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Natural Abundance ^{17}O NMR Spectroscopy of Rat Brain *In Vivo*

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Abstract

Oxygen is an abundant element that is present in almost all biologically relevant molecules. NMR observation of oxygen has been relatively limited since the NMR-active isotope, oxygen-17, is only present at a 0.037% natural abundance. Furthermore, as a spin 5/2 nucleus oxygen-17 has a moderately strong quadrupole moment which leads to fairly broad resonances ($T_2^* = 1 - 4$ ms). However, the similarly short T_1 relaxation constants allow substantial signal averaging, whereas the large chemical shift range (> 300 ppm) improves the spectral resolution of ^{17}O NMR. Here it is shown that high-quality, natural abundance ^{17}O NMR spectra can be obtained from rat brain *in vivo* at 11.74 T. The chemical shifts and line widths of more than 20 oxygen-containing metabolites are established and the sensitivity and potential for ^{17}O -enriched NMR studies are estimated.

Keywords

^{17}O NMR; natural abundance; rat brain; high magnetic field

INTRODUCTION

Oxygen is one of the most abundant elements in nature and is present in almost all biologically relevant molecules. The oxygen atom exists in three stable isotopes, ^{16}O , ^{17}O and ^{18}O , of which only ^{17}O has a nuclear spin. NMR observation of oxygen has been relatively limited since ^{17}O is only present at a 0.037% natural abundance. Furthermore, as a spin 5/2 nucleus ^{17}O has a moderately strong quadrupole moment ($Q = -2.578 \times 10^{-26} \text{ cm}^2$) which leads to fairly short T_2 relaxation times and hence broad resonances. A significant fraction of the oxygen-17 NMR literature is dedicated to solid state NMR studies describing the effects of quadrupole coupling constants and asymmetry parameters on the chemical shift. In the liquid state, the quadrupole interaction is averaged to zero and the chemical shift is determined by chemical shielding and scalar coupling interactions. However, in the liquid state the field-independent quadrupolar relaxation pathway is still the most dominant relaxation mechanism. See (1,2) for reviews.

Despite the apparently unfavorable characteristics NMR detection of oxygen-17 in the liquid state is highly desirable. The chemical shift of oxygen-17 NMR is very sensitive to pH and ion binding as the oxygen nucleus is often an integral part of the protonation or binding site. The T_1 and T_2 relaxation rates of oxygen are linearly dependent on the rotation correlation time, making oxygen-17 NMR sensitive to rotational mobility. NMR detection of oxygen-17 is aided by the fact that the oxygen-17 NMR sensitivity ($|\gamma|^3 I(I+1)$) is relatively high (2.9% of ^1H), which is especially relevant when the low natural abundance can be overcome by ^{17}O -

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enrichment methods. Despite the large line widths, NMR resonances from different oxygen groups are readily differentiated since the chemical shift range spans over 1000 ppm for all compounds and over 300 ppm for biologically relevant metabolites. Finally, the favorable T_2^*/T_1 ratio allows substantial signal averaging which can further improve the NMR detection of oxygen-17.

Here it is shown that high-quality, natural abundance ^{17}O NMR spectra can be obtained from rat brain *in vivo* at 11.74 T. Multiple resonances are readily detected and are assigned based on the chemical shift positions of over 20 compounds measured *in vitro*. The strong pH dependence of the oxygen-17 NMR chemical shift of a selected number of compounds is demonstrated after which the sensitivity of ^{17}O and ^1H NMR detection *in vivo* is compared.

MATERIALS AND METHODS

All *in vivo* experiments were performed on a 11.74 T Magnex magnet (Magnex Scientific Ltd, Oxford, UK) interfaced to a Bruker Avance spectrometer (Bruker Instruments, Billerica, MA) equipped with 9.0 cm diameter Magnex gradients capable of switching 395 mT/m in 180 μs . RF transmission and reception was performed with a two-turn 14 mm diameter surface coil tuned to the oxygen-17 NMR frequency (67.76 MHz). Two 20 mm diameter surface coils tuned to the proton NMR frequency (499.8 MHz) and driven in quadrature were used for MR imaging and shimming.

Three male Sprague-Dawley rats (218 ± 16 g, mean $\pm$ SD) were prepared in accordance to the guidelines established by the Yale Animal Care and Use Committee. The animals were tracheotomized and ventilated with a mixture of 70 % nitrous oxide and 28.5 % oxygen under 1.5 % isoflurane anesthesia. A femoral artery was cannulated for monitoring of blood gases (pO_2 , pCO_2), pH and blood pressure. Physiological variables were maintained within normal limits by small adjustments in ventilation ($\text{pCO}_2 = 33\text{--}45$ mm Hg; $\text{pO}_2 > 120$ mm Hg; pH = 7.20–7.38; blood pressure = 90–110 mm Hg). After all surgery was completed, anesthesia was maintained by 0.3 - 0.7 % isoflurane in combination with 70 % nitrous oxide. During NMR experiments animals were restrained in a head holder, while additional immobilization was obtained with d-tubocurarine chloride (0.5 mg/kg/40 mins, i.p.). The core temperature was measured with a rectal thermosensor and was maintained at 37 ± 1 °C by means of a heated water pad.

The magnetic field homogeneity was optimized over a $5 \times 5 \times 5$ mm cubic volume by manual adjustment of all first order shims using a STEAM localization method, typically resulting in 20 - 25 Hz proton water line widths *in vivo*. Oxygen-17 signal was acquired with a pulse-acquire sequence (20 μs 90° pulse) over a 60 kHz spectral width (512 complex acquisition points). The transmitter offset was typically set around 300 ppm downfield from the water resonance. Circa 1.5 million averages (180 blocks of 8,192 averages) were acquired with TR = 15 ms, leading to a measurement time of circa 6 hours. Post-mortem ^{17}O NMR spectra were acquired overnight with circa 3.5 million averages (14 hours). Processing included frequency alignment and summation of the 180 individually stored FIDs, zero-filling to 32,768 points, exponential multiplication (20 Hz line broadening), Fourier transformation and zero- and first-order phase correction.

All *in vitro* experiments were performed on a 500 MHz vertical bore high-resolution NMR system (Bruker Instruments, Billerica, MA) using a modified version of a standard Bruker $^1\text{H}/^{13}\text{C}$ probe. The modification entailed a permanent retuning of the ^{13}C coil to the ^{17}O NMR frequency. With the aid of an external tuning circuit (3) sufficient ^2H sensitivity could be maintained to allow manual shimming on the 5% D_2O signal. As a result of this particular design choice, no lock could be applied during acquisition of ^{17}O NMR signals.

However, the effect of temporal magnetic field variation was minimized by separately storing ^{17}O FIDs (8,192 averages) and performing a post-acquisition frequency alignment of the ^{17}O water resonance.

All ^{17}O NMR spectra were obtained from 200 mM samples in pure water. Chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) at 98+% purity. The 5% D_2O added in the earlier experiments was omitted in the final experiments because (1) the presence of deuterium led to increased ^{17}O line widths and because (2) the shimming remained constant over the nearly identical samples (1.0 mL in a 5.0 mm NMR tube, ^1H water line width = 8.0 ± 0.9 Hz over 35 samples). ^{17}O NMR spectra were acquired with a pulse-acquire sequence ($8 \mu\text{s}$ 90° pulse) over a 71.5 kHz spectral width (2,048 complex acquisition points). The transmitter offset was typically set around 300 ppm downfield from the water resonance. Depending on the compound between 32 and 128 blocks of 8,192 averages were acquired with $\text{TR} = 30$ ms, leading to measurement times between circa 2 and 8 hours. Processing included frequency alignment and summation of the 32 to 182 individually stored FIDs, zero-filling to 32,768 points, exponential multiplication (10 Hz line broadening), Fourier transformation and zero- and first-order phase correction.

To compare the ^1H and ^{17}O NMR sensitivities of the water signal on rat brain *in vivo*, two different MR methods were implemented, namely a 3D MRSI method and a single-volume STEAM sequence. The 3D MRSI scan was executed with a $500 \mu\text{s}$ adiabatic half passage excitation pulse, followed by a $500 \mu\text{s}$ phase-encoding gradient and signal acquisition. Oxygen-17 and proton signal was acquired over a $\text{FOV} = 24 \times 24 \times 24$ mm (data matrix = $24 \times 24 \times 24$) with $\text{TR} = 20$ ms and 120 ms, respectively. The proton TR was purposely made very short in order to decrease the overall experimental duration and to allow a similar receiver gain setting as the oxygen-17 scan. A T_1 saturation correction factor was applied post-acquisition. The total duration of the ^{17}O and ^1H 3D MRSI scans was identical (circa 60 min). A single MRSI voxel from each dataset positioned at an equal distance from the coil was used for the signal-to-noise determination.

The STEAM method was executed with $320 \mu\text{s}$ Gaussian excitation pulses and $\text{TE} = 1.76$ ms, $\text{TM} = 0.88$ ms and $\text{TR} = 4T_1$ for both nuclei. The number of averages was adjusted to give a total acquisition time of 120 s. Despite the short TE and TM times, relaxation correction factors for oxygen-17 were significant ($T_1 = 4.92 \pm 0.23$ ms measured with a 6-point inversion recovery method, $T_2 = 3.01 \pm 0.31$ ms measured with a 5-point spin-echo method, mean $\pm$ SD as measured over three animals). The voxel location was chosen at an equal distance from the coil in both scans. ^1H NMR signal was acquired with a separate single-turn, 14 mm diameter surface coil tuned to the proton NMR frequency (499.8 MHz). In order to ensure consistent signal-to-noise ratio (SNR) determination, the acquisition time was set to $3T_2^*$ for all measurements. No post-acquisition line broadening was used. The SNR was determined as the peak height over the root-mean-square noise level of the absorption NMR spectrum. The ^1H -to- ^{17}O NMR sensitivity of the water signal was calculated as $S(^1\text{H})/[2 \times S(^{17}\text{O}) \times \text{NA}]$, where $S(^1\text{H})$ and $S(^{17}\text{O})$ are the T_1 and T_2 -corrected signal intensities of the proton and oxygen-17 signals and NA is the oxygen-17 natural abundance ($= 0.037\%$). The factor of 2 accounts for the two protons against one oxygen atom in water.

RESULTS

Figure 1 shows pulse-acquire ^{17}O NMR spectra from rat brain *in vivo* (top) and post mortem (bottom). The spectra represent the total of circa 1.5 million (*in vivo*) and 3.5 million (post mortem) averages, totaling 6 and 14 hours, respectively. Besides the limited active volume of the surface coil no additional localization was applied. The post mortem spectrum was acquired from pure brain tissue, following the removal of all extracranial tissues and skull. The

oxygen-17 NMR resonances were assigned to specific metabolites based on chemical shift measurements of pure compounds *in vitro* (see Figure 2 and Table 1). Furthermore, the expected changes in metabolite levels post mortem confirmed the assignments of phosphocreatine, lactate and inorganic phosphate. The average SNR (peak height over root-mean-square noise) ratios of taurine and phosphocreatine were 6.1 ± 1.2 and 4.3 ± 1.4 (mean $\pm$ SD over 3 animals), respectively.

Figure 2 shows ^{17}O NMR spectra of cerebral metabolites acquired *in vitro* at 298 K and pH 7.2. The chemical shifts and line widths, as extracted by Lorentzian line fitting of these ^{17}O NMR spectra, are summarized in Table 1. With water placed at 0.0 ppm, the biologically relevant nuclei are roughly grouped in three chemical shift regions; oxygen in phosphate groups (90 - 120 ppm), sulfate groups (170 - 180 ppm) and carbonyl groups (260 - 290 ppm). Oxygen in hydroxyl groups (e.g. glucose) were not observable at physiological temperatures, confirming the previously described results of Gerothanassis et al (4). Spectral line widths varied strongly, from <150 Hz for acetate, inorganic phosphate, phosphocreatine and taurine to >250 Hz for glutamate, glutamine and NAA. The chemical shift separation of the different oxygen-containing groups in ATP and glutathione is small, such that a broadened peak containing all singlet resonances is observed. Scalar coupling between phosphorus-31 and oxygen-17 nuclei could be visually observed in inorganic phosphate, phosphocreatine, phosphocholine and phosphoethanolamine. While absolute determination of $^1\text{J}_{\text{PO}}$ is difficult due to the broad resonance lines, $^1\text{J}_{\text{PO}}$ was estimated to be in the range 80 - 120 Hz. It is interesting to note that the ATP resonances completely disappeared in the presence of magnesium ($\text{ATP}:\text{Mg}^{2+} = 1:1$) ions, demonstrating the sensitivity of ^{17}O NMR towards rotational mobility.

The chemical shift of many compounds shown in Figure 2 display a strong pH dependence around the pK of the corresponding chemical group. Figure 3 shows the pH dependence of the chemical shift of acetate (Figure 3A), bicarbonate (Figure 3B) and inorganic phosphate (Figure 3C). The dots represent the measured data, referenced relative to water, whereas the solid line represents the best fit to a Henderson-Hasselbach relationship. The fitted pK, protonated chemical shift δ_{HA} (in ppm) and unprotonated chemical shift δ_{A} (in ppm) are (pK, δ_{HA} , δ_{A}) = (4.60, 257.8, 281.3) for acetate, (9.99, 171.1, 190.3) for bicarbonate and (6.65, 86.5, 98.1) for inorganic phosphate. The large oxygen-17 chemical shift difference of $98.1 - 86.5 = 11.6$ ppm for inorganic phosphate compared to the 2.5 ppm difference obtained by ^{31}P NMR (5) demonstrates the superb sensitivity of oxygen-17 NMR to pH. Note that the chemical shift of bicarbonate below a pH of circa 6.0 (gray line in Figure 3B) could not be reliably determined due to the low signal-to-noise ratio as a result of excessive carbon dioxide boil-off. Although the chemical shift of many other compounds, like glutamate, is constant in the physiological pH range, the chemical shift shows a strong dependence around the corresponding pK value as reported by Gerothanassis et al (6). The maximum signal intensity in the post mortem spectrum resonates at 91.6 ppm. Under the assumption that the inorganic phosphate is the dominant contributor to this signal, the intracellular pH can be calculated as 6.54.

Finally, when comparing the signal-to-noise ratio of the water resonance in ^1H and ^{17}O NMR spectra of rat brain *in vivo* acquired over the same time span (5 minutes) and with comparable acquisition parameters, it appeared that the ^1H and ^{17}O NMR sensitivity per detected nucleus was roughly similar. This somewhat unexpected finding warranted additional experiments, as detailed in the Materials and Methods section, to compare the ^1H and ^{17}O NMR sensitivities. When the experimentally determined SNRs are scaled for the number of detected nuclei, the ^1H -to- ^{17}O sensitivity ratio (i.e. $\text{SNR}_{^1\text{H}}/\text{SNR}_{^{17}\text{O}}$) for water in rat brain *in vivo* becomes 3.9 and 3.4 for the 3D MRSI and single voxel scans, respectively.

DISCUSSION

Here the feasibility of acquiring natural abundance ^{17}O NMR spectra from rat brain *in vivo* has been demonstrated. A large number of resonances could be readily detected in the time span of hours.

While the low absolute sensitivity of natural abundance ^{17}O NMR spectroscopy may limit its applications, the high NMR sensitivity (SNR per detected nucleus) together with the wide chemical shift range opens the door to a variety of experiments with ^{17}O -enriched compounds. Zhu et al (7) have shown that ^{17}O NMR in combination with inhalation of enriched $^{17}\text{O}_2$ gas allows the quantitative measurement of the cerebral metabolic rate of oxygen (CMRO_2) in animal and human brain *in vivo*. Incorporating the ^{17}O isotope in molecules like glucose or acetate would allow the detection of a wide range of metabolic pathways with a greatly enhanced sensitivity when compared to the more established ^{13}C NMR methods.

The majority of ^{17}O NMR spectra presented here were acquired with pulse-acquire methods for simplicity and to maximize the sensitivity. However, several more sophisticated NMR methods can be implemented to further improve the sensitivity or spatial specificity. While the short T_2 relaxation times of ^{17}O -containing molecules prevent the use of typical spatial localization methods like PRESS, the similarly short T_1 relaxation times allow the rapid acquisition of 3D MRSI data, as performed here and by others (7,8).

While heteronuclear ^1H - ^{17}O scalar coupling could not be resolved for any of the >20 metabolites studied, it may still lead to significant line broadening. For water this is especially true around neutral pH levels where the proton exchange rate is relatively slow. Broadband proton decoupling is expected to lead to significant enhancements in spectral resolution as has already been shown by Earl and Niederberger (9) for water. In all ^{31}P containing compounds (except for GPC due to the broader lines) scalar coupling between ^{31}P and ^{17}O nuclear spin could be visually resolved. Broadband ^{31}P decoupling should give close to a factor of two improvement in SNR for those compounds.

Despite the low gyromagnetic ratio ($\gamma_{^{17}\text{O}} = 0.136\gamma_{^1\text{H}}$) the sensitivity of ^{17}O NMR is high and was experimentally determined to be 3 to 4 times lower than the ^1H NMR sensitivity. While the sensitivity of ^1H NMR largely originates from the high gyromagnetic ratio, the sensitivity of ^{17}O NMR is increased due to the high spin ($I = 5/2$) and the favorable T_2^*/T_1 ratio. The fact that the sample (i.e. rat head) presents a moderate load to the ^{17}O NMR coil ($Q_{\text{unloaded}} = 175.3$, $Q_{\text{loaded}} = 115.9$) suggests that, at 11.74 T, the coil no longer represents the dominant noise source. In that case NMR sensitivity analysis (10) predicts that the sensitivity of ^{17}O above 11.74 T will increase approximately linear with magnetic field strength. At lower magnetic fields, where the coil loading is less, the sensitivity should increase quadratically with magnetic field as has been verified experimentally by Zhu et al (8). Furthermore, since the sample becomes the dominant noise source at 11.74 T, a substantial increase in the SNR of the detected ^{17}O NMR signal can not be expected upon cooling of the ^{17}O NMR coil and preamplifier to liquid nitrogen temperatures (11).

One caveat that requires detailed further study is the *in vivo* NMR visibility of oxygen-17. As a spin 5/2 nucleus, oxygen-17 exhibits a quadrupole moment. Depending on the rotational mobility of the oxygen-containing molecule several of the single-quantum transitions may become so broad that they can no longer be distinguished from the spectral baseline (12,13). In addition to short relaxation times, the ^{17}O NMR resonances may also be broadened by exchange processes (12,13).

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Figure 1. Pulse-acquire ^{17}O NMR spectra from rat brain *in vivo* (top) and post mortem (bottom). The spectra represent the total of circa 1.5 million (*in vivo*) and 3.5 million (post mortem) averages, totaling 6 and 14 hours, respectively. Besides the limited active volume of the surface coil no additional localization was applied. The post mortem spectrum is acquired from pure brain tissue, following the removal of all extracranial tissues and skull.

Figure 2. Pulse-acquire ^{17}O NMR spectra from 200 mM solutions *in vitro* of the indicated metabolites. Depending on the compound, the ^{17}O NMR spectra represent between 2 and 8 hours of signal averaging. Chemical shift referencing as well as amplitude scaling was performed relative to the water signal as 0.0 ppm. For display purposes the water resonance has been removed from all metabolite ^{17}O NMR spectra by a SVD algorithm.

Figure 3.

pH dependence of the chemical shift of (A) acetate (NaCH_3COO in water), (B) bicarbonate (NaHCO_3 in water) and (C) inorganic phosphate (NaH_2PO_4 in water). The solid dots represent the measured data ($T = 298 \text{ K}$), whereas the solid line represent the best fit to a standard Henderson-Hasselbach pH relationship. Determining the chemical shift of bicarbonate at $\text{pH} < 6.0$ (gray solid line) was difficult due to excessive carbon dioxide boil-off and hence low signal-to-noise of the remaining, dissolved carbon dioxide.

Table 1Chemical shifts, line widths and multiplicities for ^{17}O -containing cerebral metabolites ^a

Compound	Group	Chemical shift (ppm)	Line width (Hz)	Multiplicity ^b
Acetate ^c	$^1\text{COO-}$	281.2	130	s
<i>N</i> -acetyl aspartate (NAA)	$^1,^4\text{COO-}$	279.1	450	s
	$^1\text{C=O}$	265.9	410	s
	P=O	103.9	800	m
Adenosine triphosphate (ATP) ^c	$^1\text{COO-}$	265.5	230	s
Alanine	$^1\text{COO-}$	276.8	300	s
γ -amino-butyric acid (GABA)	$^1\text{COO-}$	266.7	270	s
Aspartate	$^4\text{COO-}$	280.3	350	s
	HCO_3^-	171.1	130	s
Bicarbonate ^c	$^1\text{COO-}$	270.4	400	s
Creatine	$^1\text{COO-}$	268.2	370	s
Glutamate	$^5\text{COO-}$	277.4	270	s
	$^1\text{COO-}$	270.7	350	s
	$^5\text{C=O}$	283.1	320	s
Glutamine	CO	271.8	740	m
Glutathione	P=O	85.3	420	d
Glycerophosphocholine	P=O	95.5	210	d
Inorganic phosphate (P_i) ^c	$^1\text{COO-}$	260.5	270	s
Lactate	$^1\text{COO-}$	270.6	380	s
Phosphocreatine	P=O	117.8	210	d
	P=O	99.1	270	d
Phosphocholine	P=O	99.1	270	d
Phosphoethanolamine	S=O	173.2	90	s
Taurine	H_2O	0.0	100	s
Water				

^a All chemical shifts are referenced against water at 0.0 ppm.^b Multiplicities are defined as: singlet (s), doublet (d), multiple resonances (m).^c Chemical shift displays a pH-dependence in the physiological pH range. See text for more details.