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Detection of Proton Chemical Exchange between Metabolites and Water in Biological Tissues

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Metabolites in proton chemical exchange with water were detected via the water proton signal using saturation transfer techniques in model systems and biological tissues. The metabolites were selectively saturated and the resulting decrease in the much larger water proton pool was used to monitor the metabolite. This indirect detection scheme can result in a several orders of magnitude increase in sensitivity for metabolites over direct detection methods. A control irradiation scheme was devised to compensate for macromolecular/water magnetization transfer. Using this approach, significant chemical exchange regions at ~1 and 2.5 ppm were detected in kidney medulla. Using a difference imaging technique between a control irradiation above (−1.74 ppm) and below (+1.74 ppm) the water resonance, a chemical exchange image of the kidney was calculated. These data revealed a linear gradient of chemical exchange increasing from the cortex to the medulla. Studies on medullary acid extracts and urine revealed that the exchange observed in the kidney was predominantly with low molecular weight metabolites. Urea (1 ppm) was identified as contributing to the kidney/urine chemical exchange; however, other unidentified metabolites may also contribute to this effect. These studies demonstrate that tissue metabolites can be detected and imaged via the water protons using the signal amplification properties of saturation transfer in the presence of water/macromolecule magnetization transfer. © 1998 Academic Press

Key Words: **urea; ammonia; saturation transfer; pH; urine; kidney; MRI; muscle; chemical exchange.**

INTRODUCTION

Proton chemical exchange between water and metabolites is a common biological process. Metabolite/water proton chemical exchange can range from fast to slow depending on the chemistry of the exchange sites, temperature, pH, and many other factors. NMR saturation transfer has been extensively used to evaluate proton and other nuclide chemical exchange, *in vitro* and *in vivo,* providing unique insights into a variety of chemical reactions (*1*, *2*). In addition, several strategies have been presented to image the distribution of chemical exchange using saturation transfer in the magnetization preparation period of an imaging sequence (*3*–*5*). Saturation transfer (ST) is most effective under slow to intermediate exchange conditions where the exchanging spins can be adequately resolved and sufficient exchange occurs between the molecules, relative to T_1, to detect a transfer. This limitation reduces the number of reactions that can be detected with ST; however, it may improve the specificity of this measurement in complex biological tissues. Studies using ST in the fast exchange domain have also proven useful *in vitro* using a model fitting routine (*6*).

Previous studies showed that by saturating the exchangeable protons on small metabolites (i.e., ammonia) the associated decrease in water proton intensity results in orders of magnitude higher sensitivity when compared to direct detection (*5*). This increase in sensitivity occurs because even a 1% decrease in the ~100 M water signal with metabolite saturation results in a 1 M water proton signal associated with the metabolite. The increase in signal-to-noise using this indirect detection scheme was shown to be adequate to image the distribution of low-concentration metabolites *in vitro.* However, no information on the use of this approach in intact tissue has been provided.

The purpose of this study was to explore the use of ST for the measurement and imaging of proton chemical exchange between metabolites and water in biological tissues. There are numerous problems in attaining this goal as pointed out in earlier studies (*5*). The magnetization exchange between the broad proton resonance in many macromolecules and water (*7*) has a very wide frequency bandwidth that will interfere with transfer associated with a metabolite. In addition, the potential spectral overlap and near-fast exchange rates of many metabolites may make the specific assignment of a detected exchange difficult. Environmental conditions such as pH, ionic composition, and temperature may also alter the metabolite exchange rate, complicating the detection or quantitation of metabolites. The focus of this work was threefold: (1) to evaluate methods of compensating for the broad macromolecule/water magnetization transfer in ST detection of proton chemical exchange between water and free metabolites, *in vitro;* (2) to determine if significant metabolite chemical exchange can be detected in biological tissues and begin to identify the molecules involved;

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and (3) to attempt to image the distribution of metabolite/water proton exchange in tissues.

To accomplish these goals, experiments were performed on model systems to validate techniques as well as numerous biological tissues.

MATERIALS AND METHODS

Model System

The model system consisted of a 3% purified agarose gel made up in saline with a 25 mM phosphate buffer (Sigma Chemical Co., St. Louis, MO). Agarose has been previously shown to provide a large magnetization transfer effect with water similar to biological tissues (*8*). Different concentrations of ammonia (10, 100, 300, and 500 mM) in agarose were prepared to determine the effects of macromolecule magnetization transfer on the detection of chemical exchange between water and ammonia. Experiments were also conducted in the absence of agarose. To optimize the chemical exchange processes, the pH was adjusted to 5 (*5*). The pH of the gel was confirmed using a universal optical pH indicator included in the gel (Fisher Scientific Co., Fair Lawn, NJ). A pH of 5 will not significantly affect the magnetization transfer between agarose and water (*8*).

Tissue Preparation

Male Sprague–Dawley rats (350–400 g: Taconic, Germantown, NY) were anesthetized with an intraperitoneal injection of 0.8 mg ketamine (100 mg/ml), followed by a 1.5-ml injection of heparin (1500 units/ml IP: AJ Buck, Owens Mills, MD) and decapitation. Rabbit kidneys were obtained from male New Zealand White rabbits (2.0 to 4.0 kg: Hazelton Research Products, Denver, PA). The rabbits were initially anesthetized with an intramuscular injection of a mixture of ketamine/acepromazine (180 mg/2 mg), intubated, and placed on a Siemens 900c ventilator (Siemens Medical Systems, Danvers, MA). Anesthesia was maintained with 2% isoflurane. Animals were sacrificed with 6 meq of KCI IV. Animal tissues were harvested and samples placed into 5-mm tubes for NMR spectroscopy studies, with the exception of some rabbit kidneys which were used for MRI experiments; see next section. All experiments were conducted at room temperature.

Perchloric acid (PCA) extraction was used to precipitate the large molecular weight proteins from the tissue homogenates. PCA extraction was performed by mixing together a 15% PCA solution with an equal volume of tissue homogenate at 5°C. After centrifugation, the supernatant of this mixture was neutralized and the PCA precipitated out with KOH. After another centrifugation the supernatant was used as the final protein free sample.

NMR METHODS

The spectroscopic experiments were conducted on a Bruker AC 300 spectrometer with an observe frequency of 300.13 MHz. The observation frequency was set on the water peak and the decoupler was used to apply the off-resonance saturation. Studies were conducted under steady-state conditions with the irradiation time three times the measured T_1 ($\sim$15 s). The chemical shift of the exchanging protons was determined via direct observation or by sweeping the irradiation frequency from +333 to −333 ppm from water as previously described for detecting the enzyme intermediates in phosphorus exchange experiments (*9*). Frequency steps were varied depending on the experimental goals and results. Typically, these data were plotted in the form of water amplitude versus irradiation frequency at a constant irradiation power. These action spectra of the off-resonance irradiation are referred to as magnetization transfer spectra. Once the irradiation frequency for the metabolite had been determined, the irradiation power was varied to find the optimal power setting at the metabolite resonance frequency relative to the control irradiation on the opposing side of the water resonance. This generally resulted in irradiation B_1 fields on the order of 1×10^{-6} T. Specific irradiation field strengths are provided in the figure legends. These experiments, in the presence and absence of agarose or ammonia, provided the data to devise a strategy for compensating for the macromolecule magnetization transfer in the metabolite chemical exchange detection.

The spin–lattice relaxation T_1 was measured with an inversion recovery experiment on the Bruker system. The range of inversion delays (T_i) was between 0.001 and 27 s, with a 15-s predelay. The water peak amplitudes were fitted to a single exponential to determine T_1. The T_1 in the presence of metabolite saturation (T_{1sat}) was determined to estimate the rate of chemical exchange. T_{1sat} was measured with an inversion recovery experiment with irradiation of the metabolite. In these experiments the magnetization was prepared with a 15-s off-resonance irradiation before beginning the inversion recovery. After the 180° pulse the irradiation was again applied to maintain the spin saturation during the inversion recovery time. The range of T_i values for these experiments were the same as those given above.

Magnetic Resonance Imaging

Rabbit kidneys were inserted into a 25-mm NMR tube and placed in a MRI microscope system. Experiments were performed at 500 MHz on a Bruker 500 DX equipped with imaging gradients. A gradient recalled echo acquisition sequence was modified to include an off-resonance presaturation pulse with the decoupler channel. This pulse was 5 s, 1.5×10^{-6} T, and $\pm$1.74 ppm from the water protons. The power and frequency was determined from previous spectroscopic studies on kidney samples at this frequency. An irradiation

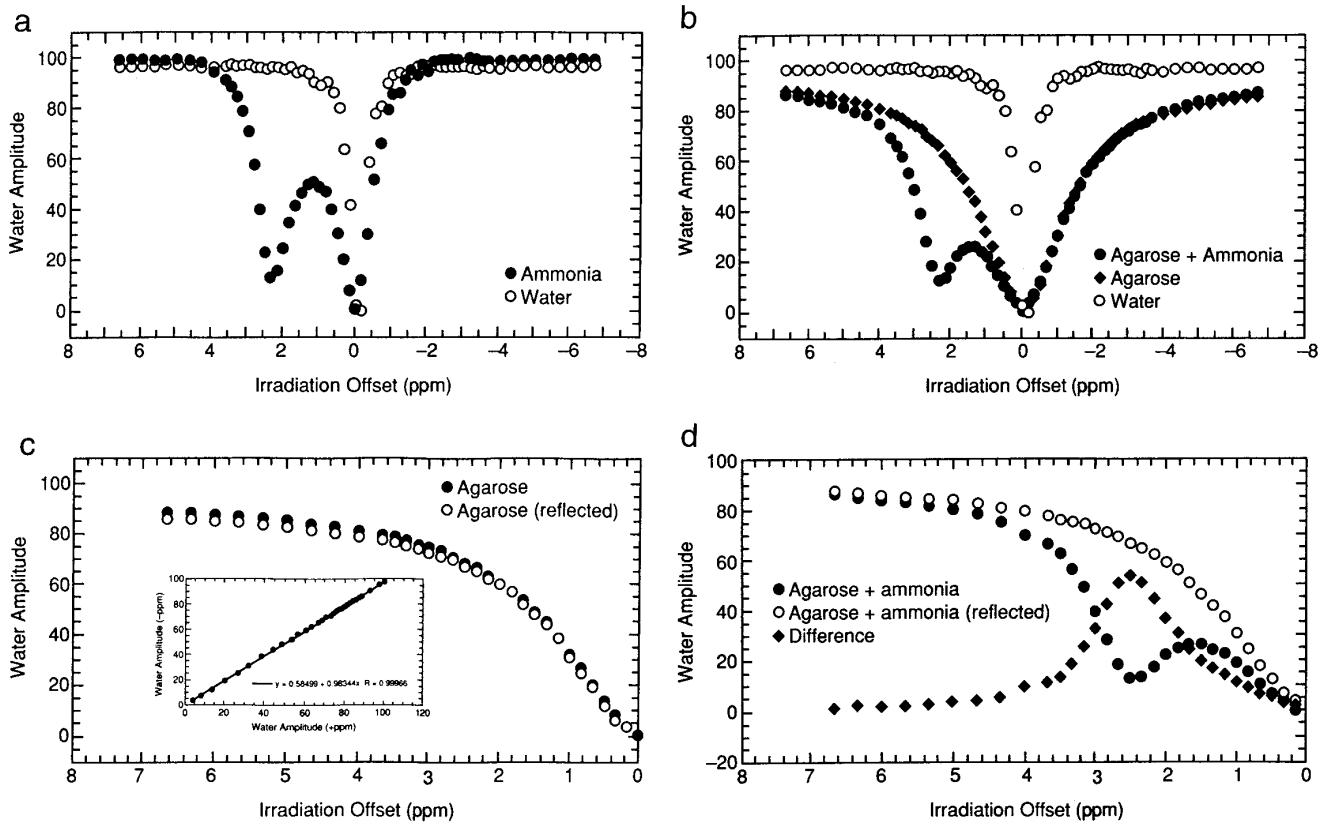

FIG. 1. Magnetization transfer spectrum of model systems. (a) Pure water and water plus 500 mM ammonia at pH 5.0. (b) Tap water, 3% agarose with 500 mM ammonia at pH 5.0. (c) Data from (b) where the higher frequency irradiation data is reflected on the lower frequency data around the water resonance frequency. The insert plot is the linear regression of these two data sets arising from the irradiation above and below the water resonance ($r^2 > 0.99$). (d) Data from (b) where the same treatment as in (c) was applied to the agarose–ammonia data. The difference spectrum is the higher frequency irradiation data—the lower frequency data. This is the same convention used in the remaining difference spectra presented in this manuscript.

frequency of ± 1.74 ppm provided the largest difference in the MT spectra at 500 MHz. The images were collected with the following parameters: FOV 25 mm, 128×128 digital resolution, 4 averages per phase encode, TR = 7 s and TE = 3.16 ms.

RESULTS

A magnetization transfer spectrum for pure water and a solution of 500 mM ammonia (pH 5) is presented in Fig. 1a. Zero ppm corresponds to the water resonance frequency where water was directly irradiated and the amplitude is reduced to near zero. The second minimum in the ammonia sample, at approximately 2.4 ppm, corresponds to the ammonia resonance. The selective decrease in water proton magnetization at 2.4 ppm irradiation is due to the chemical exchange of ammonia protons with the water. The same experiment performed in the presence of agarose is shown in Fig. 1b. Despite the broadening of the magnetization transfer spectrum due to both magnetization transfer with agarose and changes in water relaxation times, the ammonia effect can still be detected as a specific decrease in the 2.4-ppm region.

We noted that agarose magnetization transfer spectrum was highly symmetrical around the water resonance. To emphasize this effect, the data points from irradiation frequencies above water were reflected around the water resonance onto the lower frequency irradiation points (Figs. 1c and 1d). The overlap of the reflected data suggest a symmetry around the water resonance. Performing a linear regression on these two data sets results in a slope of 0.98 and a R value >0.999. Finally, by performing a subtraction between the lower and higher frequency data, the spectral characteristics of the asymmetry can be directly accessed (Fig. 1d). Using this approach, the ammonia contribution was found to be at ~ 2.4 ppm. These results suggested that a control irradiation equal in frequency above the water resonance should provide an adequate control for the agarose or macromolecular effects on the lower frequency irradiation. The method of reflecting the high frequency irradiation data on the lower frequency data and difference spectra of this data set will be the standard presentation of the magnetization transfer spectra throughout the remainder of this paper.

We attempted to quantify the concentration of ammonia in this system by estimating the pseudo-first-order rate constant

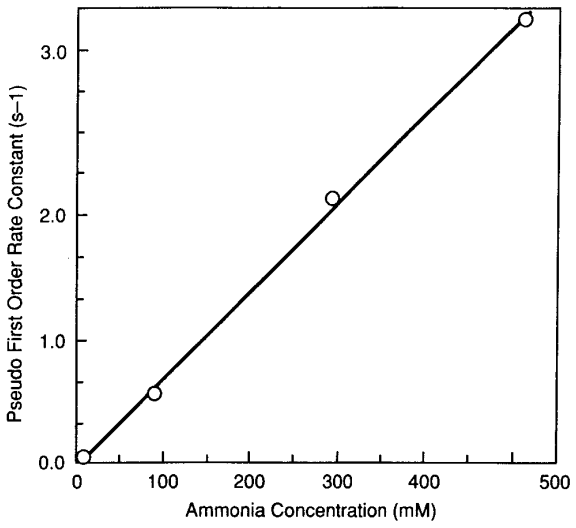

FIG. 2. The calculated first-order rate constant for ammonia water exchange versus the concentration of ammonia in 3% agarose at pH 5.0. The line is fit by linear regression with $r^2 > 0.99$.

for the ammonia water exchange rate to compensate for changes in the relaxation rate. Since the macromolecule was being equally irradiated on either side of the water resonance, we used the same approach presented by Ugurbil (*10*) to reduce a multisite exchange problem to a simple two-site exchange process. Requirements for this simplification include (1) that the macromolecule be equally saturated in control and experimental conditions; (2) that fraction of total spins in the metabolite pool be small relative to the water pool of protons; and (3) that the spin–lattice relaxation of the metabolite protons be identical to the spin–lattice relaxation rate of water, which implies that the lifetime of the metabolite proton is dominated by its rate of conversion to water, and not by its own spin–lattice relaxation process. In these samples all of these requirements were met. First, the macromolecule effect was symmetrical around water, resulting in identical saturation conditions with the control and experimental irradiation periods (Fig. 1c). The water proton pool is much larger that the metabolites. The T_1 of 100 mM ammonia was found to be indistinguishable from that of water at 2.81 s. Using this approach this multisite exchange process reduced to a two-site exchange process (*1*),

$$k_A = 1/T_{1sat}(1 - Ms/Mo), \qquad (1)$$

where k_A is the pseudo-first-order rate constant for water chemical exchange with ammonia, T_{1sat} is the T_1 in the presence of ammonia (and macromolecule) saturation, Mo is the water signal with macromolecular saturation, and Ms is the water signal with ammonia and macromolecule saturation. Figure 2 is a plot of k_A as a function of $[NH_3]$ in 3% agarose using this approach. T_{1sat} was determined as described in the Methods section. The pseudo-first-order rate constant was linear with $[NH_3]$ and was quantitatively similar to previous studies in solution in the absence of agarose (*5*). Varying the agarose concentration from 1.5 to 5% had no effect on the calculated k_A (not shown), suggesting that the measurement was independent of the degree of macromolecular magnetization transfer with water. The limitations of this quantitative approach will be further reviewed in the Discussion section.

At physiological pH values, we reasoned that most of the detectable exchange processes would be in amines, amides, or ammonia with exchangeable protons with resonant frequencies in the range 3 to 1 ppm from water. Thus, an asymmetry in the magnetization transfer spectrum, with more water saturation occurring with the low-frequency irradiation, was initially taken as a method of screening different biological tissues for metabolite/water chemical exchange. Experiments were conducted on the rat brain, muscle, spleen, heart, and liver, as well as the cortex and medulla of the rabbit kidney. Some of these data are presented in Figs. 3 and 4. The largest asymmetry in the magnetization transfer spectra was observed in the rabbit kidney medulla (Fig. 4c). A significant effect was also observed in the heart. The kidney cortex, liver, and brain showed an asymmetry with irradiation frequencies >5 ppm from water.

To better visualize the frequency dependence of the asymmetry, difference spectra between the data collected with irradiation above and below the water resonance were evaluated. As seen in Fig. 4a the heart had a maximum at $\sim$4 ppm from water. The kidney medulla had a more diffuse effect in the magnetization transfer spectrum with maximum effects at $\sim$2.6 and $\sim$1 ppm from water (Fig. 4b). These chemical shifts are not outside of the chemical shift of nitrogen containing metabolites found in urine (*11, 12*) and may correspond to ammonia and urea, respectively. Because of the large effect in the renal medulla and high concentration of metabolites, we concentrated our efforts to further characterize this effect in this tissue.

To ensure that the magnetization transfer spectrum asymmetry was due to free metabolites, and not due to an asymmetry in the macromolecule magnetization transfer characteristics, PCA extracts of the medulla were analyzed. A PCA extract will not contain the denatured proteins and structural elements. The magnetization transfer spectrum of the PCA extract revealed almost the identical asymmetry in the magnetization transfer spectrum (Fig. 5) as detected in the intact medulla with maxima at $\sim$2.4 and $\sim$0.9 ppm. Note that the macromolecular contribution to the transfer has been eliminated and the off-resonance irradiation effect is essentially gone above 6 ppm. These data suggest that the asymmetry in the magnetization transfer spectrum is due to the small metabolites concentrated in the medulla and not due to macromolecular structures.

Since the urine contains many of the metabolites found in the kidney medulla, we assayed urine samples from the same animals. As seen in Fig. 6, the asymmetry in the magnetization

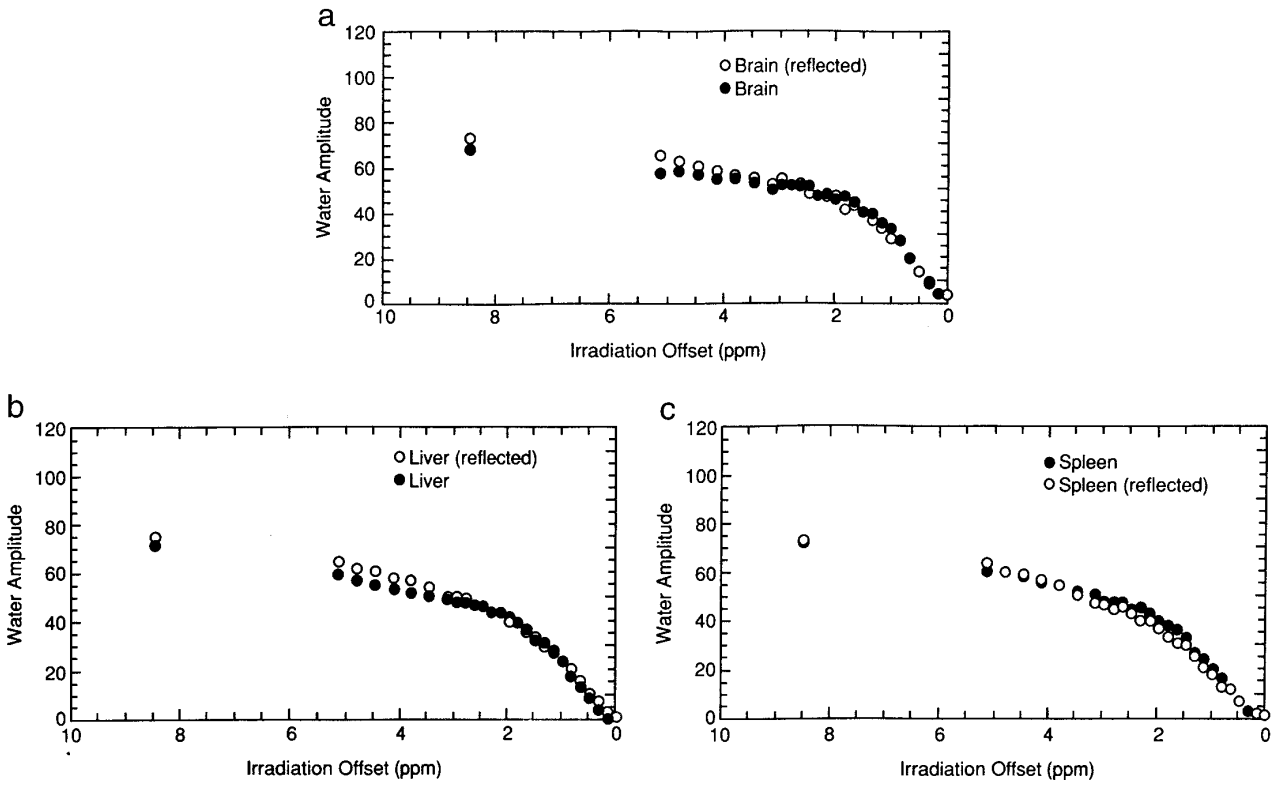

FIG. 3. Magnetization transfer spectra for rat tissues *in vitro.* (a) Brain, (b) liver, and (c) spleen. Again the reflected data is from the higher frequency data points reflected around the water resonant frequency.

transfer spectrum was also found in the urine. Again peak effects were observed at ~2.4 and ~1 ppm. All of these data collected in the renal medulla and urine are consistent with low molecular weight metabolites contributing to the asymmetry observed in the magnetization transfer spectrum in the rabbit medulla.

We focused on urine samples because urine could be collected in large volumes and data were available on its composition. Some of the metabolites with exchangeable protons in this region of the proton spectrum are ammonia, urea, creatine, creatinine, histidine, indoxyl sulfate, and hippuric acid. The pH dependence of the irradiation effect was investigated to help identify the types of compounds contributing to the reaction in urine. The pH was varied by titration over several pH units. The results for a urine sample and urea sample are shown in Fig. 7. At acid pH values (pH 5 or lower), the water and urea approach intermediate exchange rates, broadening the water line. However, an analysis of the difference spectra reveal that the ~1-ppm signal from urea can still be observed. In urine samples a similar pH dependency was observed in the ~1-ppm region. These results suggest that urea is contributing to the 1-ppm component of the asymmetry in urine. At ~2.5 ppm from water in urine, the off-resonance effect was smaller but increased with increasing pH, the opposite of the urea. These results suggest that the 2.5-ppm asymmetry is caused by a base-catalyzed reaction (i.e., increasing with base concentration) such as ammonia (*5*). The 2.5-ppm asymmetry was not observed in the urea standard (Fig. 7a). The pH dependency of the 2.5-ppm asymmetry is consistent with ammonia contributing to the water magnetization transfer spectrum.

To further assess the contribution of urea to the magnetization transfer spectrum in urine, urea was removed using the enzyme urease. The addition of urease decreased asymmetry in the magnetization transfer spectrum but did not eliminate it (Figs. 8a and 8b). In the difference spectrum, urease decreased the 1 ppm contribution with little effect on the 2.5 ppm asymmetry consistent with the removal of urea from the sample.

Magnetization transfer spectra were collected for creatine, creatinine, histidine, indoxyl sulfate, and hippuric acid solutions of 10 to 50 mM. No significant asymmetry was observed in the individual solutions, or in combinations of these compounds alone or together at physiological urine pH values. Additions of these compounds, in the millimolar range, to urine did not increase in the magnetization transfer spectra asymmetry, suggesting that no catalyst for their water proton exchange (see *13*) exists in urine.

The large and specific asymmetry in the magnetization transfer spectrum of water seen in the renal medulla suggested that this effect could be imaged in the intact kidney. Using the rabbit kidney in a 500-MHz microimaging system, MRI experiments were conducted with the off-reso-

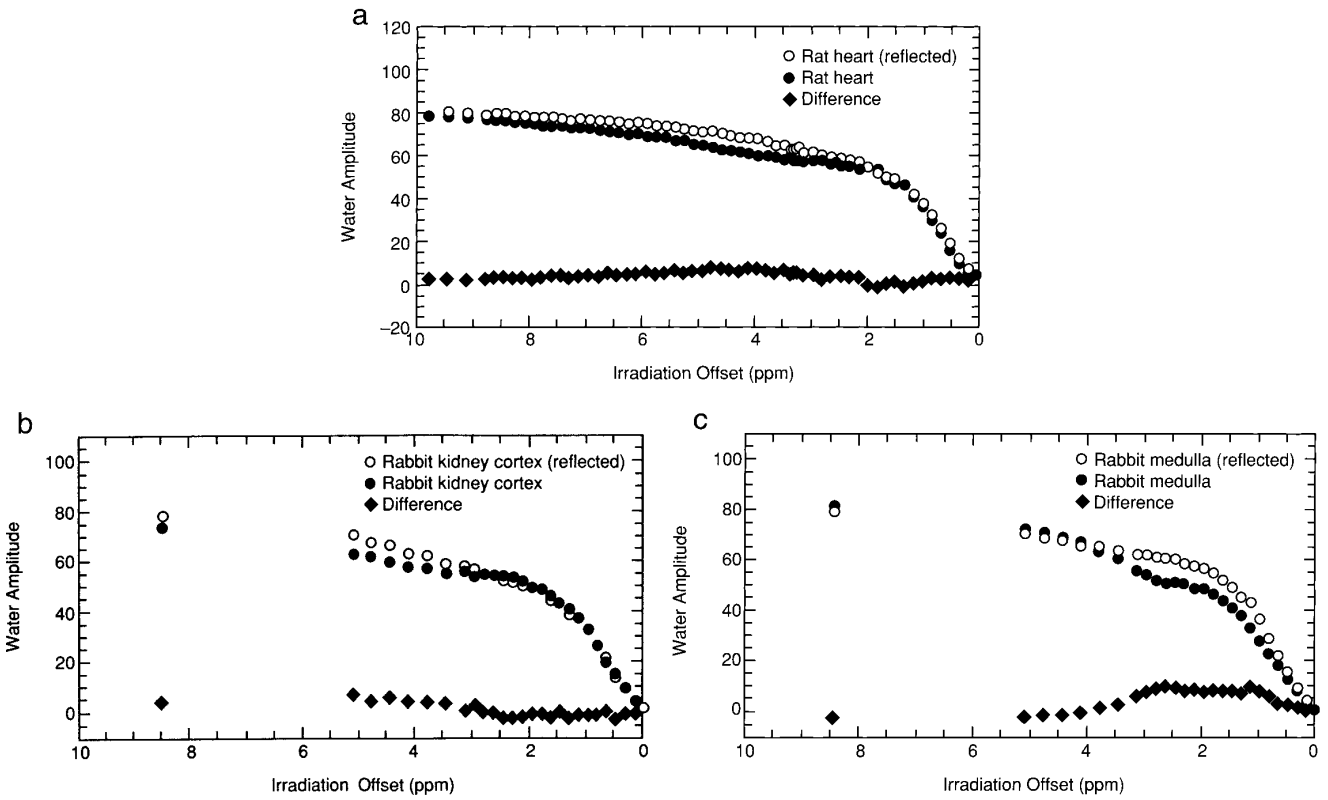

FIG. 4. Magnetization transfer spectra from the rat heart and regions of the rabbit kidney. Difference spectra between high-frequency irradiation and low-frequency data. (a) Rat heart, (b) rabbit kidney cortex, and (c) rabbit kidney medulla.

nance irradiation applied to the center of the asymmetry observed in the magnetization transfer spectrum at ~1.74 ppm from water or the control at −1.74 ppm. The power of the irradiation was adjusted to attain the maximum difference between these off-resonance effects in image projections. A difference of these two images was used to visualize the distribution of the chemical exchange process. These images are presented in Fig. 9a. A decrease in signal intensity corresponds to regions with chemical exchange or an asymmetry in the magnetization transfer spectrum. As seen in these images, most of the chemical exchange was limited to the medulla in agreement with the earlier fractionation studies. The decrease in signal amplitude outside of the kidney in the region of the medulla is believed to be due to the direct suppression of intrarenal fat during the control −1.74-ppm irradiation. The effect through the medulla was analyzed by evaluating the signal intensity along a column from the center of the medulla out to the cortex as shown in

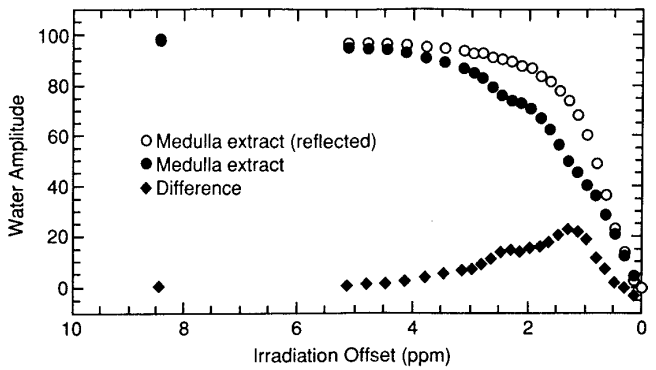

FIG. 5. Magnetization transfer spectrum from a perchloric acid extract of a rabbit kidney medulla. Tissue was prepared as described in the Methods section.

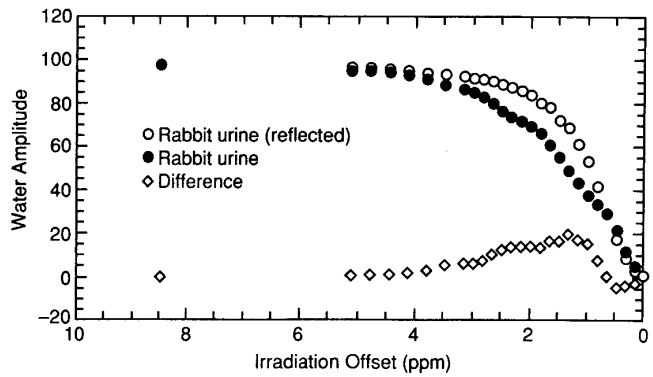

FIG. 6. Magnetization transfer spectrum from rabbit urine, pH 7.8.

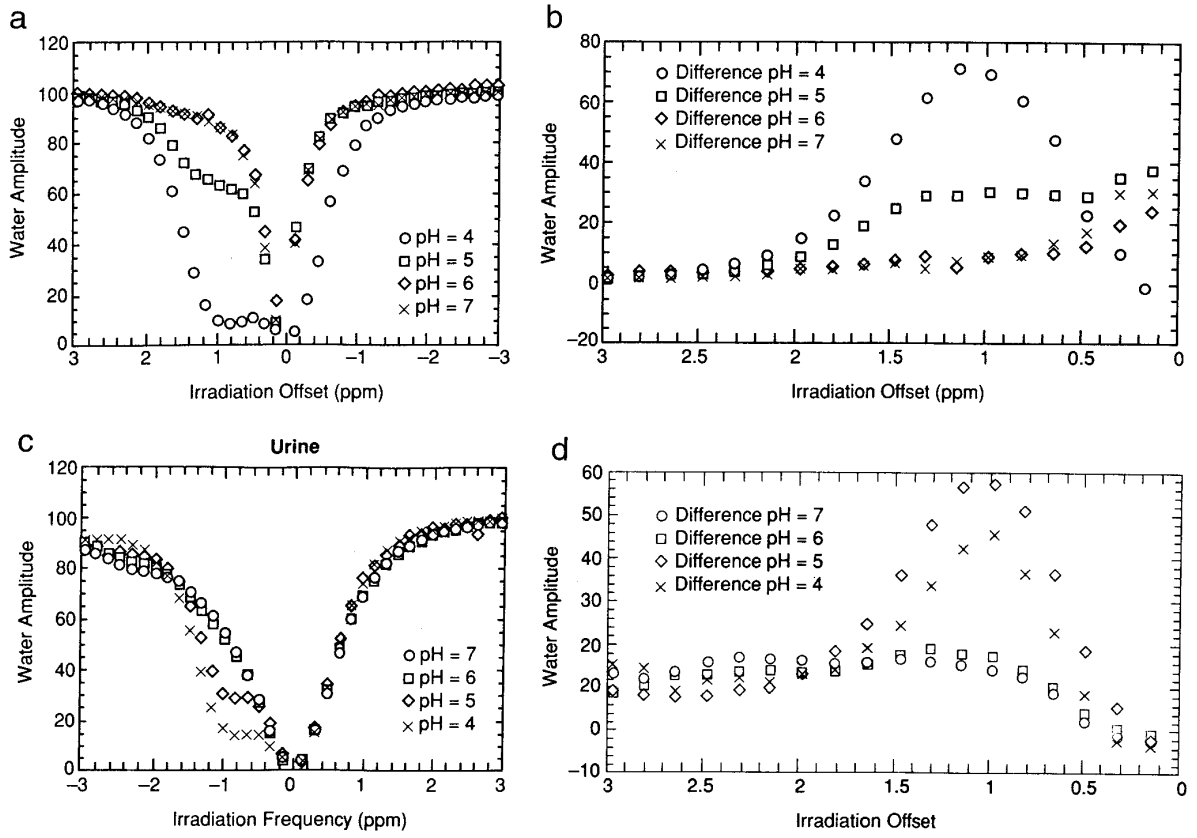

FIG. 7. The pH dependence of the magnetization transfer spectrum of urea and rabbit urine. (a) The pH dependence of the entire magnetization transfer spectrum for a 500 mM solution of urea. (b) The difference spectra, (high-frequency data) − (low-frequency data), for the data presented in (a). (c) The pH dependence of the entire magnetization transfer spectrum of rabbit urine. (d) The difference spectra for the data in (c).

Fig. 9b. The chemical exchange effect linearly increased within the medulla from the cortico-medullary region to the papillary tip. This is a distribution very similar to that found for urea, sodium, and other molecules concentrated by the countercurrent system in the kidney (for example, see *14*). These results are consistent with asymmetry in the magnetization transfer spectrum being associated with one of the metabolites that is concentrated in the renal medulla.

DISCUSSION

These studies show that ST can detect metabolites in proton chemical exchange with water in the presence of water/macromolecule cross-relaxation. Quantitative measures of the ammonia concentration in agarose gels were made using a compensation scheme for the underlying water/macromolecule cross-relaxation process. Qualitative measures of chemical exchange were made in

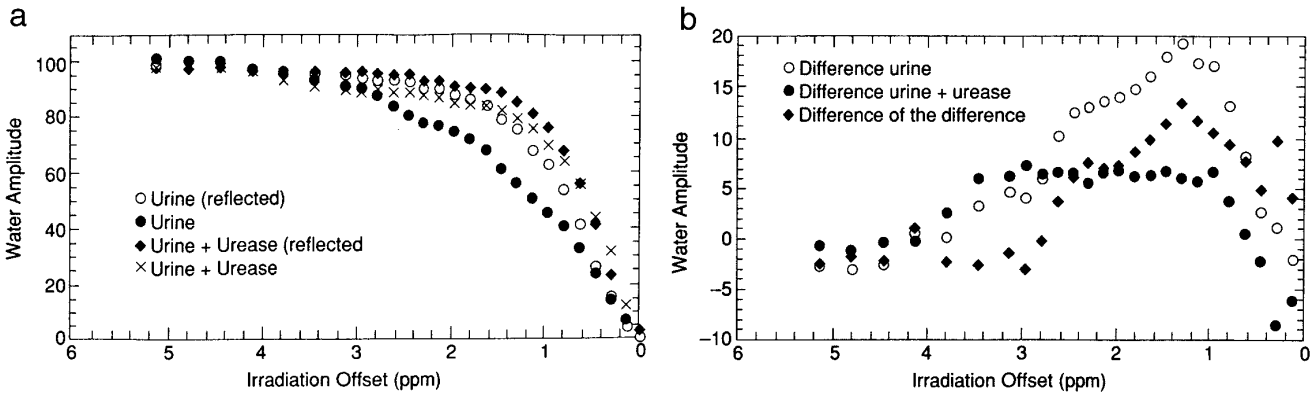

FIG. 8. The effect of urease on the magnetization transfer spectra of rabbit urine. (a) Magnetization transfer spectra. (b) Difference spectra.

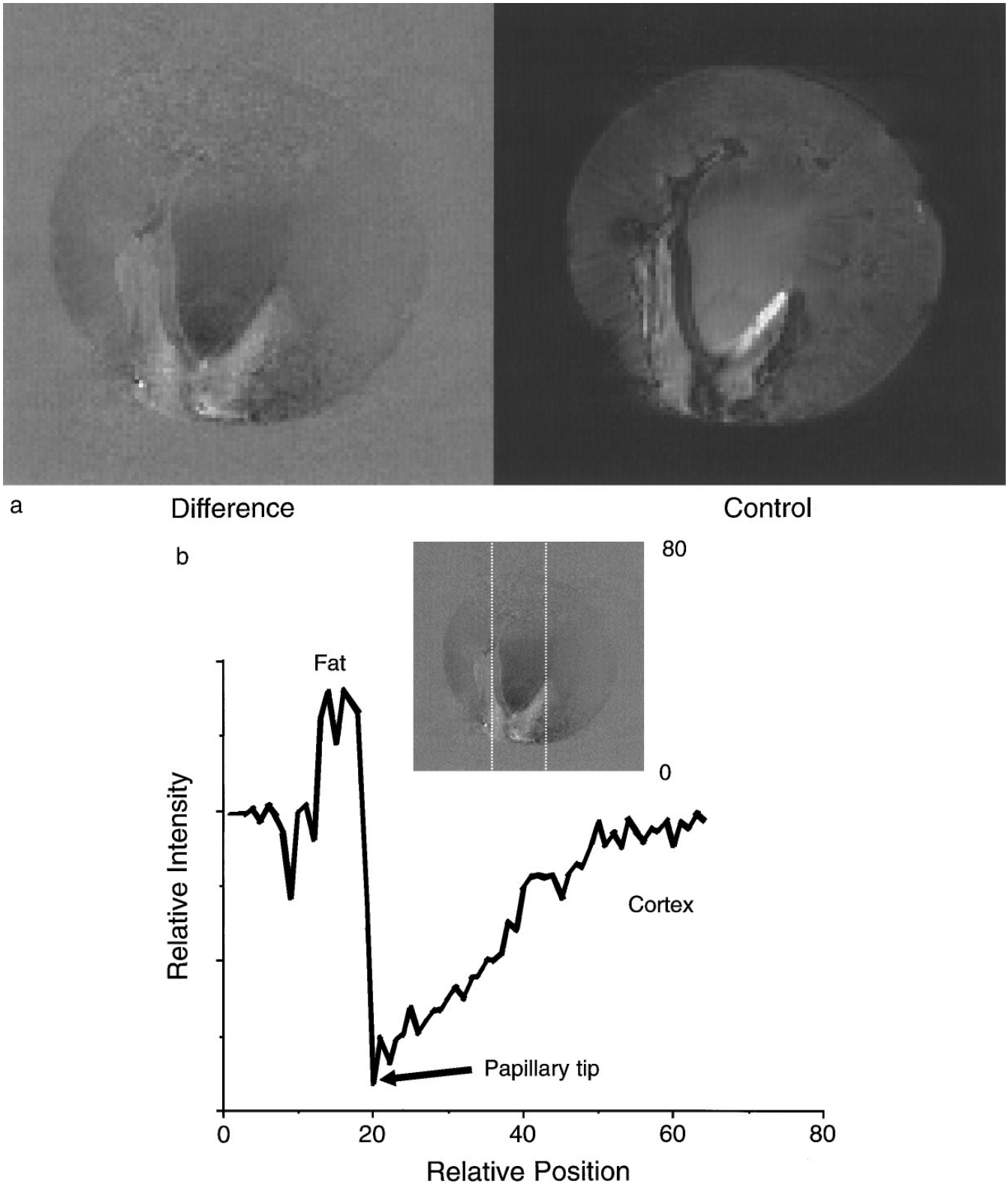

FIG. 9. Chemical exchange imaging of the rabbit kidney at 500 MHz. (a) Control and difference image of the experimental irradiation (1.74 ppm at 1.5×10^{-6} T) minus the control irradiation (-1.74 ppm). All images were collected with a gradient recalled echo sequence with details presented in the Methods section. (b) Pixel amplitude along the cortico-medullary axis of the kidney. Pixels were averaged along the column outlined in the difference image in the inset. Pixel from 0 to 20 correspond to the irradiation of fat in the control, pixels 20 to 40 correspond to the medulla, and pixels 40 to 80 represent the cortex.

several tissues and urine by determining the asymmetry of the magnetization transfer spectrum. Most of the asymmetry could be ascribed to a larger decrease in the water amplitude when irradiated at frequencies below the water resonance. Exploiting this asymmetry, water–metabolite chemical exchange images of the mammalian kidney were collected at 500 MHz.

The water macromolecule magnetization transfer spectrum was symmetrical around the water resonance out to ± 5 ppm in agarose as well as many of the biological tissues studied (see Figs. 2 and 4). An asymmetry in the magnetization transfer spectrum was present in tissues and urine caused primarily by the irradiation frequencies below water resulting in a larger decrease in water amplitude than the same offset above water. This effect was noted in two general spectral regions, a broad effect farther than 5 ppm from water and more localized effects from 4 to 1 ppm.

Pekar *et al.* (*15*) found a small diffuse asymmetry in the magnetization transfer spectrum at 4.7 T in the cat brain *in vivo,* which was most pronounced -20 ppm from water, using much higher irradiation power. Very little asymmetry was observed within ~ 2 ppm of water, consistent with our *in vitro* data on rat brain (Fig. 3). Most tissues studied did have an increase in the off-resonant irradiation effect at frequencies >5 to 10 ppm from water. This effect in brain, renal cortex, and liver may be due to chemical shift in the macromolecule protons since the chemical shift (>5 ppm) is at the limits of small metabolites. Potentially intermediate exchange with a metabolite could asymmetrically broaden the water line beyond the chemical shift of the metabolite as observed with urea at acid pH values (Fig. 7). Further studies will be required to localize the origin of this >5-ppm component of the magnetization transfer spectrum.

The largest asymmetry at 3 to 1 ppm was found in the renal medulla and was the focus of most of the attention in this study. The topology of the asymmetry in the magnetization transfer spectrum was assessed using MRI and tissue fractionation studies. The asymmetry was localized in a linear gradient in the renal medulla similar to that existing for ions and metabolites concentrated in the urine. Nearly identical asymmetries in the magnetization transfer spectrum were found in medullary PCA extracts and the urine, both free of macromolecules as confirmed by the magnetization transfer spectrum (i.e., lack of a broad component exchange, Figs. 5 and 6). These data suggest that the magnetization transfer spectrum asymmetry in kidney medulla is caused by water proton chemical exchange with water soluble low molecular weight compounds in the cellular or extracellular space.

The most obvious candidates at these chemical shifts with exchangeable protons are urea and ammonia that are concentrated in the renal medulla and urine (*14*). It is important to note that urea and ammonia are present in both the cellular and extracellular compartments of the kidney and the differentiation of these two compartments would be difficult. Studies suggesting that urea is contributing to the 1-ppm magnetization transfer spectrum component are based on the chemical shift of the effect, pH dependence in urine, and the effect of urease. However, several lines of evidence suggest that other compounds are also involved: (1) The magnetization transfer spectrum of urea in water has a maximum effect at ~ 1 ppm from water, whereas a component at ~ 2.5 ppm was consistently observed. (2) At pH values above 6, no ST was observed in urea standards, but ST in the 1-ppm region was observed in urine samples (Fig. 7). (3) The pseudo-first-order rate constant of urine versus the concentration of urea did not pass through the origin (data not shown) as it should if urea was solely responsible for the exchange. (4) The removal of urea with urease reduced the asymmetry in the magnetization transfer spectrum urine, but did not eliminate the effect, especially in the 2.5-ppm region. These data suggest that urea does play some role in the asymmetry detected either directly or as a catalyst (*13*), but all of the asymmetry in the magnetization transfer spectrum cannot be ascribed to urea alone.

The effect at ~ 2.5 ppm from water has the appropriate chemical shift for ammonia (Fig. 1) which is present in low concentrations in the urine of rabbits. In addition, the pH dependence of the 2.5-ppm region is apparently base catalyzed (i.e., increasing rate with increasing base concentration) as previously shown for ammonia (*5*). These two results suggest that ammonia may contribute to the 2.5-ppm effect but does not rule out other compounds. It should also be pointed out that the spectral resolution of these studies is quite poor because of the spectrally broad effects of the off-resonance irradiation that is a function of both the T_1, T_2 (*16*) and chemical exchange rate. Therefore, chemical shift alone is not definitive in the assignment of the magnetization transfer effects.

To identify compounds, other than urea and ammonia, which may contribute to the chemical exchange asymmetry in kidney medulla, a study of known urine compounds in the millimolar concentration range was performed. All of these metabolites by themselves, in various combinations, or added directly to urine did not show a significant effect at urine pH values. These results implied that any other compound or compounds contributing to the asymmetry are in very low concentrations (below millimolar), where hundreds of candidate compounds may exist. A very low concentration metabolite could cause the ST effects observed as long as its rate constant was significant relative to the water T_1. A survey of the literature with high-resolution proton spectra of urine reveal that many small resonances are present in the 2.5- to 3-ppm region which have yet to be assigned (for examples, see *11*, *12*). Indeed, an unknown compound may be near intermediate exchange rates broadening its resonance, making its direct detection difficult even under high-resolution conditions. Further studies will be required to sort out the origin of this chemical exchange process in urine and the kidney to establish whether other compounds are contributing to the effect. One interesting study may be to collect a series of images with different offset frequencies to establish the distribution of the different components of the magnetization transfer spectrum asymmetry throughout the kidney. In the current study only one ‘mean‘ frequency was evaluated.

The use of the high-frequency control irradiation provided a method of compensating for the background magnetization transfer with the macromolecule. This approach was used to quantitate metabolites in gels and qualitatively in MR imaging experiments. A compensation scheme was presented for the quantitation of ammonia in agarose gels. However, to quantitate other metabolites in tissues, or even fluids, will require appropriate calibrations between metabolite concentration and pseudo-first-order rate constants as a function of pH, ionic environment, temperature, and so forth. As shown in Fig. 5 for urea, which is only 1 ppm from water, as exchange approaches intermediate rates at acid pH values, the linewidth increase in urea will defeat the simple quantitation approach used in this study since the metabolite will effectively encroach on the control irradiation region. Under these conditions a more sophisticated modeling scheme will be required (*6*). This is one of the reasons that ammonia, 2.5 ppm from water, was used as our initial model system.

Another concern may be exchangeable protons at higher frequencies than water in complex biological tissues (e.g., hydroxyl groups) or other sites which would distort this region of the spectrum. In addition, fat protons may be selectively saturated during the control irradiation, which could influence images created from both fat and water protons, as shown in the rabbit kidney. Fat proton effects on images may be minimized by using nonsaturating fat suppression approaches (for example, see *17*) in combination with the off-resonance irradiation. Outside of fat protons, no evidence of compounds in the +ppm range was found in the magnetization transfer spectrum of the tissues studied. These data suggest that these exchange processes are either too slow or fast to contribute significantly at these frequencies.

The magnetic field inhomogeneity of the sample could also be a critical factor when using a simple two-frequency approach to create an image. Differences in the distribution of the magnetization transfer effect could be due to topological variations in the water resonance frequency and not chemical exchange. This could be a significant problem in the body where field variations of up to 2 ppm are commonly observed. An evaluation of this potential problem could be made from field plots; a correction may be performed by collecting a series of images with different irradiation offsets to find the water minima and make the appropriate corrections for the field distribution. Based on the correlation between the MR images, tissue fractionation, and extracts, it is unlikely that sample inhomogeneity contributed significantly to the distributions observed in the kidney.

Parameters other than the simple concentration of metabolites in the cytosolic or extracellular space could contribute to the magnetization transfer effect observed. The pH is clearly a very important factor as shown in the urine and model studies. All of the tissue samples evaluated in this study were likely more acidic than normal *in vivo* tissue because of the prolonged ischemia they experienced. This is true even if the extracellular space is buffered. Thus, all of the spectra provided should be considered ischemic tissue at room temperature. How closely these spectra will track normal tissue at physiological pH and temperature in unknown. No information on *in vivo* conditions is provided in this initial study. The ischemia-induced acidification is most likely also affecting the *in vitro* kidney images collected. Since the urea exchange rate was more effective in generating a saturation effect at acid pH values, urea is predictably dominating the effect in these images. In addition, some of the topological distributions within the kidney could also be due to the distribution of pH and not solely the distribution of metabolites. Other factors can also serve as catalysts for proton–water exchange that could alter the magnetization transfer effect in addition to the simple concentration of the metabolite (*13*).

Relatively small magnetization transfer spectrum asymmetries were observed in tissues other than the kidney. These results suggest that similar metabolite–water chemical exchange may be occurring in these tissues which may warrant further study, specifically the 4-ppm component in heart. We did not pursue these other tissues because of the large effect detected in the kidney, which provided the largest signal to work with in this preliminary study. However, because of the large gain in signal obtained using ST effects on water, this approach may be worth pursuing further in biological tissues if the specificity issue can be better addressed.

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