-albumin while total water volumes are determined using freely diffusable radiolabeled compounds such as [^{3}H]water and [^{14}C]ethanol [1–5]. Recently, various attempts have been made to determine cellular volumes and intracellular water space using NMR [6–9]. Here we present a method for the determination of intracellular water space based on ^{59}Co and ^2H or ^1H NMR.

2. EXPERIMENTAL

Human blood was either freshly drawn into heparinized tubes or less than 10 days old from the blood bank. $K_3Co(CN)_6$ was commercially obtained. $Co(imidazole)_6^{3+}$ [$Co(Im)_6^{3+}$] was prepared by H_2O_2 oxidation of $Co(Im)_6^{2+}$ and separation by column chromatography (G. Navon and R. Panigel, to be published).

NMR spectra were taken using a Bruker AM 360-WB NMR spectrometer equipped with a variable frequency 10 mm probe. All measurements were conducted at a temperature of 20 ± 2°C. T_1 was measured using the 180–90° inversion recovery method. Osmolalities were measured using a Viescor 5100C vapor pressure osmometer. Cobalt analysis was done using a Perkin-Elmer 403 atomic absorption spectrophotometer.

3. RESULTS

The basic idea of our method for the determination of intracellular water space is similar to that of the radioactive isotope dilution method, i.e. a simultaneous determination of the quantities of 2 compounds; one, which is membrane impermeable, determines the extracellular volume and the other, which is freely diffusable, the total water volume. We selected cobalt(III) complexes as the membrane-impermeable compounds and D_2O as the freely diffusable compound. The amounts of these compounds are determined from their ^{59}Co and ^2H NMR signal intensities respec-

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tively. In this method a small amount of a solution containing both the cobalt complex and some D_2O is added to the cell suspension. Two samples, those of the cell suspension and of its supernatant, are taken for the NMR measurement. Let us denote the integrals of the ^{59}Co and ^{2}H NMR signals of the sample containing the cell suspension as I_c^{Co} and I_c^{D}, respectively, and those of the sample containing the supernatant as I_s^{Co} and I_s^{D}. Since the ^{59}Co signal intensity of a membrane-impermeable compound is proportional to the external water space, V_w^{ex}, while that of the ^{2}H to the total water space, $V_w = V_w^{in} + V_w^{ex}$, the ratio of the internal water space, V_w^{in}, to that of the total water space is given by:

$$\frac{V_w^{in}}{V_w} = 1 - \left(\frac{I_c^{Co}}{I_s^{Co}}\right) \Big/ \left(\frac{I_c^{D}}{I_s^{D}}\right) \quad (1)$$

It should be emphasized that since eqn 1 depends on the ratios (I_c^{Co}/I_c^{D}) and (I_s^{Co}/I_s^{D}) and since both the ^{2}H and ^{59}Co NMR measurements are performed on the same samples, any correction due to unequal volumes of the cell suspension sample and the sample of the supernatant cancels out. Also, any loss of ^{59}Co signal intensity caused by the suspending medium cancels out in the ratio (I_c^{Co}/I_s^{Co}). The total water space may be also estimated from the ^{1}H NMR signal intensities. In this case one uses the proton decoupling coils, and thus avoids the need to tune the spectrometer to the ^{2}H frequency. However, since the measurements are made by 2 different coils some dependence on the volume of the samples is found.

The criteria for the selection of Co(III) complexes include their stability, impermeability, lack of binding to the cell and narrow ^{59}Co NMR signals. We have tested a number of cobalt(III) complexes in suspensions of erythrocytes in Ringer solutions. As the linewidth in such cell suspensions has a large contribution from field inhomogeneities we have used the ^{59}Co T_1 of the cobalt(III) complexes to assess their binding to the outer surface of the erythrocytes. Since one of the uses of our method is the determination of intracellular concentrations of Na^+ and K^+ in the presence of dysprosium tripolyphosphate ($Dy(TPP)_2^{7-}$) [7,8,10–13], we have also measured the effect of cells on the ^{59}Co T_1 in the presence of this shift reagent. On the basis of the above criteria, one anionic, $Co(CN)_6^{3-}$, and one cationic, $Co(Im)_6^{3+}$, complex was selected. They both have relatively narrow ^{59}Co signals and long T_1 relaxation times which are influenced very little by the presence of cells suspended in a Ringer solution or in solutions containing the $Dy(TPP)_2^{7-}$ shift reagent. The ^{59}Co relaxation times become shorter in plasma, presumably due to binding to some of the plasma proteins. Furthermore, for $Co(CN)_6^{3-}$ there may be some loss of intensity in plasma. However, as shown above, such an intensity loss does not interfere with the determination of the intracellular water space.

Experiments were done to test whether $Co(CN)_6^{3-}$ and $Co(Im)_6^{3+}$ penetrate erythrocytes. Thus erythrocytes were washed 4 times with Ringer solutions containing 2 mM of the Co(III) complex incubated at room temperature for 8 h and the ^{59}Co concentration was then measured in the supernatant. The same procedure was also repeated in the presence of 4 mM $Dy(TPP)_2^{7-}$ shift reagent. The recovery was 98.0 ± 2.0 SD and 98.5 ± 2.0 SD for $Co(CN)_6^{3-}$ and $Co(Im)_6^{3+}$, respectively. Thus we conclude that no significant penetration of these complexes occur within a time much longer than that required for a typical NMR experiment. Experiments were performed to determine if there is an intensity loss of ^{59}Co NMR signal of $Co(CN)_6^{3-}$ in highly packed erythrocytes. Thus 3 mM $K_3Co(CN)_6$ was added to whole blood which was then centrifuged to various degrees of cell packing. The ^{59}Co NMR signal in the packed cells and the supernatant was recorded. Cells were then washed 4 times with saline and the amount of cobalt in the combined washings was measured by atomic absorption. Our results indicate that there is no detectable loss of intensity in packed erythrocytes containing up to 89% cells.

To exemplify our method we tried to measure intracellular water volume in erythrocytes as a function of the osmolality of the suspending medium. The osmolality was varied by the addition of sucrose solutions of various concentrations to cell suspensions in a Ringer solution. The results are shown in fig.1. The decrease of the intracellular water space with increasing osmolality is clearly seen. The results are in good agreement with those previously obtained using the isotope dilution method [5].

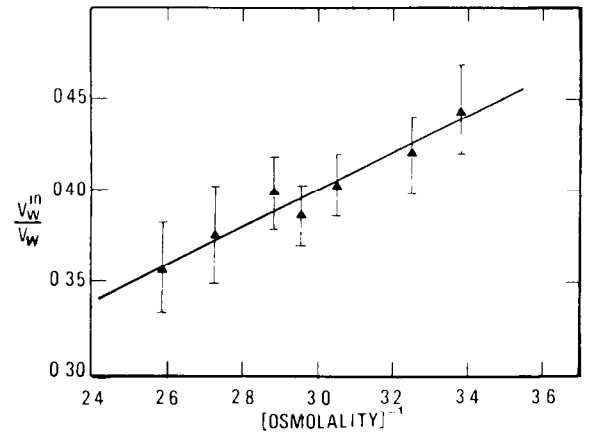

Fig.1. Relative intracellular water space of erythrocytes suspended in media with different osmolalities. Cells were washed 3 times with a Ringer solution, then, $K_3Co(CN)_6$ in D_2O was added to final concentrations of 2 mM and 2%, respectively, and to each portion of 8 ml, 0.92 ml of sucrose solutions of different concentrations was added. After 30 min of equilibration at room temperature 2 ml cell suspension were removed, the sample was centrifuged and 2 ml were taken from the supernatant. The ^{59}Co and ^{2}H NMR signals of the 2-ml samples were recorded. Each point in the figure is an average of 5 identical samples and the error bars are the standard deviations.

4. DISCUSSION

The method suggested here for the determination of intracellular water space may substitute the isotope dilution method in all experiments where the use of radioactive materials is preferably avoided. This is particularly convenient in NMR experiments, since the intracellular water space can be determined on the same samples using the same NMR probe as the other NMR measurements. The NMR frequency of ^{59}Co is conveniently located near that of ^{13}C and ^{23}Na; its NMR sensitivity is very good, 28% of that of protons, so that only 1 or 2 mM of the cobalt compound may be added, with negligible effect on the total osmolality. Another advantage of the ^{59}Co NMR is the fact that unlike ^{31}P and ^{13}C NMR, there is no interference from naturally occurring signals in the biological samples. The lower sensitivity of ^{2}H (1% of that of protons) does not pose any difficulty since it may be added in higher concentrations without any significant perturbation to the biological system. In fact, a strong ^{2}H signal is obtained in solutions containing less than 1% D_2O. Ogawa et al. [6] measured intracellular water space by comparing ^{1}H signal intensities of water with that of 0.25 M added mannitol. Some of the problems of their method were the major change of osmolarity by the addition of the high concentration of mannitol and the difficulty of measuring the mannitol signals in the presence of a large water signal. Other NMR methods suggested previously, based on ^{23}Na [7] and on ^{59}Co NMR [8], measure the fraction of the volume occupied by the cells and not the intracellular water space obtained by our method. Also, their accuracy is diminished by the dependence on sample volumes. It has been noted previously that the ^{23}Na NMR signal of extracellular Na^+ is significantly broadened in packed erythrocytes in the presence of $Dy(TPP)_2^{7-}$ shift reagent [8]. Moreover it was reported recently that there is an intensity loss of the extracellular $^{23}Na^+$ NMR signal [13,14], thus limiting the applicability of ^{23}Na NMR in determining cellular volumes at high hematocrits. Cowan et al. [9], in work aiming at the determination of intracellular Na^+ concentrations, have estimated intracellular water space on the basis of ^{1}H and ^{23}Na chemical shifts. Their method requires the presence of a shift reagent and thorough oxygenation of the erythrocytes. A complicating factor in the method, i.e. the determination of the shift reagent concentration from ^{23}Na chemical shifts, may in fact be avoided by measuring the ^{1}H chemical shift of the supernatant. However, it is tacitly assumed that the intracellular ^{1}H chemical shift relative to a capillary does not depend on the presence of the shift reagent, thus neglecting the bulk susceptibility correction. The advantages of ^{59}Co over ^{23}Na or ^{1}H NMR for volume determinations are that (i) the presence of shift reagents is not obligatory for ^{59}Co NMR, (ii) it can also be used in systems where Na^+ transport during the experiment cannot be ignored, (iii) the degree of oxygenation of the hemoglobin is not important, and (iv) there is no intensity loss of extracellular $^{59}Co(CN)_6^{3-}$ at high hematocrits.

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