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Homonuclear ^1H double-resonance difference spectroscopy of the rat brain *in vivo*

(brain metabolism/NMR/glutamate/γ-aminobutyric acid)

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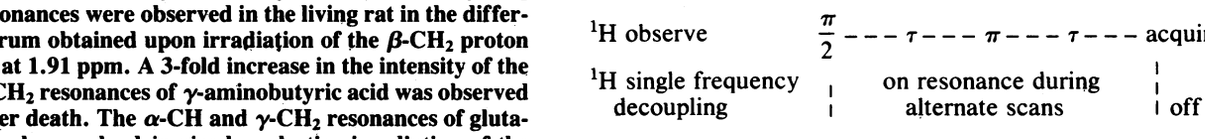
ABSTRACT **We have used ^1H homonuclear double-resonance difference spectroscopy at 360.13 MHz to resolve specific metabolite resonances in the brains of intact rats. Metabolite resonances resolved include previously obscured proton resonances of alanine, γ-aminobutyric acid (GABA), glutamate, and taurine. The γ-aminobutyric acid α- and γ-CH_2 proton resonances were observed in the living rat in the difference spectrum obtained upon irradiation of the β-CH_2 proton resonance at 1.91 ppm. A 3-fold increase in the intensity of the α- and γ-CH_2 resonances of γ-aminobutyric acid was observed 30 min after death. The α-CH and γ-CH_2 resonances of glutamate were also resolved *in vivo* by selective irradiation of the β-CH_2 protons to which they are spin-coupled. In addition, this technique was used to observe the β-CH_3 protons of lactate through the intact scalp of a rat. Large lipid signals arising from scalp tissue were eliminated in the difference spectrum, revealing the lactate β-CH_3 resonance.**

^1H NMR spectra of the brain in the living rat and rabbit have shown that it is possible to utilize the high sensitivity of ^1H NMR to detect changes in concentration of substrates, intermediates, and products of carbohydrate and amino acid metabolism (1–3). Studies of the rat brain (1, 2) at 360.13 MHz in which the intense H_2O peak was suppressed have revealed the resonances of numerous metabolites including aspartate, *N*-acetylaspartate, glutamate, glutamine, and lactate. The intense proton resonances of lipids contained in extracranial tissues (skin and muscle) were eliminated in these studies by surgically removing those tissues from the sensitive volume of the surface coil. Despite the good spectral resolution obtained with cerebral tissues, the resonances of many metabolites cannot be adequately resolved in the ^1H NMR spectrum due to their overlap with the resonances of endogenous brain lipids or other metabolites. The much more intense fatty acid peaks of other mammalian organs (4, 5) have presented even more serious problems of resolution. Recently, we described the use of ^1H homonuclear double-resonance difference spectroscopy to separate overlapping resonances in AX-spin systems (two spin-coupled resonances where $\delta >> J$) in a variety of lipid-containing excised tissues (6). Here we report the use of this technique to resolve specific metabolite resonances in the intact rat brain both before and after death. This technique has enabled us to resolve the α- and γ-CH_2 resonances of γ-aminobutyric acid (GABA) *in vivo* and to follow their postmortem increase.

METHODS

Spectroscopy. High-resolution ^1H NMR spectra were obtained using a WH 360 wide-bore spectrometer (Bruker Instruments, Billerica, MA) operating at 360.13 MHz for ^1H in the pulsed Fourier-transform mode. The ^1H channel of a double-tuned $^1H/^{31}P$ surface-coil probe was used in these experiments. The coil and probe circuitry has been described elsewhere (2, 7).

Spectra were acquired using a homonuclear double-resonance difference sequence (6):



Multiplet resonances, which arise from spin–spin (or *J*) coupling to neighboring protons, are phase-modulated relative to a singlet resonance (8, 9). This phase modulation can be inhibited by single-frequency irradiation of the resonance to which the multiplet resonance is coupled (10). In the pulse and decoupling sequence given above, the phase modulation of a multiplet resonance is removed by single-frequency irradiation during alternate blocks of scans. A delay τ is chosen ($1/2J$ for doublets and quartets, $1/4J$ for triplets) that, in the absence of decoupling, results in the selected multiplet being π radians out of phase relative to a singlet. Subtraction of alternate blocks of scans produces a spectrum containing only the decoupled multiplet. Since the proton–proton couplings are small (3–10 Hz), low decoupling power is sufficient. Hence, the off-resonance decoupling is limited to ≈0.2 ppm, which retains most of the selectivity.

^1H spectra were acquired with 4096 data points and a spectral width of 7000 Hz. Magnetic field homogeneity was optimized by shimming on the tissue water resonance. The pulse length that gave the greatest tissue water signal intensity was used for the $\pi/2$ pulse (25–30 μsec). The large tissue water resonance was presaturated by 1–2 sec of 50 mW single-frequency irradiation (11). Single-frequency irradiation of 20 mW was used for homonuclear ^1H decoupling during the spin–echo delay periods. The decoupling was switched off-resonance to 8.00 ppm during alternate blocks of eight scans. The phase of the π pulse was cycled 180° (+x, −x) relative to the $\pi/2$ pulse (+x) to eliminate phase and intensity errors due to H_1 inhomogeneity (12, 13). Blocks of scans obtained with and without decoupling were stored in separate memory locations. Chemical shift values were assigned relative to the methyl resonance of the *N*-acetyl moiety of *N*-acetylaspartate at 2.023 ppm (1–3).

Animal Preparation. Rats (Charles River strain, 220–240 g) were tracheotomized under enflurane anesthesia and ventilated with 33% O_2/67% N_2O. Extracranial tissues were retracted from the calvarium as described (2) unless noted otherwise (see Fig. 4). Enflurane anesthesia was discontinued 30–50 min prior to collection of spectra; ventilation with 33% O_2/67% N_2O was continued. Death due to anoxia was brought about by ventilation with 100%N_2O. The electrocar-

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Abbreviation: GABA, γ-aminobutyric acid.

diogram was recorded through wire leads (electrically filtered) to the left extremities. The surface coil was positioned over the exposed calvarium.

RESULTS

A ^{1}H NMR double-resonance difference spectrum of the brain of a live rat is shown in Fig. 1 (spectrum A). Coherent radiofrequency irradiation was applied to decouple the β-CH_2 resonance of glutamate at 2.10 ppm. A τ delay of 34 msec was used, which is approximately $1/4J$ for the first-order triplet couplings of the α-CH and γ-CH_2 resonances of glutamate. The difference spectrum contains only the α-CH and γ-CH_2 resonances of glutamate, at 3.77 and 2.35 ppm, respectively. In contrast to the difference spectrum, the glutamate resonances in the 34 msec τ spin–echo spectrum (Fig. 1, spectrum B) are difficult to resolve because of spectral overlap and phase modulation due to spin–spin coupling.

When the irradiation was applied to the β-CH_2 of GABA at 1.91 ppm in the ^{1}H spectrum, the triplet resonances of the γ- and α-CH_2 protons of GABA were detected in the difference spectrum at 3.01 and 2.27 ppm, respectively (Fig. 2*b*). After complete anoxia (0% O_2/100% N_2O) in the rat, both GABA resonances increased, reaching 3 times the control values 30 min after death (Fig. 2*a*). GABA levels rise during the anaerobic conditions incurred postmortem because complete catabolism of GABA would require the oxidation of succinic semialdehyde to succinate—a step that is blocked by anoxia (16, 17). The α- and γ-CH_2 resonances of GABA are resolved cleanly in the difference spectrum (Fig. 2*b*) in contrast to the 34-msec τ spin–echo spectrum (Fig. 2*c*) where they are overlapped by more intense resonances, due mainly to the methyl protons of creatine (-HN-CH_3, 3.04 ppm) and to the methylene protons of glutamate (γ-CH_2,

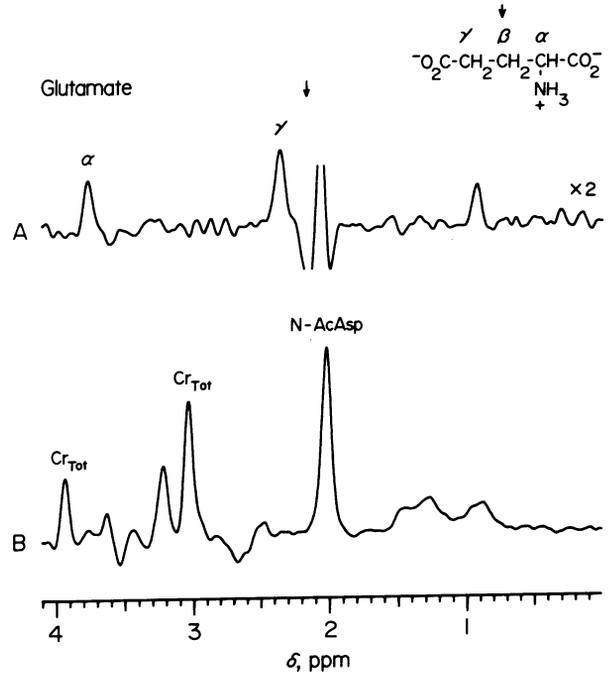

FIG. 1. Spectrum A: ^{1}H NMR double-resonance difference spectrum of the glutamate α-CH and γ-CH_2 proton resonances in the brain of a live rat. Single-frequency irradiation was applied to decouple the β-CH_2 proton at 2.10 ppm (arrow). The difference spectrum is the result of 64 transients with a 2-sec recycling time. The resonance at 0.9 ppm was not identified. Spectrum B: 34-msec τ spin–echo spectrum that is the nonirradiated component of the difference spectrum A. Resonances of creatine and phosphocreatine (unresolved, Cr_{tot}) and *N*-acetylaspartate (N-AcAsp) are labeled. The 34-msec τ delay ($1/4J$) results in the outer lines of the α and γ triplets being 180° out of phase relative to the center lines. Because the spin coupling (≈7 Hz) is less than *in vivo* line widths (≈25 Hz), the intensities of the triplets in spectrum B are reduced. In the decoupled spectrum (not shown), these lines are in phase. Hence, the difference spectrum (spectrum A) is the sum only of the outer lines of the α-CH and γ-CH_2 triplets. The center lines cancel during subtraction since they have the same phase in both the decoupled and nondecoupled subspectrum. Broad components in spectrum B were removed by Gaussian multiplication of the free induction decay (14) and convolution difference (15). Identical processing was used for spectrum A, although resolution enhancement was not required.

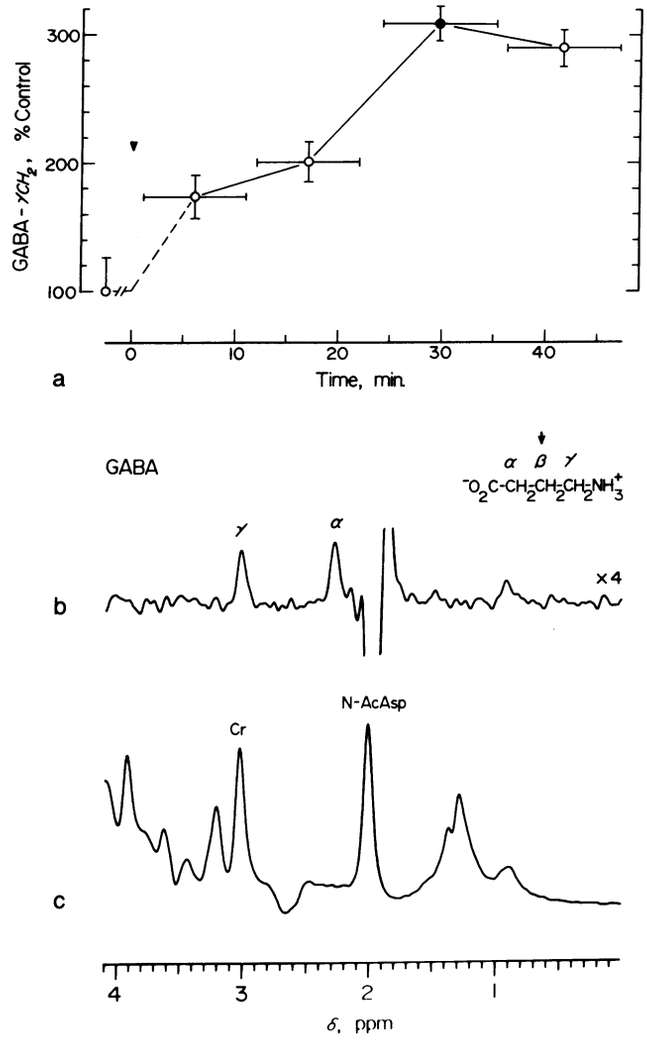

FIG. 2. (*a*) Relative intensities of the GABA γ-CH_2 resonances (δ 3.01 ppm) are plotted as a function of time after terminal anoxia was initiated (arrowhead). The resonance areas in the difference spectrum were measured with a planimeter. The GABA intensity measured while the rat was alive is assigned a value of 100 and the intensities measured postmortem are scaled relative to this value. Horizontal bars on plot points indicate the time per spectrum. Vertical bars show the intensity uncertainty derived from rms noise in each spectrum. (*b*) Difference spectrum of GABA obtained 29 min after terminal anoxia. The corresponding point in *a* is indicated by a filled circle (●). The spectrum is the result of 256 transients with a 2-sec recycling time. A τ delay of 34 msec was used (≈$1/4J$ for the GABA α-CH_2 and γ-CH_2 triplet resonances). Single-frequency irradiation was applied at 1.91 ppm to decouple the β-CH_2 protons. (*c*) The 34-msec τ spin–echo spectrum which is the nonirradiated component of the difference spectrum. Note that the γ-CH_2 resonance of GABA has been resolved in the difference spectrum in *b* from the strong overlapping creatine resonance (Cr) at 3.03 ppm. Spectra in *b* and *c* were processed as were the spectra in Fig. 1. N-AcAsp, *N*-acetylaspartate.

2.35 ppm) and glutamine (β-CH_2, 2.11 ppm).

We have identified several other metabolite resonances in the intact rat brain using the double-resonance difference sequence. These resonances include those due to the protons of the β-CH_2 of taurine (-CH_2-NH_3^+) (Fig. 3, spectrum B), the β-CH_2 of glutamate (Fig. 3, spectrum A), the β-CH_3 of alanine (Fig. 3, spectrum D), and the β-CH_3 of lactate (Fig. 3, spectrum E). The ^1H resonances of the β-CH_2 protons of glutamate and the β-CH_3 protons of alanine were obtained in the same difference spectrum because they are both coupled to α-CH groups, which resonate near 3.77 ppm. The taurine β-CH_2, alanine β-CH_3, and glutamate β-CH_2 resonances cannot be resolved in an ordinary spin–echo spectrum (Fig. 3, spectrum F) because of resonance overlap but are well resolved in the double-resonance difference spectra.

The phase modulation of multiplet resonances in the intact rat brain will deviate from the values predicted for a spin–echo sequence with $\pi/2$ and π radian pulse angles due to the H_1 inhomogeneity of a surface coil (9). The effect of imperfect pulses will be that the phase-modulated lines of a multiplet will not be completely inverted in the nonirradiated subspectrum. Since the irradiated subspectrum is not affected by spin–spin coupling, the intensity reduction in the difference spectrum arises entirely from the nonirradiated subspectrum.

We have observed, however, that for the resonances of glutamate γ-CH_2 and lactate β-CH_3, both of which can be partially resolved in either the nonirradiated or irradiated subspectrum and thereby compared with the difference spectrum, the intensity reduction in the difference spectrum was at most 20%. The good multiplet inversion that was obtained is shown by the intensity of the inverted lactate β-CH_3 resonance (Fig. 3, spectrum F), which is only 8% less than the intensity of the lactate β-CH_3 resonance in the corresponding difference spectrum (Fig. 3, spectrum E). By measuring the intensity difference one can calculate that the inverted lactate β-CH_3 resonance in Fig. 3 (spectrum F) achieved 85% of the intensity that would be obtained with perfect pulses (6, 9).

To evaluate the ability of this method to detect brain metabolites in the presence of lipid resonances arising from surrounding tissue, we obtained double-resonance difference spectra of a dead rat with an intact scalp. The large broad lipid signal in a pulse–acquire spectrum (Fig. 4, spectrum C) is significantly reduced in a 34-msec τ spin–echo spectrum (Fig. 4, spectrum B). In the double-resonance difference spectrum, of which Fig. 4 (spectrum B) shows the nonirradiated component, the lipid resonances have been completely eliminated (Fig. 4, spectrum A), thus revealing the lactate β-CH_3 proton signal at 1.33 ppm. Based upon re-

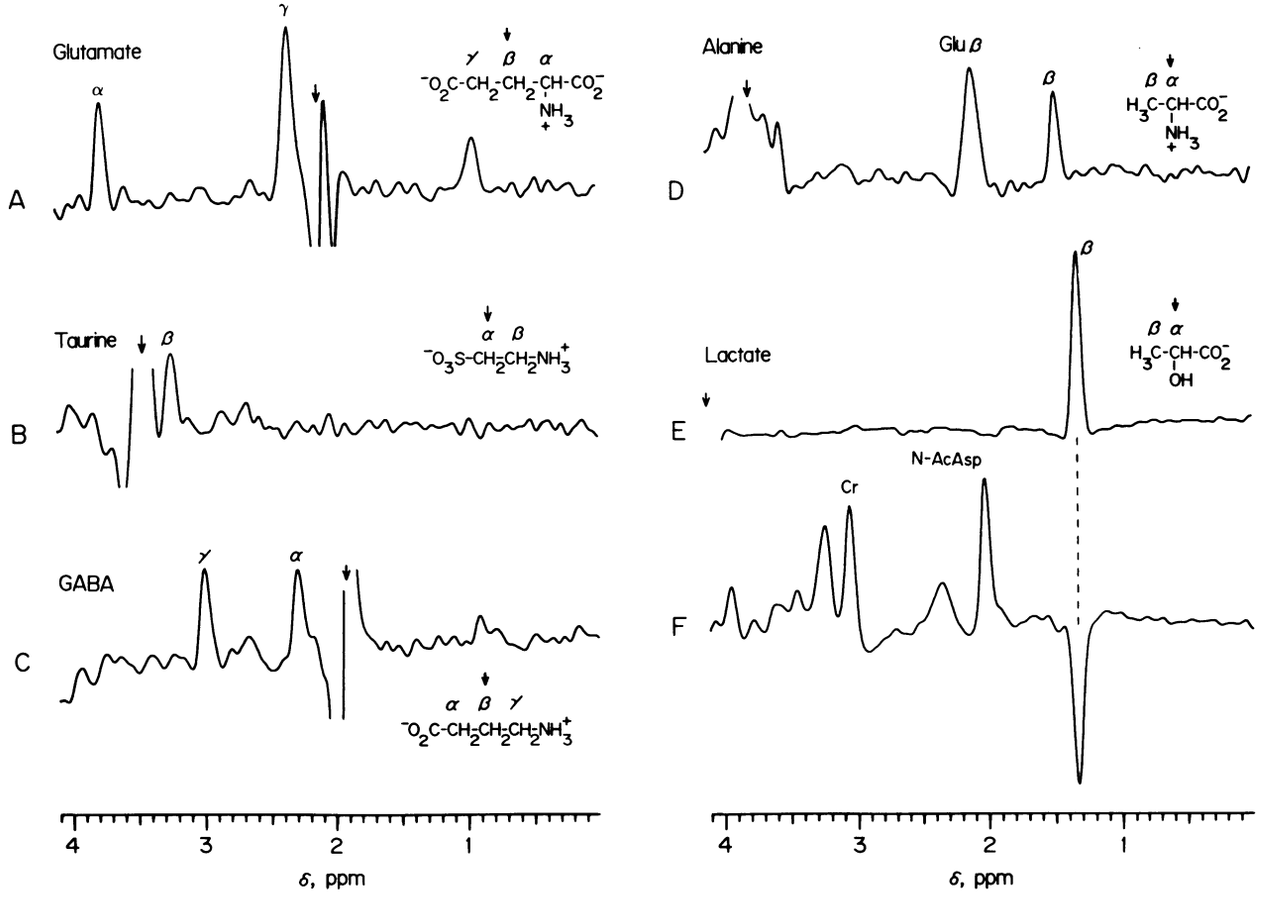

FIG. 3. ^1H double-resonance difference spectra of the brain of a dead rat. Spectra were accumulated in 64 transients with a 1.5-sec recycling time and processed as the spectra in Figs. 1 and 2. Spectra A–C show the resonances of triplets and are on the same intensity scale. They were acquired with a 34-msec τ delay ($\approx 1/4J$ for the resonances shown). Spectrum A: glutamate α-CH (δ 3.77 ppm) and γ-CH_2 (δ 2.35 ppm). Irradiation (arrow) was applied at 2.10 ppm to decouple the β-CH_2 protons. Spectrum B: taurine β-CH_2 observed at 3.26 ppm. Irradiation (arrow) was applied at 3.46 ppm to decouple the α-CH_2 protons. Spectrum C: GABA α-CH_2 (δ 2.27 ppm) and γ-CH_2 (δ 3.01 ppm). Irradiation (arrow) was applied at 1.91 ppm to decouple the β-CH_2 protons. Spectra D and E: doublet resonances. A τ delay of 68 msec was used ($1/2J$ for the alanine and lactate β-CH_3 doublet resonances). Spectrum D: alanine β-CH_3 observed at 1.48 ppm and glutamate β-CH_2 observed at 2.10 ppm. Irradiation (arrow) was applied to their α-CH resonances at 3.77 ppm. Spectrum E: lactate β-CH_3 observed at 1.33 ppm. Irradiation (arrow) was applied to the α-CH proton at 4.10 ppm. Spectrum F: the nonirradiated component of difference spectrum E. Resonance intensities in spectra E and F can be directly compared while intensities in spectrum D must be divided by 4 to be compared with those in spectrum F. Cr, creatine; N-AcAsp, *N*-acetylaspartate.

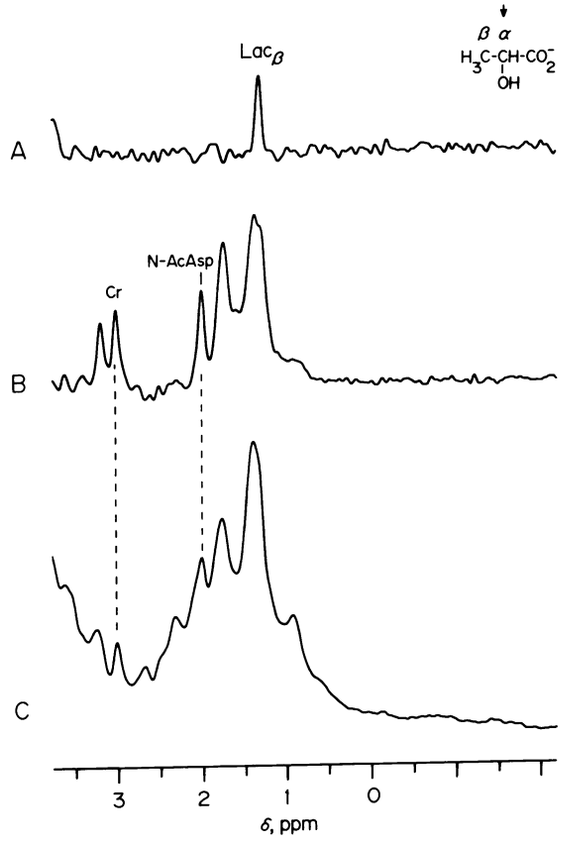

FIG. 4. ^{1}H NMR spectra of the head of a dead rat with its scalp left intact. Spectrum A: double-resonance difference spectrum obtained by irradiation at 4.10 ppm to decouple the lactate α-CH (arrow) proton resonance. The large signal at 1.33 ppm is the β-CH_3 resonance of lactate (Lac_β). Spectrum B: 34-msec τ spin–echo spectrum, which is the nonirradiated component of spectrum A. Spectra A and B are on the same intensity scale. Spectrum C: conventional acquisition (pulse–acquire–delay) spectrum obtained using the same $\pi/2$ pulse duration (15 μsec) as was used to obtain spectra A and B. All three spectra were processed identically by Gaussian multiplication of the free induction decay (14). Cr, creatine; N-AcAsp, *N*-acetylaspartate.

ported postmortem spectra (1, 3), the relative intensities of the lactate methyl proton signal in Fig. 4 (spectrum A) and the *N*-acetylaspartate methyl proton signal (2.023 ppm) in Fig. 4 (spectrum B) indicate that most of the lactate signal is from cerebral tissue.

DISCUSSION

The homonuclear double-resonance difference sequence significantly increases the number of metabolite resonances in the intact rat brain that can be resolved by ^{1}H NMR. By eliminating spectral overlap, this technique allows resonance intensity changes to be measured with greater accuracy than that achieved in prior ^{1}H NMR studies of the rat brain (1, 2). In addition, it is a powerful *in vivo* resonance assignment technique, identifying resonances by three criteria: (*i*) chemical shift, (*ii*) spin–spin coupling, and (*iii*) chemical shift of the irradiated resonance.

As shown for the lactate β-CH_3 proton resonance, good multiplet inversion occurs despite the imperfect pulses obtained from a surface coil. The good inversion observed is probably due to the "depth pulse" characteristics (12, 13) of a phase-cycled spin–echo sequence, which result in most of the observed signal coming from sample regions receiving a refocusing pulse of $\approx\pi$ radians. To improve resonance quantitation, we are developing pulse sequences that will allow a correction for incomplete multiplet inversion to be determined and applied *in vivo*.

In combination with ^{13}C-labeled substrates, the double resonance difference sequence can be used to measure metabolite fluxes by observing ^{13}C sidebands (6, 18, 19). The advantages of ^{1}H NMR over ^{13}C NMR (20, 21) are its greater sensitivity and the ability to measure both the labeled and unlabeled fractions of a metabolite—thereby allowing concentration changes to be distinguished from isotopic turnover (18).

The resolution of the lactate β-CH_3 proton resonance from fatty acid signals originating predominantly from the scalp and other extracranial tissues suggests an application of the double-resonance difference sequence in the spatial localization of resonances during ^{1}H NMR studies of humans. When looking at resonances from metabolites peculiar to the brain (e.g., *N*-acetylaspartate or GABA) the spectral resolution should improve the spatial resolution. Even at the lower field strengths available for human NMR studies (1.5–2.0 Teslas), several proton resonances of brain metabolites have enough AX character to be resolved by the double-resonance difference sequence. These resonances include those due to lactate β-CH_3, alanine β-CH_3, glutamate and glutamine α-CH and β-CH_2, and GABA γ-CH_2. However, off-resonance decoupling will be more severe at lower fields and may limit the achievable selectivity.

In summary, it has been shown that ^{1}H homonuclear double-resonance difference spectroscopy of the rat brain *in vivo* retains the high sensitivity of ^{1}H NMR while achieving a selectivity which, by taking advantage of homonuclear spin coupling, is considerably better than that obtained *in vivo* using other nuclei. These results have been obtained with a surface coil so that the method should be generally applicable to *in vivo* ^{1}H NMR studies. Taking into account the good suppression of the tissue water resonance and the high sensitivity achieved in other recent *in vivo* ^{1}H NMR studies of the rat brain (1, 2), we suggest that ^{1}H NMR can now significantly increase the range of NMR studies of metabolism.

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