Determination of the Intracellular Sodium Concentration in Perfused Mouse Liver by ^{31}P and ^{23}Na Magnetic Resonance Spectroscopy

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A combination of ^{31}P and ^{23}Na NMR spectroscopy has been used to quantify the concentration of intracellular sodium, $[Na]_{IC}$ in the isolated and perfused mouse liver. The ^{31}P resonances of dimethyl methylphosphonate and $LaDOTP^{5-}$, markers of total tissue space and extracellular space, respectively, were used to determine the intracellular liver volume. For a mean wet weight of 1.7 ± 0.3 g, the intracellular liver volume as measured by ^{31}P NMR averaged 1.2 ± 0.2 ml. The amount of intracellular sodium was measured from the baseline-resolved intracellular ^{23}Na resonance during perfusion of the shift reagent, $TmDOTP^{5-}$. These two measurements resulted in an NMR-determined value for $[Na]_{IC}$ of 29.0 ± 5.2 m*M*. Separate measurement of total tissue Tm and Na by atomic absorption spectroscopy on the same samples provided an AAS-determined value for $[Na]_{IC}$ of 32.1 ± 7.4 m*M*. These results indicate that intracellular sodium in the isolated, perfused liver is 100% visible by ^{23}Na NMR spectroscopy.

Key words: NMR; liver; phosphorus space markers; shift reagent.

INTRODUCTION

In normal cells, the maintenance of a transmembrane Na^+ gradient is a vital process carried out by the Na/K-ATPase pump. In the liver, this pump controls the membrane potential in hepatocytes, thereby affecting internal pH, intracellular free calcium, cytoskeletal activity, and secondary active transporters, which in turn may regulate bile transport (1). Since dysfunction of this pump may be associated with diseases such as hypertension or cholestasis, there is considerable interest in developing methods that would measure intracellular sodium *in vivo*.

Due to its noninvasive nature and relatively high sensitivity, ^{23}Na nuclear magnetic resonance spectroscopy is an attractive method for *in vivo* applications. Unfortunately, intracellular (IC) and extracellular (EC) sodium cannot be easily distinguished by NMR without the aid of a paramagnetic shift reagent (SR) (2–4). Recently, the thulium (III) complex of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis (methylenephosphonate) ($TmDOTP^{5-}$) has been successfully used to produce baseline-resolved IC and EC ^{23}Na resonances in the *in vivo* rat liver at relatively low doses (5). $TmDOTP^{5-}$ does reduce mean arterial pressure when infused into live rats (presumably due to chelation of some Ca^{2+}) but does not alter heart rate, blood gases, or electrolytes (6–8). Although this SR allows a qualitative observation of IC and EC ^{23}Na in biological tissues, a quantitative determination of $[Na^+]$ in the EC and IC compartments remains elusive because the volumes of the two tissue spaces detected by the NMR coil is unknown. In a recent study, Clarke *et al.* (9) quantified the molar IC concentrations of several phosphorus metabolites in the rat heart using ^{31}P NMR spectroscopy and phosphonate markers. Given its distribution in both the EC and IC compartments (10, 11), dimethyl methylphosphonate (DMMP) was used as a marker of total water space while phenylphosphonic acid (PPA) labeled only the EC volume (12, 13). These same phosphorus markers have been applied to other organs with different results. In the case of the liver, it has been demonstrated that PPA is not an accurate EC marker since it partially enters the IC compartment (14). Recently, we have used the ^{31}P resonance of $TmDOTP^{5-}$ to mark the EC space in the *in vivo* rat liver (15). However, this compound is less convenient to use than diamagnetic compounds because the ^{31}P signal of the complex is shifted far upfield (-350 ppm) from tissue phosphorus metabolites (-20–10 ppm) due to the strong paramagnetic effect of the thulium ion. In this work, the complex formed between the diamagnetic ion La^{3+} and DOTP ($LaDOTP^{5-}$ (^{31}P chemical shift = 21.5 ppm)) was used to mark the EC space.

The present study focused on the use of phosphonate markers and ^{31}P NMR spectroscopy to measure tissue volumes and SR-aided ^{23}Na NMR spectroscopy to determine the IC sodium concentration in the isolated and perfused mouse liver. Identical results were obtained independently by atomic absorption spectroscopy, thereby verifying that IC sodium is 100% visible by ^{23}Na NMR in the perfused mouse liver.

MATERIALS AND METHODS

Liver Perfusion

Fed FVBN male mice (n = 9) with an average body weight of 35 g were used in this study. The animals were anesthetized by intramuscular injection of 1 mg of ketamine·HCl (Ketaset, Aveco, Fort Dodge, Iowa) using a protocol approved by the University's Animal Care Committee. Before removal of the livers, 50 UI heparin (Elkins-Sinn, Cherry Hill, NJ) was injected intraperitoneally to prevent formation of intravascular clots. The liv-

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ers were then isolated using techniques previously described (16). Briefly, the livers were perfused through the portal vein with a slightly modified phosphate-free Krebs-Henseleit solution kept at 37°C in a water-bath and continuously saturated with carbogen (95% O_2/5% CO_2) to give a final pH of 7.4. The composition of the buffer was NaCl 118 m*M*, KCl 4.7 m*M*, $MgSO_4.7H_2O$ 1.2 m*M*, $CaCl_2.6H_2O$ 4.0 m*M*, Na_2EDTA 0.5 m*M*, $NaHCO_3$ 25 m*M*, and glucose 5.5 m*M*. EDTA was used as a chelator of free paramagnetic ions that could affect the quality of the NMR spectrum. Thus, $[Na^+]_{total}$ in this buffer was 144 m*M*. Each liver was first perfused with non-recirculating buffer for 10 min to completely remove blood from the vessels. Thereafter, 5 m*M* DMMP and 0.75 m*M* $LaDOTP^{5-}$ were added to the perfusion fluid as respective markers of the total and EC volumes, and 100 ml of buffer was recirculated continuously.

NMR Measurements

NMR spectra were collected using an 11.75 T Bruker GN-500 spectrometer with livers centered in an 18-mm thin-walled tube and bathed in the perfusion medium. A spherical external reference bulb containing 120 μl of 0.1 *M* methylphosphonic acid, pH 10 (MPA, Aldrich, Milwaukee, WI), 0.85 *M* Na^+, and 40 m*M* $TmDOTP^{5-}$ was positioned in the homogeneous region of the coil so that both the liver and the bulb were entirely within the coil. The magnet was shimmed using the ^{23}Na FID. The sweep widths were 12 and 8 kHz for ^{31}P and ^{23}Na, respectively. 8K data points were used for the observation of both nuclei. The data were processed using NUTS NMR data processing software (NUTS, Acorn NMR, Fremont, CA). All ^{31}P and ^{23}Na FIDs were baseline-corrected and a 20-Hz line broadening was applied to the ^{31}P FIDs. Longitudinal relaxation times of the phosphorus nuclei of DMMP and $LaDOTP^{5-}$ in the perfusion fluid and of MPA in the reference were measured at 202.42 MHz and at room temperature using a standard inversion-recovery sequence.

Experimental Protocol

A partially saturated ^{31}P spectrum was acquired using a 1 s repetition delay (*TR*), 45° flip angle (θ), and 600 scans (NA) after the stabilization period following surgery and at the end of each experiment to check the metabolic status of each liver. The concentration of hepatic ATP was determined from the relative areas of the β-ATP and MPA resonances (17). IC pH was determined from the chemical shift of inorganic phosphate.

A single, fully relaxed ^{31}P spectrum (*TR* = 60 s, θ = 45°, and NA = 60) was also collected on each liver to evaluate tissue volumes. Given the known volume of the reference bulb and the concentration of MPA in the bulb and the respective concentrations of DMMP and $LaDOTP^{5-}$ in the perfusion fluid, the total (V_T) and EC (V_{ECS}) space volumes were easily determined from integration of the marker resonances. The volume of the IC space (V_{ICS}) was calculated as the difference between V_T and V_{ECS}. After the acquisition of the fully relaxed ^{31}P spectrum, 5 m*M* $TmDOTP^{5-}$ was added to the perfusion fluid. Thirty ^{23}Na spectra (*TR* = 0.3 s, θ = 45°, and NA = 200) were recorded over the next 30 min. The amount of sodium in the IC and EC spaces was calculated from the area under the respective resonances on the SR-resolved ^{23}Na spectrum with respect to the known amount of sodium in the external reference bulb. The amount of IC sodium determined by ^{23}Na NMR (in μmol) divided by the IC volume (in ml) provided a direct measure of $[Na]_{IC}$ (in m*M*).

To compare the tissue distribution of DMMP, PPA, and $LaDOTP^{5-}$, livers were perfused with DMMP (10 m*M*), PPA (5 m*M*), and $LaDOTP^{5-}$ (1 m*M*) for 60 min to allow equilibration of the compounds in the NMR chamber and through the various spaces of the liver. Thereafter, the liver was rinsed with non-recirculating fresh buffer to remove the phosphorus markers from the tissue. A partially saturated ^{31}P spectrum (*TR* = 1 s, θ = 45°, and NA = 300) was acquired every 5 min during the last 30 min of the equilibration period and during the washout period.

Atomic Absorption Spectroscopy (AAS)

A perfusion fluid sample was collected at the end of each experiment and diluted 1:50 into 25000 ppm KCl solution for thulium and sodium determinations by AAS. At the conclusion of each experiment, livers were weighed wet, dried overnight at 67°C, reweighed, and digested in 2 ml of a mixture of concentrated nitric, sulfuric, and perchloric acids. After an overnight digestion at 60°C, the liquids were diluted to 10 ml in distilled water and 2 ml of these latter solutions were mixed with 5 ml of the 25000 ppm KCl solution. The samples were analyzed on a Varian-Spectr AA-5 atomic absorption spectrometer for Tm (372.2 nm) and Na (330.2 nm).

The procedure used to evaluate $[Na]_{IC}$ was essentially that described by Scheufler and Peters (18). Assuming that $TmDOTP^{5-}$ distributes only in the EC spaces, V_{ECS} was determined by dividing the amount of Tm detected in the dried tissue (μmol) by the known concentration of $TmDOTP^{5-}$ (m*M*) in the perfusion fluid. Total tissue water volume (V_{TOT}) is given by the difference between tissue wet weight (WW) and dry weight (DW) and, consequently, the volume of the IC space was determined as, $V_{ICS} = V_{TOT} - V_{ECS}$ (18). The amount of total tissue sodium as measured by AAS was then used to evaluate $[Na]_{IC}$ using the following relationship,

$$\text{tissue Na}(\mu\text{mol}) = V_{ECS}[Na]_{\text{perfusate}} - V_{ICS}[Na]_{IC}$$

RESULTS AND DISCUSSION

Distribution of DMMP, PPA, and $LaDOTP^{5-}$ in the Perfused Mouse Liver

The lanthanum (III) complex with DOTP was chosen to mark the EC spaces because the ^{31}P chemical shift of this complex (~21 ppm) is similar to the other phosphorus markers and hepatic metabolite resonances yet does not overlap. Figure 1 shows the evolution of the ^{31}P spectrum of an isolated mouse liver perfused with 10 m*M* DMMP, 5 m*M* PPA, and 1 m*M* $LaDOTP^{5-}$ during the last 30 min of an equilibration period and during washout of the EC space with fresh buffer. The spectrum shows the resonances from DMMP (41.7 ppm), $LaDOTP^{5-}$ (21.2 ppm), PPA (15.0 ppm), sugar-phosphates (6.6 ppm), inorganic

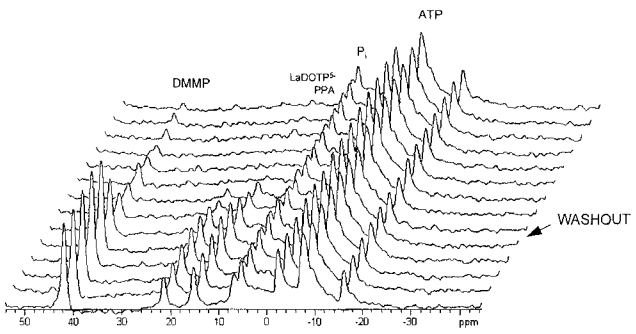

FIG. 1. Evolution of the ^{31}P NMR spectrum of an isolated mouse liver during the perfusion with DMMP 10 m*M* (41.7 ppm), LaDOTP^{5-} 1 m*M* (21.2 ppm), and PPA 5 m*M* (15.0 ppm). Each spectrum acquisition required 5 min (*TR* 1 s/NA 300).

phosphate (5.2 ppm), γ-ATP (-2.5 ppm), α-ATP (-7.6 ppm), and β-ATP (-16.2 ppm).

After 30 min of perfusion, the phosphorus markers reached their equilibrium distribution in the various spaces inside the NMR chamber, and the area under the peaks corresponding to each marker did not evolve further. At the end of the equilibration period, the perfusion line was switched to a fresh buffer free of phosphorus compounds. As expected, the intensity of the peaks corresponding to the three markers decreased progressively with time. Both PPA and LaDOTP^{5-} showed similar washout kinetics, suggesting that the tissue distribution of the two compounds was similar. Since TmDOTP^{5-} is known to remain in the EC space (15), it is reasonable to assume an EC distribution for both PPA and LaDOTP^{5-}. However, Foster *et al.* (14) detected twice as much radiolabeled ^{14}C PPA in rat liver *in vivo* as ^{3}H sucrose, suggesting that the aromatic phosphonate partially accumulated in liver. Our results in the isolated mouse liver perfused with a blood-free, KH buffer suggest that the IC fraction of PPA, if any, does not contribute significantly to the ^{31}P NMR spectrum. There are several significant differences between our experiments and those reported by Foster *et al.* (14): (1) the radiolabeling experiment is certainly more sensitive than NMR and is capable of sensing much lower levels of PPA, (2) the animals were nephrectomized in the *in vivo* experiments (14) and that procedure could modify the elimination route of PPA, and (3) albumin present in blood could aid in hepatic uptake of PPA. It is possible that a small fraction of PPA was taken up by hepatocytes in our perfused liver experiments but was quickly excreted into the bile during the washout period. Complete clearance of PPA and LaDOTP^{5-} took ~15 min. With a flow rate of 10 ml/min, this corresponded to a volume of 150 ml required to replace the fluid in the incoming perfusion line, including the bubble trap.

Conversely, washout of DMMP seemed to occur in two phases. First, the DMMP peak decreased quite quickly, at a rate similar to that seen for PPA and LaDOTP^{5-}. This likely corresponds to removal of the vascular fraction of DMMP. This was followed by a slower elimination period that continued long after the disappearance of both PPA and LaDOTP^{5-}. This second phase likely reflects biliary excretion of DMMP. After 45 min of washout, a significant fraction of DMMP was still present in the IC space. From these observations, we concluded that DMMP was indeed seeing all tissue space as expected while both PPA and LaDOTP^{5-} appeared to only distribute in the EC space in the isolated, perfused liver. However, since there is evidence in the literature for PPA entering hepatocytes *in vivo*, we used LaDOTP^{5-} as an EC space marker in all subsequent experiments.

Evaluation of the Metabolic Status of the Liver

Figure 2 shows a partially saturated ^{31}P spectrum of an isolated mouse liver at the onset of a perfusion experiment with LaDOTP^{5-} and DMMP (top) and at the conclusion of the experiment after collection of 30 ^{23}Na spectra (bottom). The ^{31}P resonance at 24.2 ppm corresponded to MPA in an external reference bulb.

At the beginning of the perfusion, hepatic [ATP] and IC pH were 7.7 ± 1.3 μmol/liver (4.1 m*M*) and 7.25 ± 0.11, respectively. At the end of the experiment 80 min later, these values were 6.6 ± 1.1 μmol/liver (3.3 m*M*) and 7.30 ± 0.12. As previously shown by Nedelec *et al.* (19) for the *in vivo* liver, our data confirm that these phosphorus markers are nontoxic in the isolated, perfused mouse liver. Our results also support those of Bansal *et al.* (5) who reported no significant effects of TmDOTP^{5-} on liver physiology *in vivo*.

Determination of the Volumes in the Total, EC, and IC Spaces

The ^{31}P longitudinal relaxation times of DMMP and LaDOTP^{5-} at 202.4 MHz in a liver perfused with Krebs-Henseleit buffer were 14.4 ± 0.1 s and 1.9 ± 0.1 s, respectively. Given these values, we used a 45° observe pulse and a 60 s repetition delay during collection of fully relaxed ^{31}P spectra to determine the saturation factors for the DMMP and LaDOTP^{5-} signals that would allow a calculation of volumes from spectra such as those shown in Fig. 2.

The average wet and dry liver weights were found to be 1.7 ± 0.3 g and 0.5 ± 0.1 g, and the D/W weight ratio was about 30%. The total volume seen by the coil and the volumes of the EC (vessels + Disse's space + liquid bathing the liver in the NMR chamber) and IC spaces determined by ^{31}P NMR were 6.7 ± 0.9, 5.5 ± 0.4, and 1.2 ± 0.2 ml, respectively. In comparison, the volumes of the EC (vessels + Disse's space) and IC spaces measured by AAS (using TmDOTP^{5-} as an ECS marker) were 0.3 ± 0.1 and 1.3 ± 0.2 ml, respectively. Thus, the IC volumes as measured by NMR and AAS were not significantly different.

Determination of the EC and IC Sodium Concentrations in the Perfused Mouse Liver

Figure 3 displays the evolution of the ^{23}Na spectrum from an isolated mouse liver perfused with 5 m*M* TmDOTP^{5-}.

In this series, the first spectrum was acquired before addition of the SR to the perfusion fluid. The large peak, arbitrarily set to 0 ppm, reflects both the EC and IC ^{23}Na, while the small peak at 15 ppm corresponds to sodium chloride in the reference bulb shifted downfield by excess SR. After the addition of SR to the perfusion medium, the EC ^{23}Na signal progressively moved downfield and stabi-

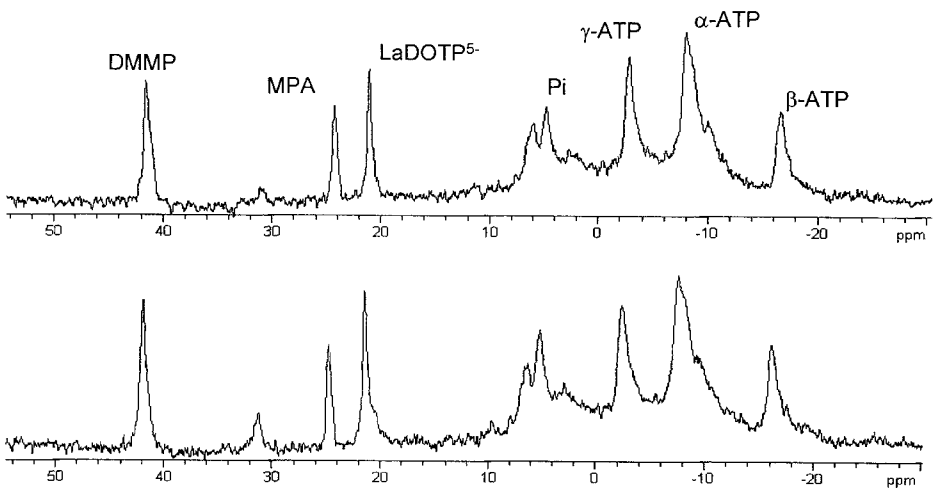

FIG. 2. Partially saturated ^{31}P NMR spectra of an isolated mouse liver after equilibration with DMMP 5 m*M* and $LaDOTP^{5-}$ 2.5 m*M* (top) and just before concluding each experiment 80 min later (bottom). Each spectrum acquisition required 10 min (*TR* 1 s/NA 600).

lized after the concentration of $TmDOTP^{5-}$ in the perfusate had equilibrated (~10 min). It should be noted that the IC ^{23}Na peak at 0 ppm remained unshifted throughout the equilibration period, demonstrating that $TmDOTP^{5-}$ did not cross the plasma membrane of hepatocytes. After equilibration, the EC and IC ^{23}Na resonances were resolved by 7 ppm. This may be compared with chemical shift differences between EC and IC ^{23}Na resonances in an *in vivo* liver using this same SR (5), where a chemical shift difference of 7 ppm corresponded to a blood concentration of about 7 m*M* (15). In separate experiments (unpublished), we also measured the concentration of free calcium in the perfusion fluid before and after addition of $TmDOTP^{5-}$. $[Ca]_{free}$, as detected by an ion specific electrode, dropped from 4 to 1.2 m*M* after the addition of 5 m*M* SR to the perfusion medium. This concentration of $[Ca]_{free}$ is comparable to that measured in blood from a live animal, with or without SR. This indicates that less SR is required to separate EC and IC ^{23}Na resonances in an isolated liver perfused with KH buffer than that required *in vivo*, perhaps due to interactions between the SR and other cationic compounds present in blood.

The amount of sodium in the EC and IC spaces was determined by integration of their respective resonance areas relative to the amount of sodium in the external reference. Given these amounts and the volumes determined previously by ^{31}P NMR, the sodium concentrations in the EC and IC spaces were easily calculated. Table 1 compares the data obtained from the NMR experiment to the values measured by AAS for eight separate livers.

As expected, the AAS results for the $[Na]_{EC}$ are close to the total sodium concentration in the buffer (159 m*M*), i.e., 144 m*M* from the Krebs-Henseleit solution plus 15 m*M* from the extra sodium added with the SR. The NMR measured values were not significantly different from those measured by AAS. Similarly, no significant differences were observed for $[Na]_{IC}$ as measured by NMR and AAS. Moreover, an average $[Na]_{IC}$ of 29 m*M* (as determined by NMR) for the perfused rat liver is quite comparable to values reported in the literature for liver *in vivo*. Blum *et al.* (20) estimated a value 26 ± 7 m*M* for $[Na]_{IC}$ from ^{23}Na NMR spectra of *in vivo* rat livers after infusion of the sodium SR, $DyTTHA^{3-}$. Even though the IC and EC ^{23}Na signals were not well resolved and several simplifying assumptions were made, i.e., that the IC and EC volumes did not change postmortem, the value for $[Na]_{IC}$ found by Blum *et al.* (20) is essentially identical to the value we report here for the perfused liver. Somewhat later, Bansal *et al.* (5) estimated a value for $[Na]_{IC}$ of 19 ± 5 m*M* from ^{23}Na spectra of *in vivo* rat liver that showed base-line resolution of IC and EC ^{23}Na signals using the same SR as used here, $TmDOTP^{5-}$. However, their calculations were based upon a literature value for the V_{ICS}/V_{ECS} ratio that may not have been appropriate for those particular experimental conditions. The value of $[Na]_{IC}$ that we found here also agrees well with literature values found for the *in vivo* liver using the EC space markers chloride (21) and inulin (22). More recently, Makos (15) used $TmDOTP^{5-}$ as an SR (for NMR measurements) and as an EC space marker (for AAS measurements) and found values for $[Na]_{IC}$ of 32 ± 2 m*M* for the *in vivo* rat liver. The close agreement of value we determined here for the perfused mouse liver and the previous *in vivo* values indicates that our perfused liver preparation was physiologically stable.

CONCLUSIONS

Phosphorus markers and ^{31}P MRS have been used previously to determine IC and EC volumes in the isolated and perfused rat heart (9). The Na^+ shift reagent, $TmDOTP^{5-}$, has also proven to be quite efficient *in vivo* for separating EC and IC ^{23}Na NMR resonances in various organs (4–8).

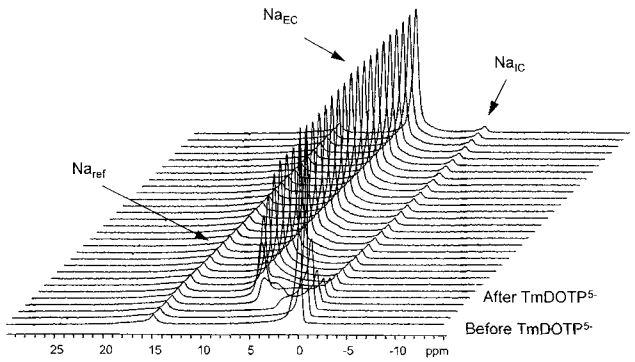

FIG. 3. ^{23}Na spectrum of an isolated mouse liver perfused with 5 m*M* $TmDOTP^{5-}$. The shift reagent was added to the perfusion fluid at the end of the first spectrum. Each spectrum acquisition required 1 min (*TR* 0.3 s/NA 200).

Table 1
Comparison of the EC and IC Sodium Concentrations in the Isolated and Perfused Mouse Liver Determined by NMR and AAS

Liver	$[Na]_{EC}$ (m*M*) NMR	$[Na]_{EC}$ (m*M*) AAS	$[Na]_{IC}$ (m*M*) NMR	$[Na]_{IC}$ (m*M*) AAS
1	162.3	162.1	24.1	21.7
2	175.0	156.8	31.8	40.2
3	173.0	168.1	29.5	35.5
4	160.0	157.3	28.0	35.0
5	138.8	162.7	27.4	27.6
6	150.3	167.6	38.8	28.2
7	140.5	165.4	23.4	25.7
8	157.0	162.2	29.1	42.7
Mean±SEM	157.1 ± 5.5	163.5 ± 4.5	29.0 ± 5.2	32.1 ± 7.4

In this study, we have combined the two methods to quantify $[Na]_{IC}$ in the isolated, perfused mouse liver. DMMP proved to be well suited for the determination of total cell volume in the perfused liver. As one of the common phosphonate markers, PPA, has been reported to enter hepatocytes *in vivo* (14), we turned to another highly charged phosphonate ligand, DOTP. Recently, Makos (15) has demonstrated that $TmDOTP^{5-}$ can be used to determine the EC space volume in the rat liver *in vivo*. Although we have used the highly shifted ^{31}P resonance of this paramagnetic chelate as an EC space marker *in vivo* (15), it is much more convenient to use a marker whose chemical shift is in the same range as other phosphorus metabolites. Consequently, we turned to the diamagnetic analog, $LaDOTP^{5-}$, with a ^{31}P chemical shift of ~21 ppm for use as an EC space marker. The NMR sensitivity and relaxation characteristics of this chelate proved to be quite suitable for such measurements.

Finally, a comparison of values found here for $[Na]_{IC}$ as determined by ^{23}Na NMR and independently by AAS indicates that all IC sodium was detected in the NMR experiment, i.e., Na_{IC}^{+} is 100% visible by ^{23}Na NMR in the isolated, perfused rat liver. The reasons that previous investigations (20) have found that Na_{IC}^{+} was less than 100% visible by NMR are numerous, including instrument dead time and inability to fully resolve the IC Na^+ signal. However, our findings reported here as well as other recent observations (15) indicate that it is possible to detect all IC Na^+ in liver by ^{23}Na NMR. This suggests that ^{23}Na NMR could be quite useful for monitoring true changes in $[Na]_{IC}$ *in vivo*.

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