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Observation of the Terminal Methyl Group in Fatty Acids of the Linolenic Series by a New ^{1}H NMR Pulse Sequence Providing Spectral Editing and Solvent Suppression. Application to Excised Frog Muscle and Rat Brain†

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ABSTRACT: A new ^{1}H NMR pulse sequence is described that combines water suppression with the selective observation of signals from coupled spin systems. The pulse sequence is easy to set up and compensates for pulse width inhomogeneity in the biological sample. Suppression of the water signal is achieved by pulses that return the water spins to their equilibrium position; spectral editing is based on the J modulation present in spin-echo spectra and its inhibition by coherent decoupling at one of the resonances of the spin system of interest. The pulse sequence, which was designed for ^{1}H NMR spectroscopy of tissue, was tested at 470 MHz on excised frog muscle and rat brain. The lactate methyl resonance of caffeine-treated frog sartorius muscle was observed selectively by irradiation at the position of its alcoholic proton. The terminal methyl signal of linolenic acid, along with other fatty acids of the linolenic series (first double bond in the ω-3 position), was observed selectively by irradiation at the position of its ω-1 methylene group. ^{1}H NMR spectra of rat brain were edited to reveal the terminal methyl of either linolenic series or all other fatty acids. The results suggest that the terminal methyl groups of fatty acids of the linolenic series (mostly docosahexaenoic acid, 22:6) have higher mobility than those of all other fatty acids.

Two major problems with ^{1}H NMR[1] spectra of cells and tissues, (i) the dynamic range barrier presented by the large water signal and (ii) the limited spectral resolution afforded by crowded spectral regions, have been dealt with successfully in recent years. Water suppression by means of a train of hard pulses (Plateau & Guêron, 1982; Hore, 1983), which obviates the need for presaturation of the water signal and its attendant cross saturation of signals from exchangeable protons, is now standard (Arûs et al., 1984a,b). The problem of overlapping resonances in tissues has been solved for coupled spin systems by using homonuclear ^{1}H double-resonance pulse sequences or "editing" sequences (Rothman et al., 1984a,b). This technique, which has been used previously to simplify spectra of proteins (Campbell & Dobson, 1975; Campbell et al., 1975) and red blood cells (Brown et al., 1977), is based on the J modulation present in spin-echo spectra; the water peak was minimized by using ^2H_2O as the solvent or by presaturating the ^1H_2O resonance. Recently, two pulse sequences have been proposed that use selective DANTE pulses (Morris & Freeman, 1978) to achieve spectral editing and water suppression (Hetherington et al., 1985; Jue et al., 1985). We explore here an alternative approach that achieves water suppression via the JR pulse sequence (Plateau & Guêron, 1982) and spectral editing via echo generation by a nonselective composite 180° pulse. We have applied this pulse sequence, which is easy to set up and provides excellent sensitivity, to studies of frog muscle and rat brain.

Most biochemical information from cells and tissues obtained by ^{1}H NMR spectroscopy has been restricted to cytosolic metabolites (Daniels et al., 1976; Brown et al., 1977; Yoshizaki et al., 1981; Agris & Campbell, 1982; Behar et al., 1983; Arûs et al., 1984a,b; Ugurbil et al., 1984; Rabenstein, 1984). However, resonances from membrane constituents also have been detected (Mountford et al., 1982; Cross et al., 1984). Recently we observed resonances from membrane phospholipids in the ^{1}H NMR spectrum of rat brain (Arûs et al., 1985). The editing pulse sequence we describe in this paper provides the means to probe further into membrane structure

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[1] Abbreviations: AQ, data acquisition; COSY, two-dimensional homonuclear correlated spectroscopy; DANTE, delays alternating with nutation for tailored excitation; EM, exponential multiplication; FID, free induction decay; FT, Fourier transform; i.d., inside diameter; JR, jump and return pulse sequence; JRE, jump and return spin-echo pulse sequence; NMR, nuclear magnetic resonance; o.d., outside diameter; rf, radio frequency; TM, trapezoidal multiplication; TT, total recycling time.

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and fluidity by selectively observing the population of polyunsaturated fatty acids of the linolenic series separately from all other fatty acids.

Experimental Procedures

Animal Tissues and Subcellular Fractions. The sartorius muscles of winter Northern American frogs (*Rana pipiens*) were dissected from pithed animals. One muscle, weighing typically about 200 mg, was mounted in a home-built perfusion apparatus that inserted into a 5-mm (o.d.) NMR tube. The muscle was perfused at 20 °C with 200 mL of recirculating Ringer's solution (115 mM NaCl/2.5 mM KCl/2.0 mM $CaCl_2$/2.15 mM Na_2HPO_4/0.85 mM NaH_2PO_4, pH 7.0) containing 10 mM caffeine for 30 min and then perfused with 200 mL of normal Ringer's solution for 1 h. Ischemia was produced by stopping the flow of the perfusate.

Rats (each about 250 g) were sacrificed by decapitation. The skull was opened and the brain excised. A hollow stainless steel rod (3-mm i.d.) was punched into the brain, and the brain tissue from the rod was delivered into the bottom of a 5-mm NMR tube with the aid of gentle air pressure. The NMR tube contained 200 μL of physiological salt solution (126 mM NaCl/3 mM KCl/2.4 mM $CaCl_2$/1.3 mM $MgSO_4$/1.2 mM KH_2PO_4/26 mM $NaHCO_3$, pH 7.4). Spectra of the excised tissue were recorded at a probe temperature of 37 °C.

Rat brain cytosol was obtained and stored as described previously (Arûs et al., 1985). Total brain lipids were extracted from freeze-dried brain according to Folch-Pi et al. (1957), dried with a stream of nitrogen, and dissolved in C^2HCl_3 to yield a solution 10 times as concentrated as the original one. Linolenic, docosahexaenoic, and arachidonic acids and cholesterol were purchased from Sigma.

NMR Spectroscopy. Spectra of samples in C^2HCl_3 were collected by means of a standard one-pulse sequence employing 90° flip angles (8 μs) unless otherwise indicated. Spectra of aqueous solutions and tissues were collected by using the JR (Plateau & Guéron, 1982) water suppression pulse sequence, as described previously (Arûs et al., 1984a,b, 1985).

The new editing pulse sequence(*J*ump and *R*eturn spin *E*cho, JRE) employs a JR pulse train for solvent suppression and a composite 180° pulse (Freeman et al., 1980) to create *J* modulation of the spectrum (Freeman & Hill, 1975; Turner, 1984). The full JRE pulse sequence is given by

$$90°_y-\tau-90°_{-y}-D1-90°_y-180°_x-90°_y-D1-\text{AQ} \quad (1)$$

where $D1$ is the evolution time required for J modulation and τ is the JR delay. In theory, a second delay should be added to the last $D1$ in the pulse sequence to allow for complete refocusing of the spin system. Since this value is insignificant (130–150 μs) compared to $D1$ (34–68 ms), it has been ignored here. The length of the $90°_{-y}$ pulse was adjusted empirically to produce maximal water suppression as judged by the FID produced by the water in the sample. The value of τ, as in the JR pulse sequence, is set equal to $1/4\Delta$, where Δ is the frequency difference between the position of the carrier and the position to be optimally excited. $D1$ was set either to $1/2J$, when a doublet resonance was edited (e.g., methyl of lactate), or to $1/4J$, when a triplet was monitored (e.g., terminal methyl of linolenic acid), where J is the coupling constant of interest (Campbell & Dobson, 1975; Rabenstein, 1984; Rothman et al., 1984a,b). Single-frequency coherent irradiation was applied in alternate pulse trains at the resonance frequency of one of the nuclei of the spin system to be edited (e.g., 2.05 ppm in the case of linolenic acid) and in the other pulse trains to a control frequency placed symmetrically on the other side of the water resonance (7.65 ppm in the case of linolenic acid). The decoupler was turned on only during the evolution period. Alternate acquisitions were averaged in different memory locations in the computer. The difference FID was obtained by subtracting the control FID from the FID in which the spin system was irradiated and then Fourier transformed to yield the edited spectrum; alternatively, the subtraction was carried out subsequent to Fourier transformation of the two FIDs. No difference was found between the two methods of obtaining the difference spectrum.

When spin systems in samples dissolved in C^2HCl_3 were edited, no solvent suppression pulse sequence was used. A Carr–Purcell (Carr & Purcell, 1954) spin-echo sequence, essentially as described by Rothman et al. (1984a) but with a composite 180° pulse, was employed instead for the refocusing pulse. Coherent irradiation was applied during the echo time, and the spectra were processed as described above for the JRE pulse sequence.

^{1}H NMR spectra were collected with either a Nicolet NT-470 (469.941-MHz) or a Nicolet NMC-360 (361.075-MHz) spectrometer. Quadrature detection and 8K data points were used, unless otherwise indicated. The sweep width was ±3012 Hz or ±2304 Hz; the total recycling time was 1.7 s at 470 MHz or 1.9 s at 361 MHz. Chemical shifts were calibrated relative to the internal residual $CHCl_3$ peak (assigned as $\delta = 7.262$) for samples dissolved in C^2HCl_3 or relative to the $=N-CH_3$ signal of creatine (assigned as $\delta = 3.022$) for tissue samples. Unless otherwise indicated, an exponential line broadening factor of 0.3 Hz was applied prior to Fourier transformation. Resolution in tissue spectra was enhanced by trapezoidal multiplication of the FID prior to Fourier transformation (Arûs et al., 1984a). No resolution enhancement was used for the edited spectra, but an exponential line broadening factor of 3 Hz was applied. The homogeneity of the magnetic field was adjusted by shimming on the FID of water. All spectra, except for the COSY experiments described below, were acquired without a field/frequency lock.

COSY data from the total lipid extract were acquired at 470 MHz by means of the conventional pulse sequence $90°-t_1-90°-t_2$ (Bax, 1982); 16 FIDs were summed for each of 512 increments in t_1. Quadrature detection was used; the sweep width was ±2000 Hz, and 1 K data points were collected. The resulting 512 × 1024 data point matrix was zero filled and Fourier transformed to 512 × 512 real data points (only the aliphatic part of the spectrum was processed). Sine-bell weighting was used for resolution enhancement both in the t_1 and t_2 domain.

COSY data for 0.3 M cholesterol in C^2HCl_3 were obtained at 361 MHz with the sum of four FIDs for each of 512 increments in t_1 and a sweep width of ±1805 Hz. Other data acquisition and processing parameters were essentially the same as used for the total lipid extract.

Results

Frog Muscle. The aliphatic part of the JR spectrum of sartorius frog muscle is shown in Figure 1A. The resonances are characteristic of a fatty acyl chain: $-CH_3$ (0.89 ppm), $-(CH_2)_n-$ (1.28 ppm), $-CH_2CH_2COO$ (1.56 ppm), $-CH=CHCH_2$ (2.05 ppm), $-CH_2COO$ (2.22 ppm), and $-CH=CHCH_2CHCH-$ (2.75 ppm). The $-(CH_2)_n$ peak contributes to the intensity of the lactate methyl peak (~1.3 ppm) and prevents accurate measurement of the intensity of the lactate signal.

This problem could be overcome by using the JRE pulse sequence as shown in Figure 1B–D. In Figure 1B (control irradiation), the methyl resonance of lactate and the resonance of creatine at 3.02 and 3.94 ppm appear 180° out of phase

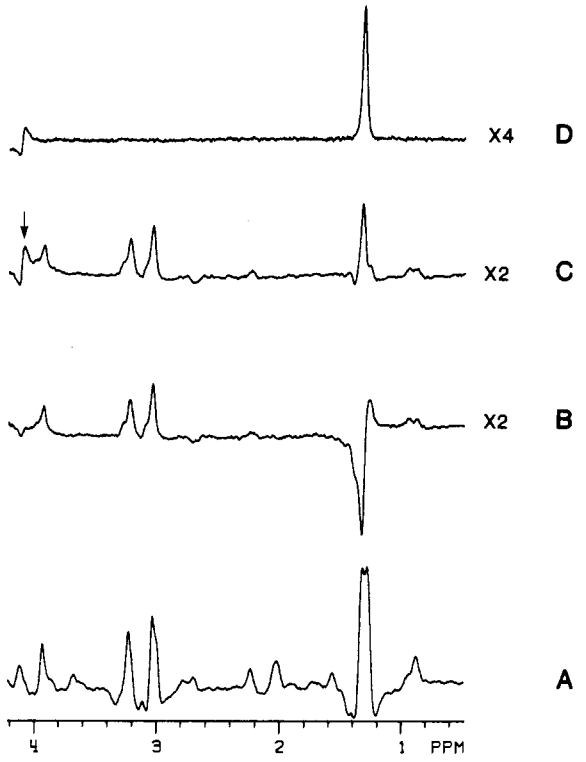

FIGURE 1: Comparison of 470-MHz ^{1}H NMR spectra (aliphatic region only) of ischemic frog muscle after caffeine treatment: (A) JR spectrum with τ = 152 μs and 64 transients; (D) edited spectrum showing the lactate methyl peak, which is the difference between JRE spectra obtained with coherent irradiation set at (C) 4.10 (arrow) and (B) 5.50 ppm (control irradiation). The JRE spectra were recorded with τ = 152 μs and $D1$ = 68 ms; 256 transients were collected for each spectrum.

with one another, as the result of J modulation caused by coupling of the CH_3 resonance of lactate (1.3 ppm) to its CH resonance (4.1 ppm). This J modulation is inhibited (Figure 1C) when a coherent radio frequency is applied at the position indicated by the arrow (4.1 ppm). The difference spectrum (Figure 1D) contains the lactate methyl peak as the only resonance.

Editing of the terminal methyl of fatty acids of the linolenic series may be demonstrated by reference to spectra of docosahexaenoic acid. The normal ^{1}H NMR spectrum is shown in Figure 2A; assignments of the resonances are listed in the figure legend. Figure 2B (control irradiation) shows that the Carr–Purcell spin-echo sequence with an appropriate delay (Figure 2B) inverts the outer components of the terminal methyl triplet (~0.95 ppm). When the peak at 2.05 ppm is irradiated (Figure 2C), the J modulation disappears, and both the central and outer components appear in phase. The difference spectrum (Figure 2D) shows a doublet in the position of the terminal methyl of docosahexaenoic acid. A solution of arachidonic acid yielded an edited doublet at ~0.90 ppm when the coherent irradiation was set at 1.3 ppm (spectra not shown).

The unique chemical shift of the linolenic series ω-1 methylene forms the basis for editing signals from the terminal methyl of fatty acids of this series from those of the other fatty acids. The terminal methyl of all fatty acids, which occurs at ~1 ppm, is coupled to the ω-1 methylene group of the chain. This methylene group resonates at ~1.28 ppm in all fatty acids except those of the linolenic series, in which the ω-1 methylene group resonates at ~2.05 ppm. Provided that the methyl correlation time is short enough (spin–spin relaxation time T_2 long enough) so that the peak remains visible after the echo

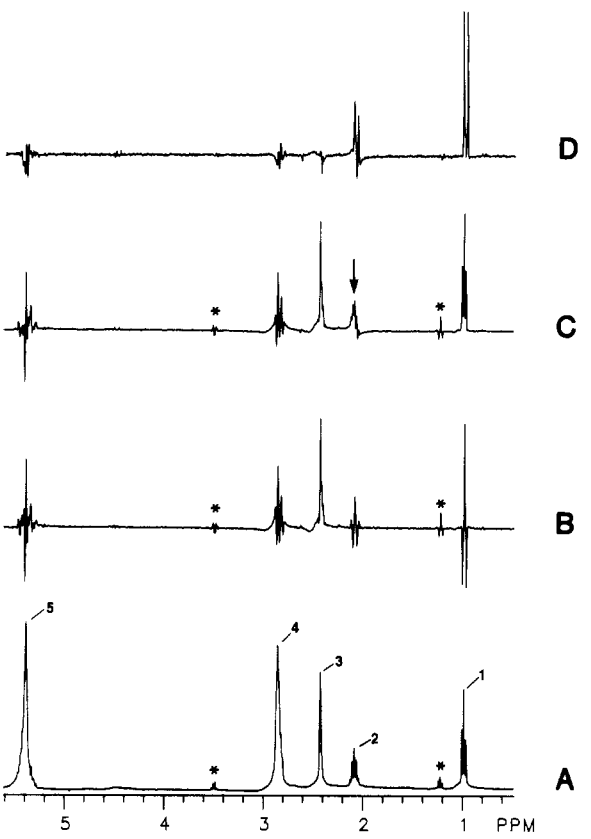

FIGURE 2: Expanded 361-MHz ^{1}H NMR spectra of 20 mM docosahexaenoic acid in C^2HCl_3. A total of 24 transients were acquired at 20 °C with a TT of 10.8 s. Spectrum A was acquired with the one-pulse sequence. Resonance assignments are as follows: 1, CH_3; 2, $CH_3CH_2CH{=}CH{-}$; 3, $={HCCH_2CH_2COOH}$; 4, $-CH{=}CHCH_2CH{=}CH-$; 5, $-CH{=}CH-$; * indicates an impurity, probably ethanol. Spectra B and C were acquired with the Carr–Purcell spin-echo sequence; $D1$ was set to 34 ms ($1/4J$ for the coupling between the terminal methyl and ω-1 methylene of docosahexaenoic acid). Coherent irradiation was set at the position indicated by the arrow: 2.05 ppm in spectrum C and 7.55 ppm for the control (spectrum B). Spectrum D, obtained by subtracting spectrum B from spectrum C, shows the edited terminal methyl resonance.

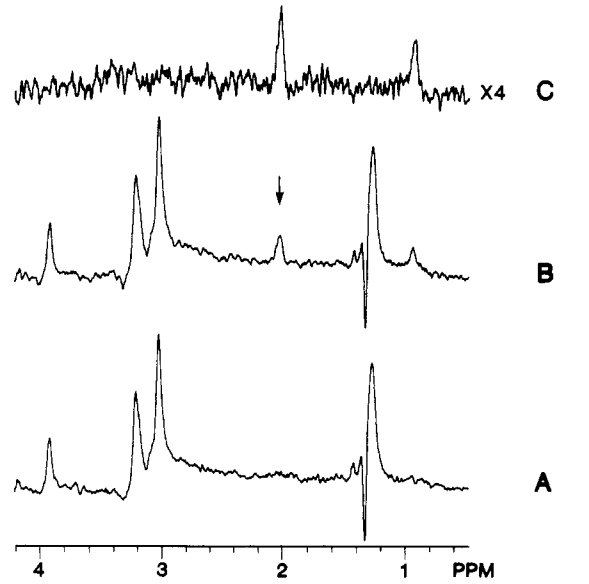

FIGURE 3: 470-MHz ^{1}H NMR spectra of caffeine-treated ischemic frog muscle acquired at 20 °C with the JRE pulse sequence, with τ = 136 μs and $D1$ = 34 ms; 256 transients were accumulated. The coherent irradiation was set at 2.05 ppm (arrow) for spectrum B and at 7.57 ppm for spectrum A. Spectrum C is the difference between spectra B and A.

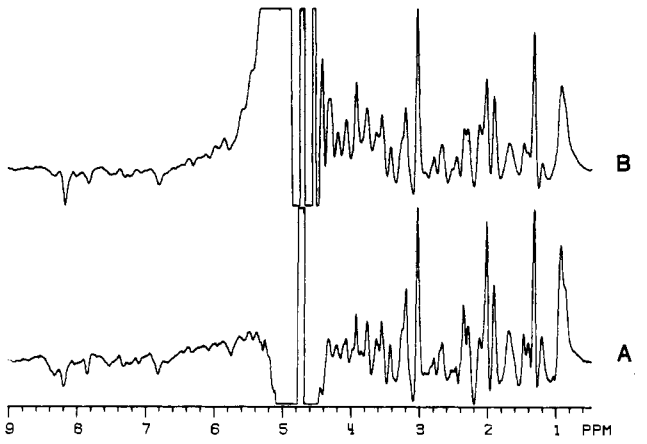

FIGURE 4: 361-MHz ^{1}H NMR spectra of (A) excised rat brain and (B) rat brain cytosol, recorded at 37 °C with the JR pulse sequence, with τ = 178 μs and 64 transients for both samples. The resolution was enhanced in spectrum A (EM factor, 5 Hz; TM with the two adjustable parameters used in the Nicolet software, T_1 = 100 and T_2 = 0). The cytosol spectrum (spectrum B) was broadened to mimic the line width in the intact tissue (EM factor, 15 Hz; TM with T_1 = 100 and T_2 = 0). For assignments see Arûs et al. (1985).

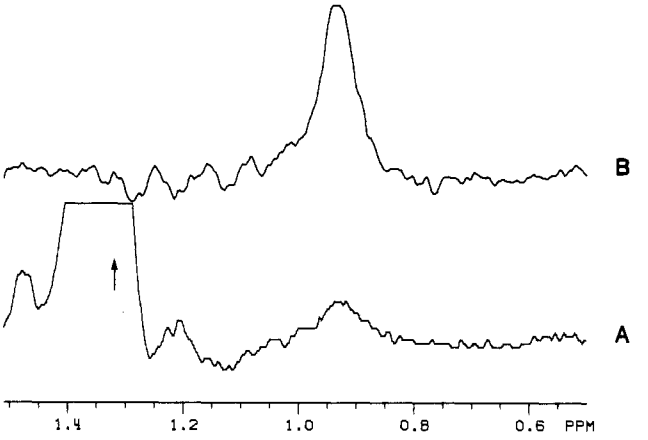

FIGURE 5: Part of the aliphatic region of the 361-MHz ^{1}H NMR spectrum of excised rat brain: (A) with the terminal methyl of all fatty acids except those of the linolenic series edited by irradiation at the ω-1 methylene at 1.28 ppm; (B) with the terminal methyl of the fatty acids of the linolenic series edited by irradiation at the ω-1 methylene at 2.05 ppm. Both spectra were derived from JRE experiments by subtracting the FIDs, as described under Experimental Procedures. τ was 178 μs, $D1$ was 34 ms, and 512 transients were acquired for each JRE spectrum.

refocusing time, the JRE pulse sequence (eq 1) should separate the signals. This is the case for frog muscle as can be seen from Figure 3. The terminal methyl of the linolenic series of fatty acids is observed at 0.95 ppm in the difference spectrum obtained by subtracting on-resonance (Figure 3B) and off-resonance (Figure 3A) JRE spectra. The 2.05 ppm peak observed in Figures 2B and 3C is an artifact caused by the experimental irradiation. In a control JRE experiment, in which the irradiation was set at 2.22 ppm, no peak was observed at 0.95 ppm (spectrum not shown). This result demonstrates that the selectivity of our decoupling field was less than 0.17 ppm.

Rat Brain. We applied the editing strategy (JRE) to gain information about the fatty acyl chain resonances contributing to the 0.9 ppm peak in the JR spectrum of excised rat brain (Figure 4A). Accordingly, we compared the edited spectrum obtained by irradiating the position of the ω-1 methylene resonance of the fatty acids of the linolenic series (2.05 ppm) (Figure 5B) with that obtained by irradiating the ω-1 methylene group of the other fatty acids (1.28 ppm) (Figure 5A).

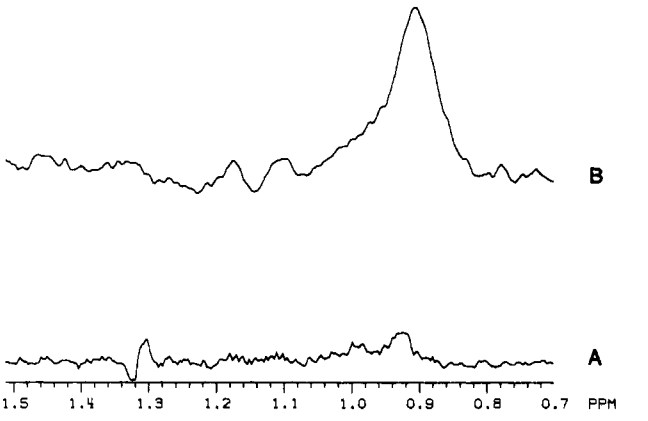

FIGURE 6: Part of the aliphatic region of the 361-MHz ^{1}H NMR spectrum at 37 °C of (A) rat brain cytosol and (B) excised rat brain tissue. In both spectra the terminal methyls of the fatty acids of the linolenic series were edited by irradiation at the ω-1 methylene at 2.05 ppm. Other parameters were as in Figure 5.

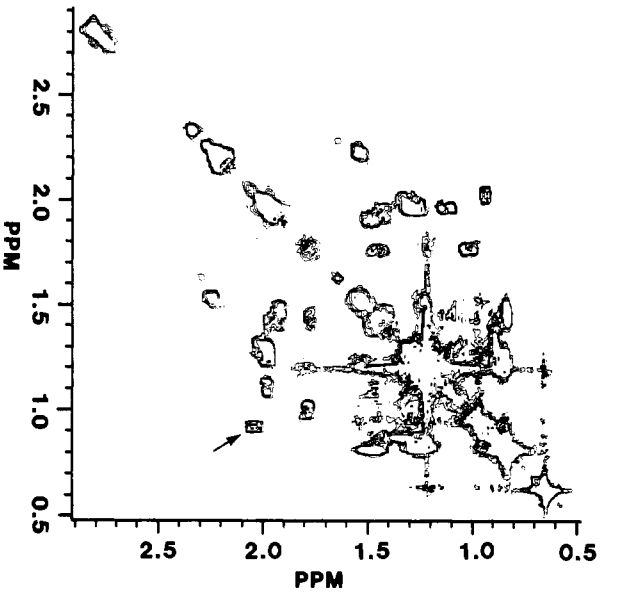

FIGURE 7: Part of the aliphatic region of the symmetrized 470-MHz ^{1}H NMR COSY plot obtained from the total lipid extract of rat brain in C^2HCl_3. The arrow points to the cross-peak assigned to the connectivity between the terminal methyl and the ω-1 methylene in fatty acyl chains of the linolenic series.

The intensity of the edited terminal methyl of fatty acids of the linolenic series was 3.2 times higher than that of all the other methyls.

Perchloric acid extracts of normoxic (Behar et al., 1983, 1985; Cerdân et al., 1985), hypoxic (Behar et al., 1985), or ischemic (Arûs et al., 1985) rat brain do not show major resonances near 0.9 ppm. Although rat brain cytosol was found to exhibit a prominent resonance at ~0.9 ppm (Figure 4B), when the JRE editing pulse sequence was applied with irradiation at 2.05 ppm (Figure 6A), only a minor peak was observed which was much smaller than that obtained with excised brain tissue under similar conditions (Figure 6B). The perchloric acid extract of rat brain cytosol did not show a major resonance at 0.9 ppm (results not shown).

We then examined whether the 0.9 ppm, 2.05 ppm connectivity is present in lipid components. Cholesterol is present in a high concentration in the total lipid extract of rat brain (Norton, 1981), but the 0.95 ppm, 2.05 ppm cross-peak was absent in the COSY contour plot obtained from a solution of cholesterol in chloroform (results not shown). In spectra of the total lipid extract of rat brain dissolved in C^2HCl_3, an

off-diagonal COSY cross-peak (labeled with an arrow in Figure 7) was found at the position expected for the connectivity between the ω-1 methylene group of fatty acids of the linolenic (ω-3) series (2.05 ppm) and their terminal methyl group (0.95 ppm).

Discussion

Recently, spectral editing pulse sequences based on semiselective and selective pulses have been employed for water suppression and editing of ^{1}H NMR spectra of tissues (Hetherington et al., 1985; Jue et al., 1985). Use of these sequences has demonstrated the advantages of spectral editing for NMR studies of living cells. However, the pulse sequences proposed employ relatively complex multiple-pulse trains. Spectral sensitivity with such a pulse sequence should be limited by pulse angle errors, caused by resonance offset and rf field inhomogeneity, which degrade the efficiency and uniformity of the excitation. The rf inhomogeneity arises from magnetic susceptibility differences throughout the sample. These techniques also suffer from the requirement to carefully calibrate the DANTE (Morris & Freeman, 1978) inversion pulse sequence. Any error in the inversion pulse will cause loss of some signal as "invisible" multiple quantum coherence. The composite inversion pulse used in the JRE pulse sequence (eq 1) reduces the field inhomogeneity problem present in tissue and also obviates the need for careful pulse calibration, since the composite inversion pulse is approximately self-compensating for inaccurate pulse lengths. The reduction in the number of pulses decreases the propagation of pulse angle errors and should increase the sensitivity and selectivity of the editing technique. The only adjustable parameters, other than the evolution time ($D1$) required for J modulation, are (i) the length of the second 90° pulse in the JR sequence, (ii) the τ value in the JR sequence, which gives some flexibility in choice of the spectral width of the excitation function, and (iii) the decoupling frequency. The last two of these parameters are insensitive to the nature of the sample and easily can be adjusted on a calibration sample. The simplicity of the pulse sequence allows the experiment to be set up quickly so that data can be collected very soon after the sample is placed in the spectrometer. Additional speed in experimental implementation might be gained, at the expense of some flexibility, by substituting the JR excitation sequence with a $1\bar{3}3\bar{1}$ excitation pulse sequence, which does not have any adjustable pulse lengths. It may be possible to improve this experiment still further by using composite pulses to compensate for field inhomogeneity effects in the JR sequence.

The performance of the JRE pulse sequence has been tested here with samples of excised muscle and brain tissue. The presence of overlapping $-(CH_2)_n-$fatty acyl chain resonances in the winter frog muscle was no obstacle to the selective editing of the underlying methyl peak of lactate. Accordingly, sequential acquisition of JR and JRE spectra should permit one to determine the concentrations of lactate and phosphocreatine and the intracellular pH (Arûs et al. 1984a,b) in superfused frog muscle despite the presence of overlapping signals from fatty acids.

Caffeine treatment was shown by ^{13}C NMR and chemical studies to partially mobilize the fatty acyl chains of membrane phospholipids without increasing the concentration of free fatty acids (Bârâny et al., 1982). Consequently, we expected that part or all of the sharp peak at 0.9 ppm observed with caffeine-treated muscle would arise from the terminal methyl of fatty acyl chains of membrane phospholipids. Subcellular fractions of frog muscle did not show resonances at 2.05 or 0.90 ppm (Arûs et al., 1984a); neither were these peaks present in the perchloric acid extract of the caffeine-treated muscle used in the editing experiment. Furthermore, the pattern of resonances observed in Figure 1A agrees with the known chemical shifts of fatty acyl chains of phospholipids in microsomes and phospholipid vesicles (Davis & Inesi, 1971; Finer et al., 1972; Michaelis & Schlieper, 1982; Neumann et al., 1985). Fatty acids of the linolenic series constitute about 21% of the total fatty acids in frog muscle (McMullin et al., 1968). We are led to conclude that the 0.95 ppm resonance showing connectivity to 2.05 ppm (Figure 3C) originates from the terminal methyl of linolenic series fatty acids, which are present mainly as esters in membrane phospholipids but to a smaller extent as unesterified fatty acids.

Application of the JRE pulse sequence to excised rat brain also produced an edited resonance near 0.9 ppm upon irradiation at 2.05 ppm. The absence of a major 0.9 ppm resonance in the perchloric acid extract from brain tissue rules out soluble metabolites as a significant source for the edited peak. Although rat brain cytosol did show a 0.9 ppm peak, a clear 0.9 ppm, 2.05 ppm connectivity was not found in the COSY spectrum. The expected connectivity was present, however, in the COSY spectrum of total rat brain lipids in C^2HCl_3 but absent in the COSY spectrum of cholesterol. Thus the fatty acyl chain in phospholipids or unesterified fatty acids appears to be the most likely source for the edited peak, and we have assigned it to the terminal methyl of linolenic series fatty acids. The 0.9 ppm peak obtained by editing for connectivity to 2.05 ppm was about 3.2 times more intense than that obtained by editing for connectivity to 1.28 ppm (Figure 5). This result was surprising, since linolenic series fatty acids constitute only 20% of the total fatty acid composition of rat brain (Cotman et al., 1969). On the basis of lipid composition, the intensity of the linolenic series methyl resonance should have been an order of magnitude higher than observed. We postulate that in excised rat brain the terminal methyl of a population of fatty acids of the linolenic series is very mobile, with correlation times probably in the range of those of cytosolic metabolites, while the methyl groups of the other fatty acids have a much more restricted mobility. The increased mobility may be a consequence of the lower gel-to-liquid-crystal transition temperature of phospholipids containing unsaturated fatty acids plus the longer average chain length of fatty acids of the linolenic series. Both saturated and unsaturated fatty acyl chains in phospholipids show increased mobility as one goes down the fatty acyl chain [see Seelig and Seelig (1980) for a review].

The extent to which free fatty acids embedded in the membrane may contribute to the edited resonances should be discussed since the amount of free fatty acids has been shown to increase upon post-mortem ischemia (Cenedella et al., 1975; Deshmukh & Radin, 1985). The concentration of free fatty acid that accumulates is small, however: only 2.86 μmol/g after 1 h of post-mortem ischemia at 37 °C (Deshmukh & Radin, 1985), i.e., about a 20 times lower concentration than the total concentration of phospholipids in rat brain [59.82 μmol/g (Cuzner & Davison, 1968)]. The proportion of free fatty acids of the linolenic series compared with the total free fatty acids, in either fresh or ischemic brain, was somewhat lower than in the brain phospholipids, 12% vs. 20% (Cenedella et al., 1975; Cotman et al., 1969). These data rule out preferential generation of free fatty acids of the linolenic series upon ischemia as the source of the 0.9 ppm, 2.05 ppm connectivity found in excised rat brain.

An unassigned peak at 0.9 ppm appeared in a ^{1}H NMR spectrum of live rat brain published by Rothman et al. (1984a).

The selectivity of their coherent decoupling was estimated to be about 0.2 ppm. It is reasonable to assume that this edited peak originated, as in our case, from the terminal methyl of linolenic series fatty acids. Since the total free fatty acids of rat brain homogenized or frozen within 30 s of decapitation is around 30 μg/g (about 0.11 μmol/g) (Bazan, 1970; Deshmukh & Radin, 1985), it is unlikely that free fatty acids contributed to the peak in live rat brain.

As much as 70% of the total fatty acids of the linolenic series in brain phospholipids is accounted for by docosahexaenoic acid (22:6), also called cervonic acid (Cotman et al., 1969). A biological function of this fatty acid in the membrane is indicated by its resistance to nutritional depletion in neural tissues [see Stubbs and Smith (1984) for a review]. ^{1}H NMR editing pulse sequences may provide a new approach for studying the function of docosahexaenoic acid in tissues of live animals.

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Registry No. 22:6, 6217-54-5; H_2O, 7732-18-5; caffeine, 58-08-2; lactate, 50-21-5; arachidonic acid, 506-32-1.

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