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Natural-abundance ^{13}C NMR study of glycogen repletion in human liver and muscle

(human metabolism)

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ABSTRACT **Optimizing the surface-coil design and spectral-acquisition parameters has led to the observation of the ^{13}C NMR natural abundance glycogen signal in man at 2.1 T. Both the human muscle and hepatic glycogen signals can be detected definitively with a time resolution of ≈13 min. A $^{1}H/^{13}C$ concentric surface coil was used. The ^{1}H outer coil was 11 cm in diameter; the ^{13}C inner coil was 8 cm in diameter. The coils were tuned to 89.3 MHz and 22.4 MHz, respectively. The ^{1}H coil was used for optimizing field homogeneity (shimming) the magnet and for single-frequency decoupling of the C_1 glycogen signal. Total power deposition from both the transmitter pulse and the continuous wave decoupling did not exceed the Food and Drug Administration guideline of 8 W/kg of tissue. Experiments were done for which healthy subjects returned to the magnets at different times for ^{13}C NMR measurement. The spectral difference between experiments was within the noise in the C_1 glycogen region. Because of the spectral reproducibility and the signal sensitivity, hepatic glycogen repletion can be followed. Four hours postprandial, hepatic glycogen increases by 3.8 times from the basal fasted state. The hepatic glycogen data correspond directly to previous biopsy results and support the use of ^{13}C NMR as a noninvasive probe of human metabolism.**

Mammalian cells store glucose as glycogen and expend the latter to meet energy demands. Despite the key role of glycogen, many questions remain about hepatic and muscle glycogen repletion and utilization in man. Our understanding has relied on animal model studies (1, 2) and has rested heavily on experimental data obtained from needle biopsy of human muscle and, sometimes, liver. Animal metabolism, of course, is quite different from human (3), and the invasive nature of needle-biopsy techniques has probably discouraged studies of human glycogen metabolism (4, 5).

Recent advances in *in vivo* NMR have emphasized ^{31}P and ^{1}H and not ^{13}C (6, 7). However, the ^{13}C studies of rats at 8.5 T (8, 9) and rabbit at 1.9 T (10) indicated that the ^{13}C NMR technique can reveal hepatic glycogen signals *in vivo*. Sillerud and Shulman (8) had shown that the ^{13}C NMR intensities originate from 100% of the glycogen atoms; the visibility was established by hydrolyzing glycogen to glucose and observing that the gain in the glucose intensities equaled the loss in the glycogen intensities. The unexpected nature of this high visibility, coming from glycogen molecules with molecular mass of $\approx 10^8$ Da, has prompted other investigators (11, 12) to repeat the experiments in live animals. Hence all glycogen molecules *in vivo* are visible; consequently, with proper calibration, their ^{13}C intensities can be used to determine concentrations.

The first natural-abundance ^{13}C glycogen signal from human muscle and liver was observed at 2.1 T (13, 14). Subsequent natural-abundance ^{13}C spectra at 4.7 T confirmed the human muscle results and monitored the changes in glycogen levels with exercise (15). However, the initial ^{13}C spectra at 2.1 T lacked the signal sensitivity to monitor physiological changes.

In this paper, we show the results of improving ^{13}C NMR techniques for detecting human muscle and liver glycogen at 2.1 T. The increased signal sensitivity allows us to follow with ≈13-min time resolution the levels of human hepatic and muscle glycogen, the normal concentrations of which are ≈230 mM and ≈90 mM glucosyl units/kg of tissue, respectively (4, 5). These results on the natural-abundance ^{13}C nuclei indicate that NMR can monitor noninvasively metabolic changes in glycogen and can measure accurately the glycogen concentrations. The ^{13}C NMR technique, thus, creates many opportunities to study the role of glycogen in human carbohydrate physiology.

MATERIALS AND METHODS

Spectra were collected on a 1-m bore, 2.1 T Biospec NMR spectrometer. A concentric $^{1}H/^{13}C$ surface coil was made from copper wire (99.9%, 2.5 mm in diameter) and connected with 5–25 pF Polyfon capacitors for the tune and match circuits: the ^{1}H coil was 11 cm in diameter; the ^{13}C coil was 8 cm in diameter. The loaded 90° ^{1}H pulse was 150 μs at the coil center; the loaded 90° ^{13}C pulse was 120 μs at the coil center. The radio frequency power was ≈400 W.

In the liver experiments, the protocol for positioning the subject was similar to the one previously reported for kidney studies (16). The subject's side, corresponding to the right lobe of the liver, was placed on top of the surface coil. A 6-mm Lucite plate separated the subject from the coil. A multislice gradient-echo image was taken to confirm placement of the coil with respect to the liver and to define the gradients. A 2-cm sphere of H_2O attached to the center of the coil served as a calibration marker. Shimming on the localized volume, defined by presaturation sychronization pulses applied in the presence of gradients, generally produced a water linewidth between 20 and 30 Hz.

For the muscle study a similar protocol was followed. Localized shimming also produced a water linewidth between 20 and 30 Hz. The gastrocnemius muscle was used.

^{13}C spectra were collected with both a pulse-acquire and l-t-$\bar{1}$ sequence. Unless specifically noted, the l-t-$\bar{1}$ pulse sequence was tailored to suppress the intense lipid signals appearing near 30 ppm and to excite optimally the C_1 glycogen region at 100 ppm. Each pulse was 75 μs, and the interpulse delay was ≈320 μs. Total repetition time was ≈22 ms. The repetition time was optimized for the C_1 glycogen signal, the T_1 of which is 240 ms and the T_2 of which is 30 ms (10). Thirty thousand scans were collected in 13 min. The final signal was 0-filled to 4 K and filtered optimally with a Gaussian-exponential function before transformation. For the ^{1}H decoupled spectra, only 11,250 scans were collected, corresponding again to 13 min of accumulation. Because of

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the very conservative Food and Drug Administration guidelines on radiofrequency heat deposition per kg of tissue, both pulse length and acquisition time parameters were not changed, but the total repetition time was increased to 80 ms. Ten watts of ^1H decoupling power was applied with a duty cycle of 30%. From our theoretical calculations, the heat deposition anywhere in the body would not exceed the recommended guideline of 8 W/kg of localized tissue (17).

For the repletion study, a male subject fasted for 30 hr. After the initial baseline spectrum, he was given a mixed meal of 50.5% carbohydrate (100 g), 30.3% fat (60 g), and 19.2% protein (38 g). The carbohydrate source was glucose; the fat was from fat emulsion (Microlipid); and the protein was from modular protein powder supplement (Propac).

RESULTS

Spectral reproducibility is examined in Fig. 1. Fig. 1, spectrum a, displays a typical natural-abundance ^{13}C spectrum of human liver collected in 13 min. The prominent peaks in the spectrum are the dominating CH_2 lipid signal at 30 ppm, the triacylglycerol moieties at 60 ppm, the olefinic carbon signals at 130 ppm, and the carbonyl resonances at 160 ppm (8, 14, 18, 19). The coupled resonance from C_1 glycogen is centered at 100.5 ppm. These peaks are split by the spin–spin coupling to the covalently bonded ^1H nucleus (J_{CH}) of 165 ± 5 Hz as previously observed in animal and human studies (14). The contribution from intercostal muscle glycogen is negligible and is confirmed by a corresponding signal decrease with reduced pulse angles. One hour after completing the first experiment, the subject was returned to the same position in the magnet. A second ^{13}C spectrum was then collected with the identical experimental protocol and acquisition parameters. Vertical scaling was kept identical. The spectral difference between the two ^{13}C spectra, Fig. 1, spectrum b, illustrates the reproducibility of the experimental data. Additional experiments confirmed this spectral reproducibility. Moreover, the spectral differences in the 100-ppm region were always within the noise. Even in the 30-ppm region these spectral differences were only a few times the noise amplitude. Our experimental procedure conveniently obtained reproducible spectral data and introduced no errors in the important 100-ppm region, where the glycogen signal appeared.

Fig. 2 displays the natural-abundance ^{13}C NMR spectra from human gastrocnemius muscle and shows the improvements obtained with a semi-selective pulse; Fig. 2, spectrum a, shows the ^{13}C spectrum obtained with a pulse-acquire experiment. The intense signals from the subcutaneous fat layer distort the baseline at 100 ppm and make signal quantitation difficult. To reduce surface signal interference, a l-t-$\bar{1}$ pulse sequence (20) was used. Fig. 2, spectrum b, shows the spectrum obtained with a l-t-$\bar{1}$ pulse sequence. The baseline about the glycogen signal is greatly improved by using semi-selective excitation. The 30-ppm lipid peak was reduced in intensity by a factor of 16. Additional experiments showed that the muscle glycogen peak at 100 ppm was also

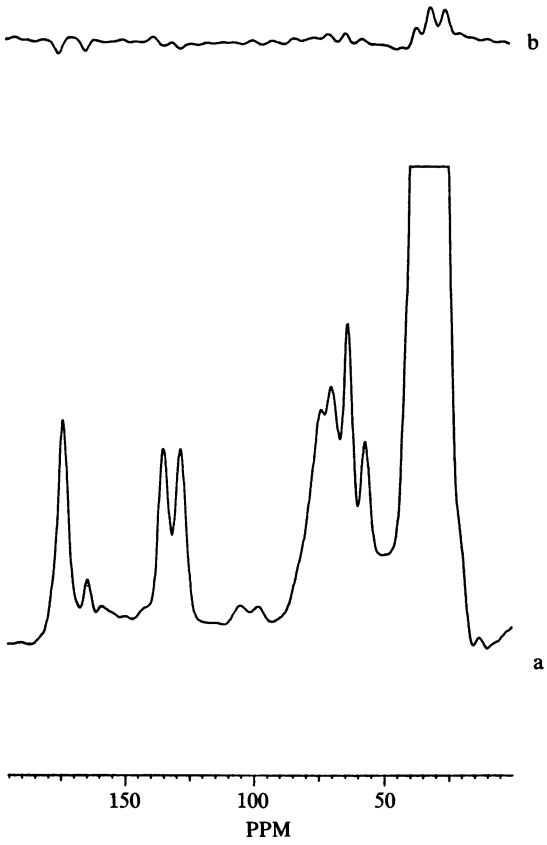

FIG. 1. Natural abundance ^{13}C NMR spectra of human liver to assess experimental reproducibility. (Spectrum a) Basal spectrum is obtained with pulse-acquire method. (Spectrum b) Difference between basal spectrum and another obtained 1 hr later when the subject was repositioned in the magnet. All spectra acquisition and processing parameters were identical. The pulse length was set at 150 μs. Thirty thousand scans were accumulated in ≈13 min.

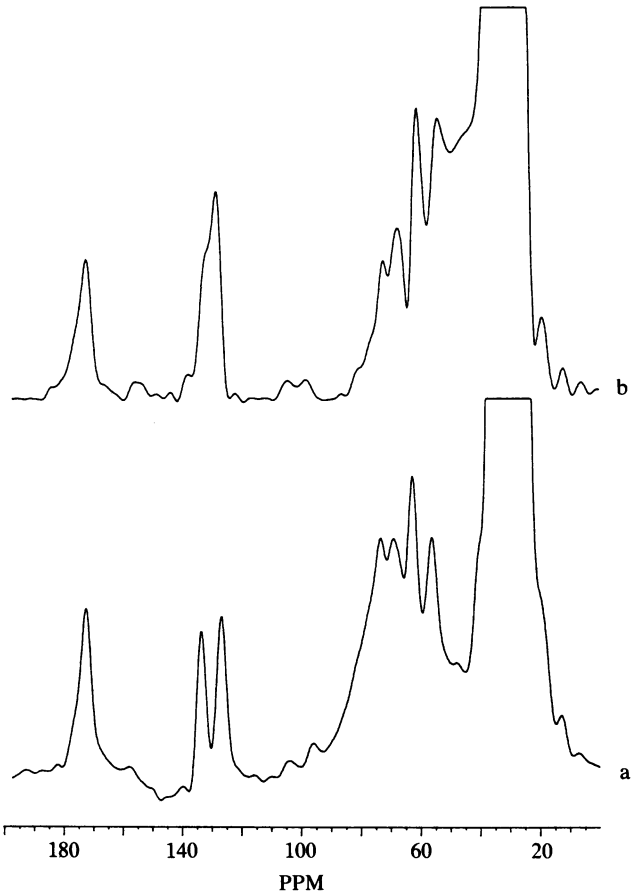

FIG. 2. ^{13}C NMR spectra of human gastrocnemius muscle. (Spectrum a) The pulse-acquire reference spectrum reveals the coupled C_1 glycogen signal at 100.5 ppm, which rests on a distorted baseline. Signal contribution from the subcutaneous fat layer spills over and distorts the baseline. (Spectrum b) A l-t-$\bar{1}$ sequence reduces the subcutaneous fat signal and straightens the baseline. The interpulse delay was set at 400 μs to minimize the lipid signal.

perfectly reproduced when the subject returned to the magnet.

The coupled ^{13}C spectra from the hepatic glycogen repletion study are shown in Fig. 3. The l-t-T basal spectrum after a 30-hr fast appears in Fig. 3, spectrum a. All subsequent spectra were scaled identically. The subject then ingested a mixed meal, described above, containing proteins, fat, and carbohydrates. Two hours postprandial, the glycogen signal increased by 2.8-fold over the control spectrum (Fig. 3, spectrum b). Four hours postprandial the glycogen signal increased 3.8-fold (Fig. 3, spectrum c). In the same experiment the corresponding ^{13}C spectra were also obtained from muscle glycogen. No signal intensity change was seen between the fasted and postabsorptive states.

Additional improvements in signal and resolution were obtained from ^{1}H decoupling (Figs. 4 and 5). Fig. 4, spectrum a, shows the coupled C_1 glycogen spectral region from human muscle. The coupled C_1 glycogen signal appears at 100.5 ppm. Another signal, which we have not yet identified, with J_{CH} of 160 ± 5 Hz appears at 117.5 ppm. This signal was not seen in ^{13}C spectra of excised human muscle and tissue extract (21). Nor does it appear in the ^{13}C spectrum of glycogen in solution. This particular signal appears prominently in females. Upon ^{1}H decoupling, the doublet collapses to give single resonance (Fig. 4, spectrum b). Upon reducing the pulse angle to emphasize the superficial signals, both peaks lose intensity, suggesting that the 117.5-ppm peak may not be signal from subcutaneous lipid.

The comparable coupled and decoupled spectra of hepatic glycogen are shown in Fig. 5. Fig. 5, spectrum a, shows the coupled signal of hepatic C_1 glycogen. Ten watts of ^{1}H decoupling was then applied with a 30% duty cycle. The resulting decoupled spectrum is shown in Fig. 5, spectrum b. The hepatic glycogen peaks are completely decoupled and give the same 45 ± 5 Hz linewidth as the coupled spectra.

DISCUSSION

Improving the spectral acquisition parameters and using a $^{13}C/^{1}H$ concentric surface coil have led to the detection of both human hepatic and muscle glycogen with sufficient sensitivity and resolution to follow glycogen repletion and other physiological changes. The concentric coil design avoids the power loss at ^{1}H frequency inherent in a $^{13}C/^{1}H$ double-tuned circuit (22). Consequently, for ^{1}H the increased rf magnetic field (B_1) strength permits shimming on a deeper sample volume for observing the ^{13}C glycogen signal. The high sensitivity of the ^{1}H coil also allows us to image deep tissue from which spectra were obtained (16).

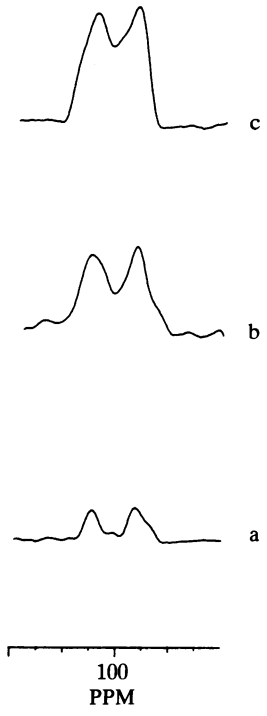

FIG. 3. ^{13}C NMR spectra from hepatic glycogen repletion study. (Spectrum a) Basal spectrum of the C_1 glycogen after the subject has fasted for 30 hr. (Spectrum b) Spectrum 2 hr after ingesting the mixed protein/carbohydrate meal. (Spectrum c) Spectrum 4 hr after ingesting meal.

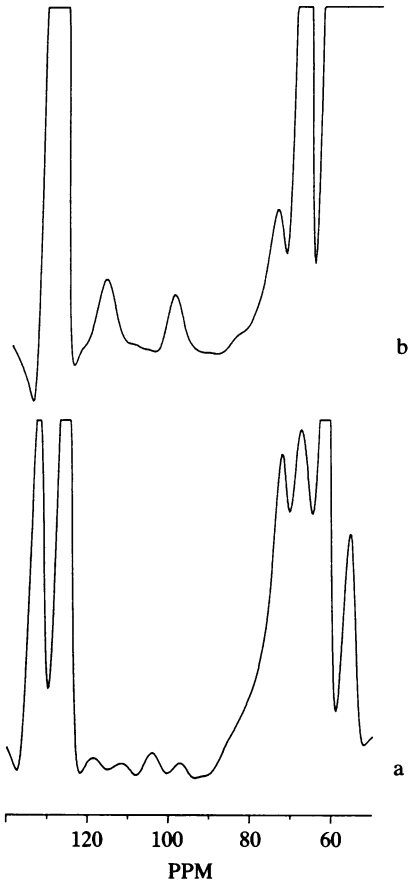

FIG. 4. Coupled and decoupled ^{13}C NMR spectra of C_1 glycogen from human muscle. (Spectrum a) The coupled glycogen C_1 signal appears at 100.5 ppm. An unidentified peak appears at 117.5 ppm with J_{CH} of 160 Hz. (Spectrum b) Upon ^{1}H decoupling with 10 W of irradiation, 30% duty cycle, both the glycogen and the unassigned peaks collapse.

Improving the acquisition parameters has enhanced both the resolution and the signal-to-noise ratio. A semi-selective excitation was used to suppress surface lipid resonances and thereby flatten the baseline around the glycogen peak. The signal-to-noise ratio was increased with rapid pulsing conditions. In a homogenous B_1 field, either the repetition time or pulse angle is adjusted to yield maximum sensitivity. For a given repetition time, $t_2 = 3T_2^*$, the optimal pulse angle is established by the Ernst angle relationship $\cos^{-1}\theta = \exp(-t_r/T_1)$, where t_r = repetition time (23, 24). In an inhomogeneous B_1 field of a surface coil, however, a range of pulse angles is produced. The low pulse-angle volume is greater than the high pulse-angle volume that is close to the coil surface. As a result, a lower ratio of t_r/T_1 preferentially samples the low pulse-angle volume and up to a certain value leads to increased sensitivity. Indeed, studies have shown that the sensitivity enhancement going from $t_r/T_1 = 5$ to $t_r/T_1 = 0.1$ is 2.2-fold and between a $t_r/T_1 = 1$ and a $t_r/T_1 = 0.1$ is 0.3-fold (25).

For the muscle glycogen experiment (13), the repetition time is set at 22 ms, corresponding to about $3T_2^*$, and the t_r/T_1 ratio is close to 0.1. On a glycogen phantom, the 22-ms

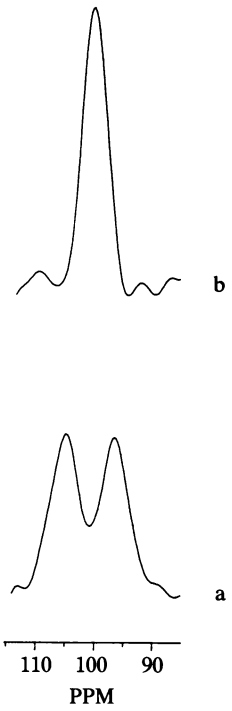

FIG. 5. Coupled and decoupled ^{13}C NMR spectra of C_1 hepatic glycogen. (Spectrum a) The coupled glycogen C_1 signal appears at 100.5 ppm. (Spectrum b) With 10 W of ^1H irradiation, 30% duty cycle, the ^{13}C glycogen signal is decoupled.

repetition time parameter yielded a 40% improvement of signal-to-noise ratio over the previously reported acquisition parameter $t_r/T_1 = 1$ (15) and should give an even greater improvement over previous parameters using longer t_r/T_1 ratios (10, 14, 19).

The signal-to-noise ratio of the C_1 muscle glycogen peak is 20/1 in 13 min. This ratio is, however, still not maximal. Because of the Food and Drug Administration heat deposition guidelines, standard conditions of ^1H irradiation to obtain nuclear Overhauser enhancement were not applied. At 1.9 T, the nuclear Overhauser enhancement is 1.3 ± 0.5 (26). Further sensitivity improvements, envisaged for a 4.0 T magnet, must also overcome the Federal Drug Administration guideline. At 4.0 T the requisite ^1H decoupling power is higher by a factor of 4 than at 2.0 T. In our experiment at 2.1 T, 10 W of single-frequency decoupling was required to collapse the C_1 glycogen doublet. Consequently, the sensitivity gain from the higher field may be compromised by loss from decoupler duty cycle requirements.

The enhanced sensitivity and improved resolution allowed us to follow glycogen repletion in man. From the NMR, the hepatic glycogen concentration increased by a factor of 2.8 over the basal level within 2 hr after refeeding and by a factor of 3.8 within 4 hr. Calibrating our glycogen signals to starting levels, based on the reported hepatic glycogen level after a 24-hr fast of ≈20 mM (5), our NMR data yield a glycogen repletion rate of 0.2 mM/min per g of liver. Data from a similar refeeding study in which a biopsy was used to measure glycogen concentration indicate that within 2 hr, the hepatic glycogen level should have increased by a factor of 2.8 from the basal level; within 4 hr the level should have increased by a factor of 3.2 (5). Our NMR results are consistent with results obtained with needle-biopsy techniques; needle-biopsy techniques have been reported to have an accuracy of 3–5% (27, 28) but are invasive and prone to sampling errors. Moreover, in time course studies, biopsy techniques are unsuitable to measure *repeatedly* the same tissue. With the present signal-to-noise ratio of the $^{13}C_1$ glycogen signal, the NMR measurement can also achieve 5% accuracy. Expected increases in signal sensitivity will also improve the accuracy limit. In contrast to the biopsy method, the NMR method is completely safe because it does not require invasive surgical procedures and can measure dynamically the same tissue undergoing metabolic changes.

Our study demonstrates that ^{13}C NMR can follow carbohydrate physiology and presents a distinct method to study the cellular regulation of muscle and hepatic glycogen metabolism in man.

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