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A ^{1}H NMR technique for observing metabolite signals in the spectrum of perfused liver

(ethanol metabolism/pyruvate/lactate/lactate dehydrogenase/alcohol dehydrogenase)

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ABSTRACT **We have developed a ^{1}H NMR technique to selectively edit the spectrum of perfused liver for specific resonances of metabolites that occur in low concentration. The method employs selective DANTE pulses, which avoid exciting the water signal and at the same time control the *J* modulation effect in the homonuclear spin-echo experiment. By difference spectroscopy, we have suppressed the background signals from lipids and water and have resolved the CH_3 resonance of lactate at 1.33 ppm. Moreover, the technique is highly selective and allows us to select the CH_3 resonance of alanine at 1.47 ppm in the presence of the CH_3 resonance of lactate at 1.33 ppm, even though the latter was much larger before editing. We have applied this technique to study the metabolic effect of ethanol in perfused mouse liver and have observed that the rate of formation of lactate from pyruvate is increased by a factor of 2.8 when ethanol is added.**

The potential sensitivity gain of ^{1}H NMR over other nuclei strongly indicates that low concentrations of metabolites could be detected *in vivo*. However, the initial promise of ^{1}H NMR becomes difficult to realize in some organs as the two major experimental problems appear: an intense H_2O resonance and strong overlapping background signals, particularly from lipids. Both dominate the weak metabolite signals and prevent their easy identification in the ^{1}H NMR spectrum.

Recent relaxation studies and ^{1}H editing schemes that select resonances of weakly coupled AX spin systems (A and X indicating nuclei) (1–4) have pointed the way towards solving these problems, particularly in the brain *in vivo*. However, perfused systems have large amounts of rapidly recirculating water, which is difficult to eliminate by presaturation. Moreover, liver contains lipid signals that are almost two orders of magnitude larger than the metabolite resonances, in contrast to brain, where the two kinds of signals are of comparable intensities. These severe problems in perfused liver studies have required a further refinement of ^{1}H editing techniques. We have developed a gated-DANTE sequence that overcomes these difficulties and allows us to edit the ^{1}H spectra of metabolites in perfused liver. It employs selective DANTE pulses (5), which minimally excite the H_2O resonance and are even more selective than the $1\bar{3}3\bar{1}$ pulses used recently (4). The present technique is easily adaptable to any AX spin system and any perfused organ studies.

We have applied the pulse sequence to measure enzyme kinetics of liver lactate dehydrogenase *in vivo* (6) and have focused specifically on ethanol's effect on liver metabolism (7–10). We are thereby able to easily obtain unique enzyme kinetics on the lactate dehydrogenase system. The results obtained from the gated-DANTE sequence increase the scope of ^{1}H NMR applications for studies *in vivo*.

MATERIALS AND METHODS

Materials. Male Swiss Webster mice (27–35 g; Charles River Breeding Laboratories) with access to food and water ad lib were anesthetized with an intraperitoneal injection of phenobarbital (50 mg/kg of body weight, Anthony Products, Arcadia, CA). The liver was surgically exposed and the portal vein was cannulated with a no. 22 Teflon catheter (Delmed, Canton, MA), which was connected to a flowing perfusion medium. The inferior vena cava was immediately severed and the liver was excised and placed in a 15-mm NMR tube.

The perfusion medium was a Krebs–Ringer buffer that was bubbled with a gas mixture of O_2/CO_2 (95:5) and was kept at 37°C and pH 7.4. After flushing the liver for 10 min, we switched to a recirculating system with a 50-ml reservoir, perfusing at the rate of 5 ml/g of liver. We monitored the oxygen consumption with a Yellow Springs Instrument 53 oxygen meter and the pH with a Corning 125 pH meter. The temperature of the perfusion medium was maintained with a Polyscience bath system. Pyruvate and lactate were obtained from Sigma and ethanol was from Publicker Chemical.

Methods. The basis for our pulse sequence is described in the early relaxation studies (1) and in several papers on ^{1}H editing *in vivo* (3, 4) and in solution (2, 11, 12). During the spin-echo evolution period, the *J* modulation of a homonuclear AX spin system can be influenced by applying to the coupled neighbor resonance either a continuous wave decoupling field (3, 11, 12) or a selective pulse (1, 4). A selective spin-echo sequence on the A resonance and a selective 0° or 180° pulse on the X resonance in synchrony with the selective A inversion pulse will result in the final magnetization of the A resonance being either parallel or antiparallel to all other ^{1}H magnetization (1, 2, 4). Adding and subtracting alternate spectra will give the edited spectrum. The vector diagram is illustrated in Fig. 1.

Our pulse sequence is generated by a DANTE, which is train of hard pulses with fixed interpulse delay time (5), and is centered on the resonance frequency at the A nucleus. During the interpulse delays, we turn on a second DANTE at the frequency of the X nucleus. This results in two selective pulses at two different frequencies (5). We can control the phase of each gated-DANTE pulse. Hence by alternating each pulse's phase by 180° or by maintaining each pulse's phase constant, we can obtain an equivalent of 0° or 180° pulse. In practice, the phase alternation scheme is equivalent to a 90°_x–90°_x or 90°_x–90°_{-x} pulse sandwich, which gives much better spectral subtraction than a simple no-pulse/180°-pulse alternation. To minimize subtraction errors, we cycle the

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Abbreviation: DANTE, delays alternating with nutation for tailored excitation.

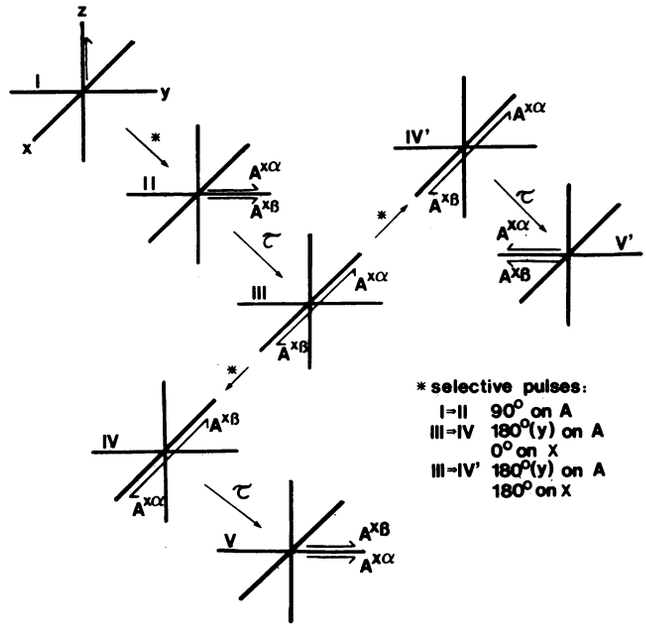

FIG. 1. Vector diagram illustrating the effect of a selective spin-echo sequence with the carrier on the A resonance: The initial 90° pulse creates transverse magnetization (II). The A magnetization coupled to X_α and X_β will precess $\pm J_{AX/2}$. After time $t = J/2$, the vectors will align antiphase along the x axis (III). If a selective 180°(y) pulse is applied only on A, then the vectors will refocus after a second time $t = J/2$ (IV, V). If the selective 180°(y) pulse on A is accompanied by a selective 180° pulse on the X, the labels of the X spin are effectively changed. In this case, the vectors will not refocus after the second $t = J/2$ (IV', V'). In fact, they will align antiphase with respect to all the other ^1H magnetization vectors. By choosing the correct delay time and by adding or subtracting the appropriate spectra, we can edit a particular AX resonance from the complex ^1H NMR spectrum.

receiver phase such that the addition and subtraction of the pertinent spectra occur while the data are being collected. Appropriate receiver phase cycling is extremely useful in achieving excellent suppression of all the other ^1H resonances in the spectrum. The pulse train is a modified Hahn spin-echo and is as follows:

A: 90°–t–180°–t–ACQ (± receiver)
X: 0°/180°,

where ACQ is acquisition.

Spectra were collected on a Bruker WH 360 spectrometer equipped with a 15 mm ^{13}C–^1H probe especially designed with an inner ^1H observe coil. The DANTE pulses had an interpulse delay time of 200 μs and the total pulse train was 20 ms long. Apodization at the natural linewidth with an exponential function improved the signal-to-noise ratio. The 3.5-kHz-bandwidth spectra were acquired with 4096 data points, which were zero-filled to 8192 before transformation. All peaks were referenced to H_2O at 4.76 ppm at 25°C.

RESULTS

Spectrum a in Fig. 2 is the ^1H NMR spectrum of a perfused mouse liver. Only a phase-cycled presaturation (13) pulse was used to suppress the H_2O signal. The spectrum represents the result of the simple pulse-and-acquire method. As shown in spectrum b Fig. 2, we can greatly reduce (by a factor of 5) the lipid signal by incorporating a spin-echo with a delay time of 68 ms. Spectrum of Fig. 2 shows the spin-echo result

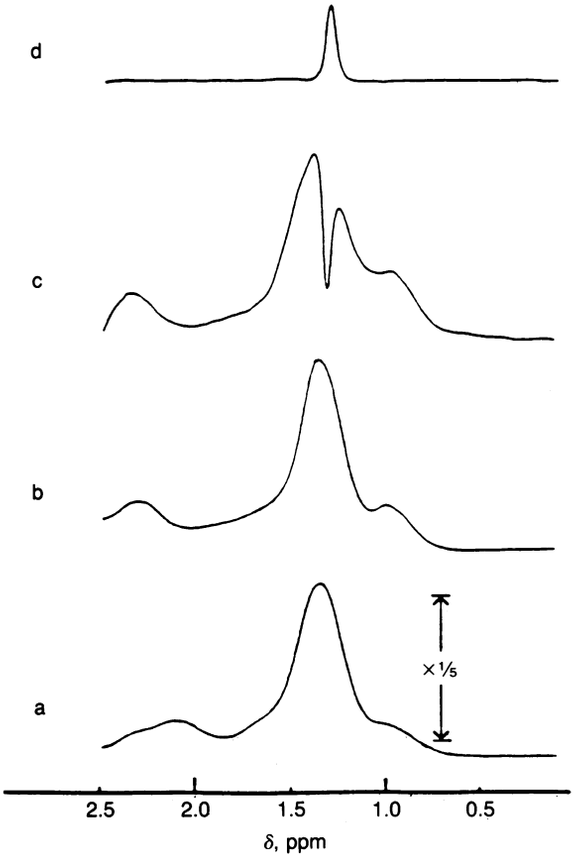

FIG. 2. ^1H NMR spectra of perfused mouse liver. Spectrum a was obtained from a nonselective pulse. The H_2O signal has been reduced with a 1-s presaturation pulse. The spectrum has been scaled down by a factor of ⅕. Spectrum b, nonselective spin-echo spectrum. A H_2O presaturation pulse was also used. Spin-echo delay time was 68 ms. Spectrum c, nonselective spin-echo spectrum with 10 mM lactate infused with $t = 68$ ms, $1/2J$. Spectrum d, the C-3 CH_3 lactate signal is clearly selected with the gated-DANTE sequence. The peak is properly phased. Spectra b–d are identically scaled.

after addition of 10 mM lactate. The CH_3 lactate signal appears at 1.33 ppm as a characteristically inverted peak when $t = 68$, ms $= 1/2J$, where J is the coupling constant between the lactate CH_3 and the neighboring CH protons. Even though the spin-echo sequence reduces the intensity of the surrounding lipid signals, it is evident that for liver studies the lipid resonances still pose a problem in quantitating the changes in the lactate peak. Spectrum d in Fig. 2 was obtained from our gated-DANTE editing sequence. Clearly, all the lipid background is removed and the lactate signal is unencumbered.

The gated-DANTE sequence excites only the pertinent resonances and thereby avoids exciting the H_2O. The H_2O signal is suppressed by approximately 10^4 as shown by spectrum a in Fig. 3, where it is about 6 times stronger than the lactate resonance (approximately 10 mM) at 1.33 ppm. Even a greater suppression may be possible in cases in which the broad water wings of the H_2O resonance are not perturbed. Lactate, however, has the C-2 proton resonance at 4.11 ppm resting on the H_2O water wing. Consequently, the H_2O signal is slightly perturbed by the gated-DANTE sequence. Our DANTE pulses cannot avoid exciting the broad water wings in our system because they are not as monochromatic as possible. They could be refined further to reduce the power spillage onto the main water line. Such improvement of the DANTE pulses would undoubtedly increase the H_2O signal suppression.

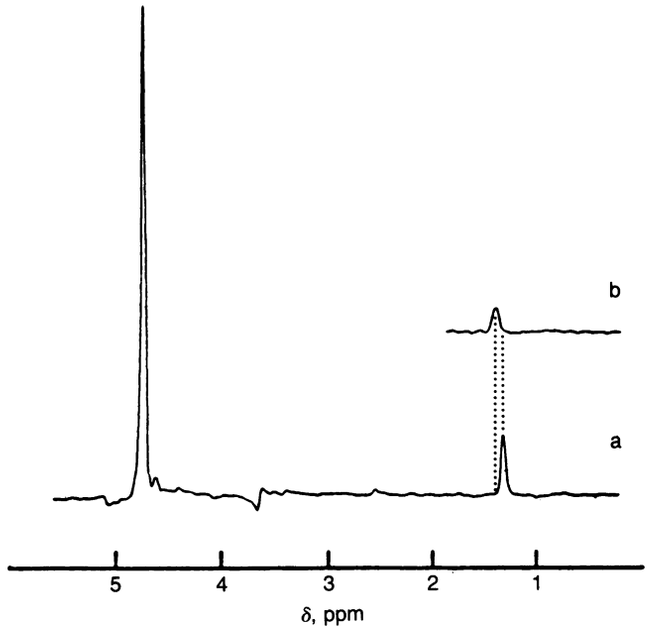

FIG. 3. ^1H NMR spectra of perfused mouse liver. Spectrum a, lactate edited spectrum, which shows that the H_2O signal has been reduced by more than four orders of magnitude. A spectrometer glitch appears at 3.8 ppm. The H_2O signal is phased to give an absorption signal. Spectrum b, alanine edited spectrum in the presence of lactate. Alanine was infused into the liver after spectrum a was taken. No lactate CH_3 signal at 1.33 ppm is detectable. Only the alanine CH_3 signal at 1.47 ppm is selected. No interference is noted from the lactate signal 54 Hz upfield at 1.33 ppm. The gain settings for both spectra were the same.

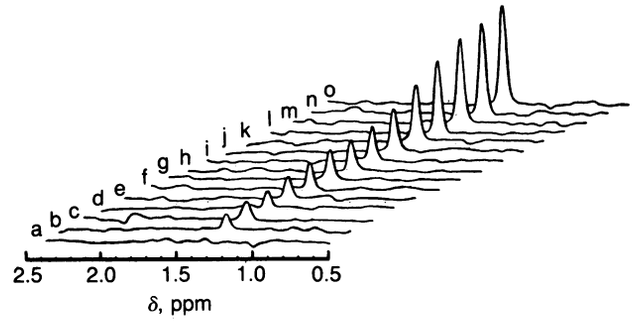

FIG. 4. ^1H NMR spectra of lactate formation in perfused mouse liver. Spectrum a, reference ^1H NMR lactate edited spectrum. The liver was initially cleaned of all metabolites. No lactate signal is detectable, as expected. The DANTE pulses are centered at the CH_3 resonance of lactate at 1.33 ppm and at the CH peak of lactate at 4.11 ppm. Spin-echo delay time is 68 ms. Spectrum b, 10 mM pyruvate was added to the system before the beginning of the accumulation. The CH_3 signal of pyruvate at 2.41 ppm is not seen in the edited spectrum. The lactate signal appears at 1.33 ppm (spectra c–i). The lactate signal continues to grow (spectrum j–o). At the beginning of the accumulation of spectrum j, 17 mM ethanol was added. The lactate formation rate increased by a factor of 2.8. Each spectrum required 128 scans and 3.5 min of data acquisition. The DANTE pulse train was 20 ms long with an interpulse delay period of 200 μs. The decoupler DANTE pulses that influence the *J* modulation were gated on during the interpulse delay time at CH resonance frequency, 4.11 ppm. The final free induction decay was apodized with an exponential function at the natural linewidth. The concentration was estimated by comparing spectra with a standard spectrum of a liver that was perfused with 10 mM lactate. All experimental conditions were kept identical. All peaks are referenced to H_2O, 4.76 ppm at 25°C.

The method's H_2O suppression properties are coupled with a finely tuned selectivity. At the end of an experiment that monitored lactate formation from pyruvate, a small amount of alanine was infused into the system. Alanine's CH_3 protons resonate 54 Hz downfield from the lactate CH_3 resonance. In the presences of alanine, the lactate methyl signal is clearly selected without any trace of the alanine or lipid signals, as shown in spectrum a of Fig. 3. Similarly, the alanine methyl signal is selected without any interference from the lactate methyl resonance, as shown in spectrum b of Fig. 3. It is clear that the gated-DANTE sequence also suppresses the natural abundant signals by at least 10^3. As shown in spectrum d of Fig. 2 and in spectrum a of Fig. 3, no lipid background signal is detectable.

By infusing 10 mM pyruvate into a perfused liver system, we can use this pulse technique to monitor the kinetics of lactate formation via the lactate dehydrogenase enzyme system (14). The time course of the kinetics is displayed in Fig. 4. Each spectrum represents about 3.5 min of data collection. Spectrum a in Fig. 4 is a reference spectrum before the addition of pyruvate. The liver has been flushed clean of any endogenous lactate. As expected, no lactate signal appears. We added 10 mM pyruvate at the time we started to accumulate spectrum b in Fig. 4. The lactate methyl signal at 1.33 ppm appears and grows. When we added ethanol to the system at the beginning of the data acquisition of spectrum j, we observed a dramatic increase in the rate of lactate formation (Fig. 4, spectra j–o), which reflects the expected shift in the NADH/NAD ratio due to the alcohol dehydrogenase reaction (7–9, 15). The time course of the reaction is plotted in Fig. 5.

After the addition of pyruvate we can easily follow the appearance of lactate at concentrations below 1 mM, as shown in Fig. 4, in which spectrum f represents about 1.5 mM lactate. We have estimated this concentration of lactate by comparing the signal intensity obtained when 10 mM lactate is perfused into a liver system with the spectrum in Fig. 4 that was obtained under identical conditions.

The signal-to-noise ratio in spectrum c of Fig. 4 is about 100:1 after 128 scans. Comparing this ratio to that from a straight one-pulse acquisition experiment on an analytical sample, we find that the sensitivity of the technique is reduced by about 30%. The loss is less severe if we consider the various factors of linewidth difference between analytical and liver samples, the incomplete population inversion, and imperfect pulses.

To illustrate the application and to demonstrate the ease

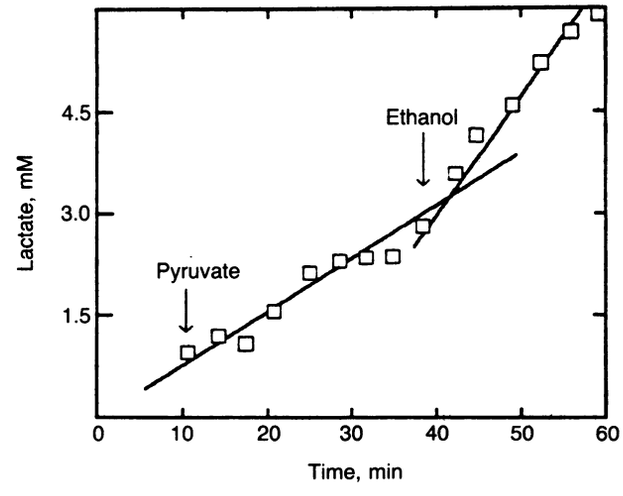

FIG. 5. Lactate formation in mouse liver perfused with 10 mM pyruvate and 17 mM ethanol. Each time point represents 3.5 min of data accumulation.

with which we can obtain kinetics with the ^{1}H NMR gated-DANTE sequence, we have measured the effect of ethanol on the rate of formation of lactate from pyruvate, as shown in Fig. 5. The lactate dehydrogenase system is well characterized (14), and the effect of ethanol on the ratio of NAD to NADH, which affects the overall lactate formation rate, is well documented (7–10, 15). The ^{1}H NMR editing technique has allowed us to follow formation of lactate from pyruvate in perfused liver. Consequently, it permits us to obtain many time points on the enzyme kinetics in one liver and to study lactate dehydrogenase kinetics *in vivo*. From the slope in Fig. 5, we calculate the rate of lactate formation in fed perfused mouse liver to be 5×10^{-2} μmol/s per g of liver. Upon the addition of ethanol, the rate increases by a factor of 2.8. Our value matches the available literature value of 4×10^{-2} μmol/s per g in fasted rat liver (16, 17). The difference between our result and the literature one may reflect experimental errors and conditions as well as the approximately 30% decrease in reported lactate utilization rate in fed liver (17). Moreover, the previously reported increase in NADH when ethanol is added to a perfused liver system is about a factor of 2.5 (9). This increase, shown in Fig. 5, is consistent with the observed factor of 2.8 rise in the lactate production rate and demonstrates a dependence of enzymatic rate upon the NADH concentration.

DISCUSSION

Campbell *et al.* (12) have demonstrated the feasibility of editing ^{1}H NMR spectra with spin-echo based sequences. They are largely predicated on alternately adding and subtracting either *J*-modulated or unmodulated spectra obtained from, for example, Carr–Purcell–Meiboom–Gill (CPMG) and Hahn spin-echo experiments (2, 12). An interesting modification of the technique involves decoupling the neighbor resonance during the evolution period in the spin-echo sequence (2, 3, 11). Such techniques are well established for solution studies and have recently been extended to *in vivo* investigations (3). However, these sequences employ nonselective pulses. In the nonselective method (3), a presaturation pulse is required to suppress the H_2O signal. For perfused systems with rapidly recirculating H_2O and with biphasic components reflecting either tissue or perfusate water, it would be difficult to suppress the H_2O signal adequately by using presaturation. Although the alternative semiselective pulse method, recently applied to *in vivo* brain studies (4), overcomes some of the dynamic range problem by employing a $1\bar{3}3\bar{1}$ semiselective excitation scheme (18) that is relatively effective even for perfused organ studies (unpublished data), it does not have the improved editing selectivity of the gated-DANTE experiment.

As a result of the selectivity, only a narrow part of the lipid region is excited. Consequently, only the excited lipid signals, which oftentimes have a lower intensity than the overall background signal, need to be suppressed by editing. Such cases demand a reduced accuracy requirement of the spectral subtraction. Furthermore, the pulse width adjustment on the X nucleus excitation that compensates for small power spillage from the semiselective pulse is also minimized (4).

The fine selectivity of the method emphasizes the contrast between ^{1}H NMR editing and simple differencing. In straight difference spectroscopy, a spectrum is subtracted from the earlier reference. The result is a difference spectrum containing peaks that change. Although the method is applicable to many simple systems, it is not always useful during *in vivo* studies in which we expect changes in many parts of the spectra. Lactate formation is accompanied by dynamic changes in the lipids, fats, and other signals that resonate in the same spectral region. The resultant spectrum could be just as complicated and would not resolve particular signals in the physiological concentration range. Even though our method incorporates differencing technique to remove all extraneous peaks, it depends on selectivity to edit a particular resonance of interest such that the signal can be detected even if no changes are occurring.

Moreover, liver has substantial mobile lipid components whose signals cannot be easily discriminated against by exploiting the metabolites' T_2 difference (19). Consequently, simple spin-echo sequences will not provide adequate spectral editing for perfused liver studies, as shown in spectrum c of Fig. 2. Neither will solvent suppression sequences by themselves (20).

It is plain that the ease with which reliable kinetics can be obtained readily underscores the power of ^{1}H NMR in perfused organ and *in vivo* studies.

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