

P-31 Nuclear Magnetic Resonance Spectroscopic Study of Obstructive Uropathy in the Rat

Joseph I. Shapiro and Laurence Chan

Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Abstract

P-31 nuclear magnetic resonance (NMR) spectroscopy of the rat kidney with ureteral ligation resulted in a rapid and major increase in a peak resonating at 7096.63 ± 0.65 Hz from the reference frequency of phosphorus (32.60 MHz). This corresponded to an increase in the concentration of the substance responsible for peak X from 0.34 ± 0.04 $\mu\text{mol/g}$ wet weight in normal kidneys to 1.45 ± 0.27 $\mu\text{mol/g}$ wet weight in unilaterally obstructed kidneys and 2.00 ± 0.34 $\mu\text{mol/g}$ wet weight in bilaterally obstructed kidneys at 3 h ($P < 0.01$). Further NMR studies performed with in vivo kidneys and tissue extracts revealed that inorganic phosphate in the urine, resonating at a lower frequency due to the acid pH environment, was responsible for the increase in this peak. These findings may prove to be of fundamental interest as well as potential clinical significance.

Introduction

Phosphorus-31 nuclear magnetic resonance (NMR)¹ has been used to study changes in the concentrations of high energy phosphate compounds and intracellular pH on isolated cells and tissues in the early 1970s (1, 2). More recently, P-31 NMR spectroscopy has been successfully used to study renal biochemistry, a topic which has been extensively reviewed (3–5). Specifically, this technique has been used to study normal renal energy metabolism as well as cellular biochemical changes occurring with systemic hypotension and ischemia, and also to study changes in extracellular acid-base status in the kidney (6–12).

When a kidney is subjected to ureteral ligation, there are major changes in renal blood flow as well as net tubular sodium reabsorption (13–19). Because of the changes in substrate supply and energy demand, we reasoned that there might be associated changes in the concentrations of high energy phosphate compounds in the kidney that occur in ureteral ligation which could be studied with P-31 NMR.

In this paper, we report dramatic changes in the P-31 NMR spectra of rat kidneys subjected to ureteral ligation.

Address reprint requests to Dr. Chan, Division of Renal Diseases, University of Colorado Health Sciences Center, 4500 E. Ninth Ave., Box C281, Denver, CO 80262.

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1. Abbreviations used in this paper: NMR, nuclear magnetic resonance; Pi, inorganic phosphate; T_1 , spin-lattice relaxation time.

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Methods

Animals

Male Sprague-Dawley rats (300–350 g) obtained from a commercial supplier (Sasco, Omaha, NE), fed on standard rat chow (Ralston-Purina Co., St. Louis, MO) and allowed free access to tap water, were used. Rats were anesthetized with pentobarbital administered intraperitoneally and then subjected to a flank incision (unilateral or bilateral). Additional anesthesia was given to the rats as needed during the experiment and postoperative period to relieve their distress. The kidney was exposed and mobilized using blunt dissection. Ureteral ligation was accomplished using two 4-0 silk ties tied ~ 1.0 cm from the renal hilus. Release of obstruction was accomplished by ureteral cannulation proximal to the point of obstruction using P-50 tubing. Urine obtained by this cannulation was studied for pH determination, phosphate concentration, and spin-lattice relaxation time (T_1) determination (see below). The rat kidneys studied by NMR spectroscopy were externalized after the flank incision, separated from the muscle and skin with surgical gauze, and covered with a thin plastic covering. Care was taken during this procedure to avoid compromising the vascular supply of the kidney.

P-31 NMR spectroscopy

In vivo P-31 NMR spectra were obtained using a 1.89 Tesla 30-cm horizontal bore cryomagnet (Oxford Research Systems) and a Biospec spectrometer (Bruker Instruments, Inc., Billerica, MA). The transmitter and receiver used was a 1.5-cm diam 2-turn solenoid tuned to the resonance frequency of phosphate in this magnet (32.60 MHz), which was built by the authors. The 90° and 180° pulse widths (15 and 30 μs , respectively) were determined on phantoms containing 100 mM inorganic phosphate (Pi) by testing the effect of increasing pulse widths on the resultant P-31 NMR spectra using one scan for each pulse width studied. The accuracy of these pulse widths were later confirmed using kidney tissue in situ. Kidney spectra were obtained by placing the solenoid over the externalized rat kidney. Spectra used for comparison were obtained using 512 scans each using a 60° pulse and a 2-s delay. Spectroscopy of urine was performed on 2-ml urine samples placed in small plastic tubes positioned within the same probe described above. Spectra of these urine samples were obtained with 32 scans of 60° using 2-s delays. T_1 values for the spectral peaks of the in situ kidneys and the major spectral peak of urine were obtained using the inversion recovery method (1, 2, 6).

P-31 NMR spectra from extracts (see below) were performed on a 4.7 Tesla vertical bore cryomagnet (Oxford Instruments, Oxford, England) using a 200-MHz spectrometer (Bruker Instruments, Inc.) and a commercial probe tuned to the resonance frequency of Pi in this magnet (80.85 MHz). Spectra from extracts are plotted as parts per million (ppm) from the resonance frequency of phosphoric acid.

Destructive chemical analysis

Kidneys from animals were freeze clamped using Wollenberger clamps cooled in liquid nitrogen and stored at -70°C before extraction. Extractions were performed using 10% perchloric acid, which was followed by centrifugation for 25 min at 12,000 g and neutralization of the supernatant with 30% potassium hydroxide. The neutralized extract was assayed for ATP using an enzymatic method that has been well described (20). Urine phosphate was determined using a standard

chemical assay (21). Urine pH was determined using a pH meter (Beckman Instruments Inc., Palo Alto).

Quantitation

P-31 NMR spectral peaks from spectra obtained from in vivo samples and extracts were quantitated by relative areas under the spectral peaks. Exponential multiplication was applied to the free induction decay with a line broadening of 10 Hz. Because of the broad phospholipid signal present on the in vivo spectra, these spectra were baseline corrected by deconvolution before peak area calculations. For each experiment, the B_0 field was shimmed using the proton signal of the sample and was brought to resonance at 80.55 MHz. Phosphate resonances are expressed as Hz from 32.60 MHz. The chemical environment of phosphate associated with the various spectral peaks was for the most part as has been previously described (1, 2). The peak at $\sim 7,096$ Hz is referred to as peak X. The absolute molar concentration of the phosphate resonating at this frequency was calculated by multiplying the relative area of peak X divided by the relative area of the beta ATP peak, by the absolute molar concentration of ATP determined by enzymatic analysis performed on the extract of that kidney (22).

Experimental design

Time course. Initially, serial spectroscopy on several animals treated with either unilateral or bilateral ureteral ligation was performed recording spectra every 20 min for the first 16–24 h after ureteral ligation. Using the data gathered from these studies, it was learned that maximal spectral changes occurred from 3 to 4 h after ligation. Therefore rats were assigned to the following groups. A, no obstruction; B, unilateral obstruction for 3 h; C, bilateral obstruction for 3 h; D, unilateral obstruction for 24 h; E, bilateral obstruction for 24 h; and F, immediately after release of obstruction present for 3 h. Kidneys from these six groups were freeze clamped immediately after the P-31 NMR in vivo spectroscopy and subsequently assayed for ATP concentration.

Characterization of peak X. Kidneys obstructed for 3 h were freeze clamped and extracted and studied with P-31 NMR as described above. Kidneys were subjected to either no obstruction or unilateral ureteral ligation. T_1 values for the spectral peaks were determined. As this determination required from 4 to 6 h, the T_1 measurement of in situ kidneys was begun ~ 1 h after ureteral ligation. Urine obtained by cannulation of ureters ligated for 3 h was studied with P-31 NMR for spectral appearance as well as T_1 value of the major spectral peak.

Statistics

Results are expressed as the mean \pm SEM unless otherwise stated. All multiple group comparisons were performed with one-way analysis of variance. Individual group comparisons were performed with the Student's t test using Bonferroni's correction for multiple comparisons (23).

Results

Time course. Shortly after ureteral ligation, all kidney spectra showed a marked accentuation of a peak present at 7096.63 ± 0.65 Hz ($n = 12$) (or 3.32 ± 0.04 ppm from the reference frequency of phosphocreatine). This peak achieved maximal height after 3 to 4 h of ureteral ligation on the unilaterally obstructed and bilaterally obstructed kidneys studied serially ($n = 5$). After 6 to 8 h in the unilaterally obstructed kidney model and 12 h in the bilaterally obstructed model, this peak began to diminish in amplitude. This peak returned to preobstruction amplitude by 24 h in the unilaterally obstructed kidneys but did not fully return to normal in bilaterally obstructed kidneys after 24 h. Representative spectra of the experimental groups are shown in Figs. 1–6. Relative peak areas are tabulated in Table I. These data show clearly that the concentration

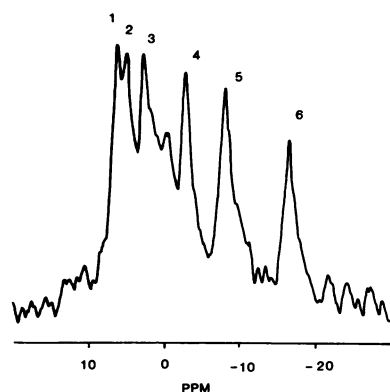


Figure 1. Group A. Normal kidney spectrum. Peak assignments: 1, sugar phosphate; 2, Pi; 3, peak X; 4, gamma ATP; 5, alpha ATP; and 6, beta ATP. Spectra obtained with 512 scans of 60° pulse with 2-s delays. Chemical shift in ppm.

of the chemical species responsible for peak X increases markedly with short periods (3 h) of unilateral or bilateral obstruction (groups B and C). This relative concentration is significantly increased compared with normal kidneys in kidneys subjected to 3 h of unilateral and bilateral obstruction. Moreover, the change in the appearance of the spectra obtained are perhaps even more impressive as peak X clearly becomes the dominant spectral peak after 3 h of obstruction (Figs. 2 and 3). This increase in peak X seen with 3 h of unilateral obstruction is reversed immediately after release of the obstruction (group F, Fig. 6). Kidneys subjected to unilateral obstruction for 24 h reestablished a normal P-31 NMR spectra (group D, Fig. 4). However, kidneys subjected to 24 h of bilateral obstruction have persistently elevated relative concentrations of peak X as well as an elevation of Pi (group E, Fig. 5). Absolute concentrations of ATP determined by enzymatic methods are indistinguishable from normal in all groups studied except the kidneys subjected to 24 h of bilateral obstruction, where it was significantly reduced (group E, Table II). The molar concentrations of peak X determined by combining P-31 NMR and destructive methodologies (see below) confirm that the concentration of the substance responsible for peak X increases markedly after 3 h of unilateral and bilateral obstruction and returns to normal after relief of obstruction or after prolonged (24 h) periods of unilateral but not bilateral obstruction (Table III).

Characterization of peak X. P-31 NMR spectra of tissue extracts ($n = 5$) reveal no significant difference in the relative quantity of phosphodiester between normal and obstructed kidneys. Table IV shows the relative peak areas obtained from tissue extracts of normal and obstructed kidneys. The relative area under the Pi peak in obstructed kidney extracts is signifi-

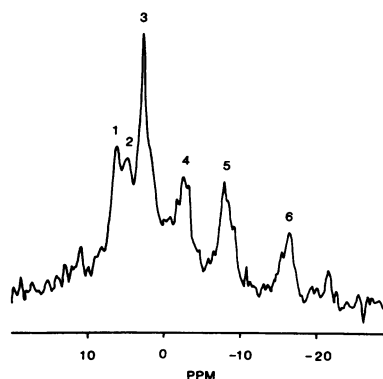


Figure 2. Group B. Spectrum of unilateral obstruction kidney (3 h). Peak assignments and acquisition parameter as in Fig. 1.

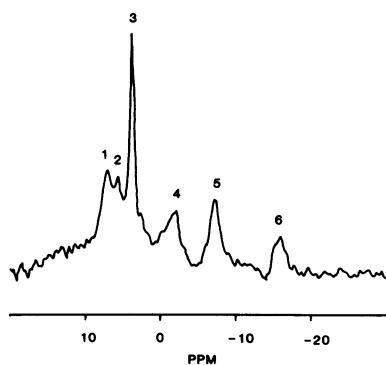


Figure 3. Group C. Spectrum of bilateral obstruction kidney (3 h). Peak assignments and acquisition parameter as in Fig. 1.

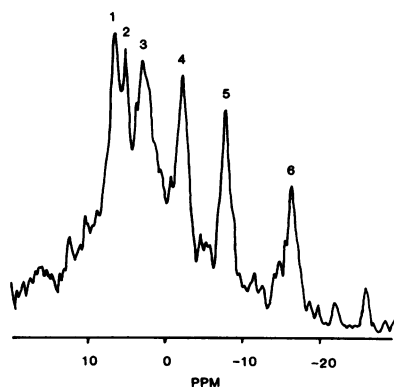


Figure 4. Group D. Spectrum of unilateral obstruction kidney (24 h). Peak assignments and acquisition parameter as in Fig. 1.

cantly greater than in normal kidneys (17.8 ± 1.7 vs. $11.6 \pm 0.9\%$, $P < 0.01$). These data were obtained with a relatively short delay (1 s). To rule out misinterpretation due to possible saturation, three normal and three obstructed kidney extracts were examined using 90° pulses with long delays (20 s). The results obtained were similar with no significant difference in the relative concentration of phosphodiester (12.6 \pm 1.2 vs. 16.2 \pm 2.3%), and higher Pi concentrations observed in the obstructed kidneys (16.2 \pm 2.3 vs. 7.0 \pm 1.6%, $P < 0.01$). Using fully relaxed (20-s delays) spectra from normal ($n = 5$) and obstructed (3 h unilateral) kidneys ($n = 5$) in vivo, peak X was found to increase 1.32 ± 2.6 $\mu\text{mol/g}$ wet weight during 3 h of obstruction. This value correlates relatively well with the increase in Pi in the fully relaxed (20-s delays) spectra from the obstructed kidney extracts (1.46 ± 0.49 $\mu\text{mol/g}$ wet weight, $n = 3$).

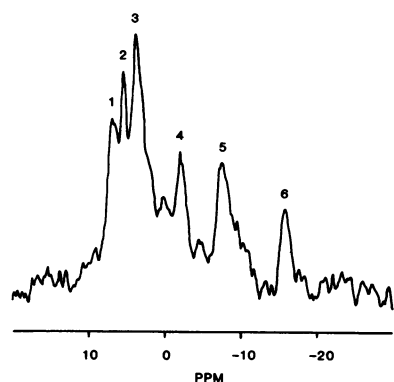


Figure 5. Group E. Spectrum of bilateral obstruction kidney (24 h). Peak assignments and acquisition parameter as in Fig. 1.

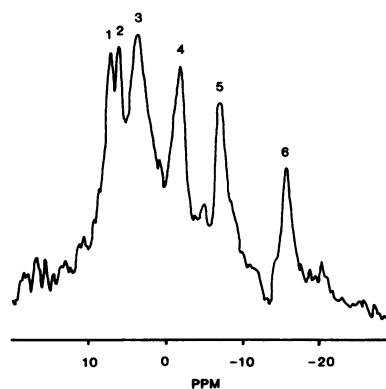


Figure 6. Group F. Spectrum of unilateral obstruction kidney (3 h) immediately after release of obstruction. Peak assignments and acquisition parameter as in Fig. 1.

Urine pH was 5.2 ± 0.2 ($n = 3$) in the samples studied. Urine phosphate was 97.4 ± 12 mM in these samples. P-31 NMR spectra obtained from these urines after relieving the ligation showed essentially a single peak, which we attributed to urine phosphate at 7096.99 ± 8.5 Hz. A single peak was observed when urine pH was increased to 10.

A comparison of the T_1 values for the spectral peaks of normal kidneys and kidneys subjected to unilateral ureteral ligation is shown in Table V. It is immediately clear that the longest T_1 belongs to peak X in obstructed kidneys. It is also clear that the 60° pulse and 2-s delay used actually underestimate the true concentration of this substance more than that of the other compounds. Of greater interest is the observation that the T_1 value of peak X of obstructed kidneys is quite similar to that obtained on urine Pi (5.52 ± 0.60 vs. 7.14 ± 2.69 s, $P > 0.10$).

Discussion

P-31 NMR spectra of rat kidneys that were exposed to either unilateral or bilateral obstruction show the rapid accentuation of a peak at $\sim 7,097$ Hz that we have designated peak X. This peak has been attributed to various compounds by previous investigators and is almost certainly under normal conditions derived from more than one biologic compound (3–8). The

Table I. Relative Areas of P-31 Spectral Peaks

Groups (N)	SP	Pi	X	Gamma	Alpha	Beta
A (5)	13 \pm 1	4 \pm 1	5 \pm 1	26 \pm 1	33 \pm 2	21 \pm 1
B (5)	9 \pm 1	3 \pm 1	18 \pm 2 [†]	21 \pm 2	31 \pm 2	18 \pm 2
C (3)	7 \pm 1	4 \pm 1	23 \pm 2 [†]	21 \pm 1*	29 \pm 2	17 \pm 2
D (3)	11 \pm 2	4 \pm 1	3 \pm 1	27 \pm 2	35 \pm 6	21 \pm 2
E (3)	5 \pm 1	8 \pm 1*	12 \pm 2*	21 \pm 1*	37 \pm 1	17 \pm 1
F (3)	16 \pm 1	4 \pm 1	6 \pm 1	23 \pm 2	34 \pm 1	18 \pm 2

Data presented as percentage of total peak area with sugar phosphate (SP) + Pi + X (peak X) + gamma (gamma ATP) + alpha (alpha ATP) + beta (beta ATP) = 100%. Results expressed as mean \pm SEM. Groups A through F are described in the Methods section. Spectra were baseline corrected with the baseline hump excluded from the calculation. It is assumed that the total phosphate in the sample is constant.

* $P < 0.05$ compared with normal (group A).

[†] $P < 0.01$ compared with normal (group A).

Table II. Tissue Concentration of ATP after Obstruction

Group (N)	ATP $\mu\text{mol/g wet weight}$
A Normal (5)	1.60 \pm 0.19
B Unilateral obstruction for 3 h (6)	1.47 \pm 0.26
C Bilateral obstruction for 3 h (3)	1.49 \pm 0.23
D Unilateral obstruction for 24 h (3)	1.39 \pm 0.05
E Bilateral obstruction for 24 h (3)	1.12 \pm 0.08*
F After release of obstruction present for 3 h (3)	1.46 \pm 0.21

Kidneys were freeze clamped and extracted with perchloric acid. The neutralized extract was assayed for ATP using an enzymatic method. Results expressed as mean \pm SEM. Experimental groups A through F are described in the Methods section.

* $P < 0.05$.

time course of the increase in this peak closely correlates with the well-described increases in tubular pressures and calyceal volumes noted in both the unilateral and bilateral models of ureteral obstruction in the rat (13–19, 24–26). Therefore, it appears that the changes in this peak may at least correlate with the changes in the collecting system that occurs with obstruction.

Examination of our T_1 data suggests quite strongly that the major chemical moiety responsible for the majority of peak X from normal kidneys is not the same as the substance resonating at 7,097 Hz in our obstructed kidneys. In obstructed kidneys, peak X is almost certainly due to a large degree to urine phosphate. In unobstructed kidneys, urine phosphate is probably responsible for only a small portion of what other authors have called the phosphodiester peak (27, 28). The increase in the urine phosphate signal seen with obstruction is probably due to stasis and the increase in urine volume as described above. The position of the peak is explained by the strong pH dependence of the chemical shift of phosphate and is quite

Table III. Molar Concentration of Peak X in Renal Tissue In Vivo After Ureteral Ligation

Group (N)	Peak X $\mu\text{mol/g wet weight}$
A Normal (5)	0.34 \pm 0.04
B Unilateral obstruction for 3 h (6)	1.45 \pm 0.27 [‡]
C Bilateral obstruction for 3 h (3)	2.00 \pm 0.34 [‡]
D Unilateral obstruction for 24 h (3)	0.18 \pm 0.06
E Bilateral obstruction for 24 h (3)	0.86 \pm 0.11*
F After release of obstruction present for 3 h (3)	0.43 \pm 0.11

Results expressed as mean \pm SEM. Experimental groups A through F are described in the Methods section. The absolute molar concentration of the phosphate resonating at this frequency was calculated by multiplying the relative area of peak X divided by the relative area of the beta ATP peak by the absolute molar concentration of ATP, as determined by enzymatic analysis performed on the extract of that kidney. The increase in peak X is underestimated because of the T_1 saturation effect.

* $P < 0.05$.

[‡] $P < 0.01$.

Table IV. Relative Peak Areas in Extracts of Normal and Obstructed Kidneys

Peak	Normal (n = 5)	Obstructed (n = 5)
Sugar phosphate	28.5 \pm 1.6	22.8 \pm 2.3*
Pi	11.6 \pm 0.9	17.8 \pm 1.7 [‡]
Phosphodiester	7.0 \pm 0.9	7.5 \pm 1.8
Gamma ATP	14.5 \pm 0.2	13.9 \pm 0.7
Alpha ATP	28.2 \pm 1.1	27.6 \pm 1.9
Beta ATP	10.2 \pm 0.3	10.4 \pm 0.4

Results expressed as percent of total peak area. Results tabulated are mean \pm SEM.

* $P < 0.05$.

[‡] $P < 0.01$.

consistent with the resonance of Pi at the urine pH's obtained (1, 2, 29). A direct correlation of the magnitude of the increase in the peak with urine phosphate concentration and a change in the chemical shift of this peak with increases in urine pH can be observed (29). Chemical method for measurement of Pi concentration in aqueous solution agrees well with the levels determined by P-31 NMR (30). This assertion is supported by the T_1 data as well as by the observations that the peak disappears immediately with release of obstruction, and that Pi is the only phosphate containing compound present in sufficient concentration in urine to generate this signal. The decrease in this peak with prolonged obstruction in the unilateral model is almost certainly due to the reabsorption of urine and normalization of tubular pressures seen in this model (13, 16, 17). The demonstrated capacity of distal tubular segments to reabsorb Pi supports this contention (31–35).

Other interesting observations can be drawn from our data. Kidneys subjected to prolonged bilateral obstruction have significantly lower tissue concentrations of ATP and higher relative concentrations of inorganic phosphate. This is most easily explained by decreases in renal perfusion, which result in relative ischemia in this model (13, 15, 16, 19, 26). Another observation is that the T_1 of Pi is significantly longer in ob-

Table V. T_1 Values for Spectral Peaks Obtained in P-31 NMR Study of Normal and Obstructed Kidneys In Vivo and Urine from Obstructed Kidneys

Spectral peak	T_1 (s)		
	Normal (n = 5)	Obstructed (n = 5)	Urine (n = 3)
Sugar phosphate	2.02 \pm 0.25	1.50 \pm 0.22	
Pi	0.99 \pm 0.03	1.43 \pm 0.08 [‡]	7.14 \pm 2.69*
Peak X	1.76 \pm 0.38	5.52 \pm 0.60 [‡]	
Gamma ATP	0.94 \pm 0.05	0.87 \pm 0.13	
Alpha ATP	0.93 \pm 0.12	1.15 \pm 0.34	
Beta ATP	0.65 \pm 0.05	0.95 \pm 0.18	

Results expressed as mean \pm SEM.

* $P < 0.05$ compared with normal.

[‡] $P < 0.01$ compared with normal.

structed kidneys than in normal kidneys. This is consistent with the chemical exchange of Pi with the gamma phosphate group of ATP being a major mechanism for magnetic relaxation of Pi. However, other physical mechanisms may be operant. Because obstructed kidneys are using very little energy for sodium transport, normally the major energy expenditure for the kidney (36), it is possible that an indirect measurement of ATP turnover rate would be different between the two groups. More accurate measurement of the differences in energy turnover rates between obstructed and nonobstructed kidneys might be accomplished with P-31 NMR by using saturation transfer techniques that were not used in this study, but will be used for future work (22, 37).

The observation that phosphate derived from the urine does not resonate at the frequency observed for intracellular Pi may partially explain the differences in the concentration of kidney tissue Pi determined by in vivo P-31 NMR and the destructive chemical techniques that have been well described by other workers (4, 22, 37–39). Explanations given to explain this phenomenon, which has been noted in other organs as well as kidney, include the rapid breakdown of other phosphate-containing compounds unavoidable with destructive techniques and the concept of most intracellular Pi being in a bound state and therefore not detectable by P-31 NMR, which measures more mobile chemical species. From our data, it appears likely that a portion of Pi detected by chemical extraction of kidney tissue is derived from urine phosphate, which does not resonate in situ at the same frequency as intracellular Pi because of the different chemical environments. It should be stressed that this observation only partially explains the discrepancy in Pi concentration measurement between in vivo P-31 NMR and destructive methods applied to the kidney, and cannot be extrapolated to other organs.

The P-31 NMR spectra obtained in this study were performed on animals which had their kidneys exposed. However, noninvasive NMR techniques are available that could allow for in situ renal spectroscopy without surgery (40–42). A great problem with these techniques is the certainty that the spectra obtained is indeed derived from the area of interest. It seems possible that the presence of this urine phosphate peak might complement the absence of phosphocreatine signal in assuring the spectroscopist that the spectra is indeed derived from the kidney. In particular, this may be helpful in human renal P-31 NMR spectroscopy. Moreover, if the findings presented here are confirmed in human obstruction, the rapid development of these spectral changes with obstruction suggests a potential clinical application. Human P-31 NMR spectroscopy studies of patients afflicted with ureteral obstruction are therefore awaited with great interest.

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