LETTER TO THE EDITOR

On the In Vivo Detection of Intracellular Water and Sodium by Nuclear Magnetic Resonance with Shift Reagents

Dear Sir:

In a recent paper, Naritomi et al. (1) describe studies in which the paramagnetic shift reagent dysprosium triethylenetetramine hexaacetic acid ($Dy(TTHA)^{3-}$) (2) is infused intravenously into gerbils, and subsequent proton and sodium in vivo spectroscopy is performed on brain and skeletal muscle. Before shift reagent infusion, one resonance (proton or sodium) is observed. After shift reagent infusion a resonance remains at the unshifted position, and one or two resonances are observed to be shifted downfield. The unshifted resonance is interpreted as the "intracellular" resonance, and the shifted resonances are interpreted to be the "extracellular" resonances. Several perturbations are performed, and the resulting spectra are interpreted as relative changes in "intracellular" versus "extracellular" protons (water) or sodium. The authors conclude that "the present paper thus confirms that in vivo separation of intra- and extracellular ^{23}Na and ^{1}H signals by the NMR method is possible by the application of $Dy(TTHA)^{3-}$ infusion and provides useful information for physiological and biochemical studies." Unfortunately, it has not yet been conclusively demonstrated that the resonances actually represent the intracellular or extracellular nuclei. In fact, previous studies utilizing shift reagents in vitro (on which their study is based) suggest that those assignments may not be totally correct.

Two characteristics of the spectra in the paper by Naritomi et al. (1) cast doubt on the assignments of intra- and extracellular resonances as such. The first is the presence of two or more resonances in the proton spectra. If shift reagent had indeed penetrated fully into the tissue, we might expect a single resonance at a position intermediate to the true intra- and extracellular positions (or the intravascular and extravascular-extracellular positions) due to the exchange of protons between these spaces. The sodium spectra are also inconsistent with in vitro studies with total shift reagent distribution into the tissue, because a $Dy(TTHA)^{3-}$ concentration large enough to produce a 6-ppm shift of the extracellular sodium would probably also shift the intracellular sodium slightly due to bulk susceptibility effects. Such a shift of the "intracellular" sodium resonance is not seen.

Examples of these effects are given in Figs. 1 and 2 in a system of a perfused heart. Proton and sodium spectra were obtained from a perfused rat heart with normal Kreb's Henseleit perfusion. The perfusate was then switched to Kreb's Henseleit modified with 10 mM $Dy(TTHA)^{3-}$ shift reagent, and the spectra were obtained again. Spectra were then obtained from the shift reagent perfusate alone.

As can be seen in Fig. 1, the addition of shift reagent to the perfused heart results in a shift of all of the proton and sodium signals. In the case of the proton spectrum, a shifted and broadened proton resonance is observed. Looking more closely, this could be a superposition of several proton resonances, with two main ones being at 1.76 and 1.43 ppm. The one at 1.76 ppm could be due to perfusate surrounding the heart, whereas the one at 1.43 ppm could represent the weighted average resonance of various intracellular, extracellular, and intravascular resonances. In the sodium spectrum, an intracellular sodium resonance, at ~1.2 ppm, is barely observable as a shoulder on the extracellular resonance. This intracellular resonance is more obvious after it increases due to ouabain exposure (Fig. 2). Note that the shift reagent concentration utilized here must be lower than that used by Naritomi et al. (1) because the shift of the extracellular resonance is lower; yet the intracellular resonance is still shifted in this case and not in the spectra shown in their paper. We have observed similar characteristics (a single shifted proton resonance and a shifted extracellular sodium resonance with a slightly shifted intracellular sodium resonance) of shift reagent spectra in other cellular systems including suspensions of red cells, in which the intra- and extracellular volumes are comparable and there is no question of full penetration of the shift reagent into the extracellular spaces.

Therefore, the assignments of the resonances as pure intracellular or pure extracellular resonances in the paper by Naritomi et al. (1) may not be correct. In reality there are probably tissues with different rates of exchange between the intra- and extracellular protons, and tissues which will have different susceptibility effects on the sodium signals. Until these effects are worked out for the in vivo situation, for the various types of tissues and vasculature, it is premature to place definitive labels on the spectra and draw physiologic conclusions.

Another possible explanation of spectra in the paper by Naritomi et al. (1) (which can also not be definitively proven from their data) is that there was a certain amount of bulk compartmentation of the shift reagent in vivo; the unshifted proton and sodium resonances would then be due to all of the nuclei in the region into which the shift reagent could not penetrate, and possibly some unshifted intracellular sodium in the regions of shift reagent penetration. The shifted resonances would be due to a weighted average of protons from the intra- and extracellular or intra- and extravascular regions in fast or intermediate exchange, and a combination of extracellular sodium and shifted intracellular sodium resonances in the sodium spectra.

It would not be surprising if the unshifted resonance reacted appropriately to the ouabain perturbation in the in vivo situation just described, in that ouabain should conceivably be able to penetrate regions more easily than the large, negatively charged shift reagent molecule, and there would be a bulk increase in the amount of sodium in regions of ouabain exposure. However, due to the large overlap of resonances in the sodium spectra, it is not clear that the increase seen by Naritomi et al. (1) is indeed in the resonance at 0 ppm; similar spectra could be obtained if there was an increase in signal around 1 ppm (a shifted intracellular resonance). Similarly, with the papaverine hydrochloride exposure, the most shifted resonance may indeed be associated with the intravascular compartment (even if it is a weighted average of

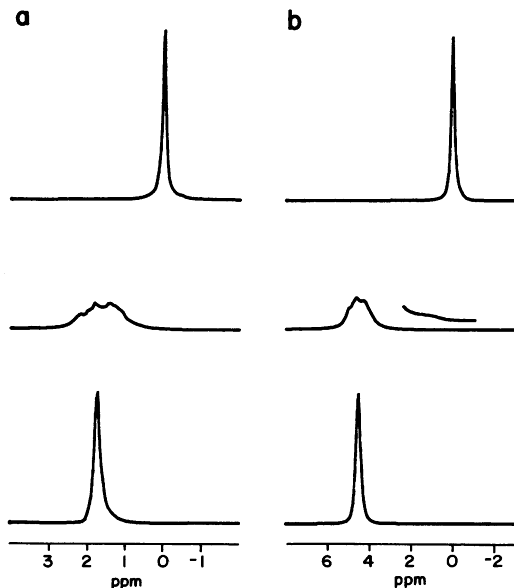

FIGURE 1 (*a*) Proton and (*b*) sodium, spectra obtained from (*top*) perfused heart with normal Kreb's Henseleit perfusate, (*middle*) heart perfused with Kreb's Henseleit perfusate modified with 10 mM $Dy(TTHA)^{3-}$, and (*bottom*) shift reagent perfusate alone. In the sodium spectrum of the shift reagent perfused heart, the region around 0 ppm is expanded vertically by a factor of 25 to illustrate the shoulder on the extracellular resonance which represents the intracellular resonance. The spectra were obtained with an 8.45 Tesla spectrometer (Bruker Instruments, Inc., Billerica, MA), operating at 360.13 and 95.26 MHz for proton and sodium, respectively. In each case, 0 ppm is defined as the position of the resonance in the Kreb's Henseleit perfused heart.

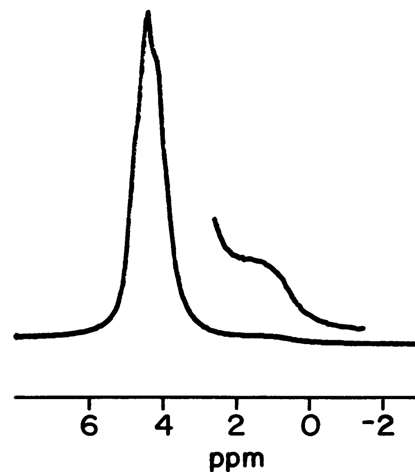

FIGURE 2. Sodium spectrum of a shift reagent perfused heart after ouabain exposure (~100 μM). The region around 0 ppm is expanded by a factor of 10 to better illustrate the intracellular resonance.

protons in the intravascular compartment and other compartments in various regions of exchange), and therefore it would be expected to respond as the authors observed.

In addition, the discussion by Naritomi et al. (1) relating to sodium "invisibility" contains inaccuracies. The references which are quoted as demonstrating invisible intracellular sodium are a combination of papers which described sodium invisibility in bulk tissue and papers describing invisibility of intracellular sodium. Some recent studies suggest total visibility of intracellular sodium in other tissue such as red blood cells (3), rabbit proximal tubules (4), and heart (5, 6). The authors also state, without references, that "... extracellular ^{23}Na is totally visible." To my knowledge, this issue is not yet resolved.

The ability to differentiate between intra- and extracellular water or sodium in vivo obviously has many possible applications for physiologic and medical studies. The relatively low toxicity of the $Dy(TTHA)^{3-}$ shift reagent makes it a promising tool in this regard. However, we must fully understand its physiologic compartmentation in vivo, the exchange rates for protons between various compartments in vivo, and in vivo susceptibility effects on the sodium signals before we can begin to label resonances as being totally intracellular, extracellular, or intravascular, and thus draw physiologic conclusions from these types of studies.

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