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Methods for Liquid- and Solid-State CP–MAS NMR Spectroscopy of Untreated Tissue Biopsies[1]

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We describe a method for NMR analysis of rapidly cooled or frozen biopsies and report its use on rat liver. Since the metabolic state of the biopsies can be expected to reflect the *in vivo* state, the method may be used as an alternative to the traditional examination of a perchloric acid extract of the biopsy. Perfusion-cooling of rat liver was applied as an efficient means of preserving the metabolic state. This method ensures very rapid cooling without interruption of the oxygen supply to the liver, and the results show that biopsies taken subsequently maintain energy metabolites near *in vivo* concentrations for at least 40–60 min. High resolution, natural abundance ^{13}C liquid-state NMR spectroscopy could be carried out within this time frame on the untreated biopsy. In addition, the biopsy was frozen for ^{13}C cross polarization–magic angle spinning solid-state spectroscopic examination, which was carried out at −40, −100, and −150°C. The solid-state spectra allowed analysis of the relative glycogen content of the intact liver tissue, which showed good correlation with chemically measured glycogen on the same samples. Furthermore it was observed that the C_1-carbon of glycogen in all liver samples splits into two resonances (5.4 ppm apart) in the solid state but not in the liquid state. This suggests that two conformational states of glycogen are populated, with rapid equilibration in the liquid state but no equilibration in the frozen state.

NMR[3] offers the possibility of measuring glycogen concentration *in vivo* in the liver and muscle by natural abundance ^{13}C NMR spectroscopy (1–7). Sillerud and Shulman (8,9) showed that the glycogen molecule is fully NMR visible, in spite of the fact that the molecular weight is 5×10^7 or more (10). By applying ^{13}C-labeled substrates dynamic changes of several other components of intermediary metabolism in addition to glycogen may be observed by *in vivo* ^{13}C NMR spectroscopy, as pioneered in perfused liver in particular by Cohen (11–13). In many instances, however, limitation in sensitivity and spectral resolution of *in vivo* NMR spectroscopy requires subsequent studies on extracts (usually in perchloric acid, PCA) of liver biopsies in order to determine concentration, exact labeling pattern, and enrichment. For several reasons the PCA extracts cannot, however, be expected to provide a faithful representation of the *in vivo* situation, i.e., in terms of the free concentration of some metabolites, as is the case for ADP (14,15), and indeed with respect to compounds which are incompletely extracted or which may be partly hydrolyzed during the PCA procedure, like fatty acids and phosphocreatine, respectively.

We describe here a method which constitutes a useful alternative to the use of tissue extraction and report its use on intact liver tissue, using ^{13}C high-resolution liquid-state and CP–MAS solid-state NMR spectroscopy. A preliminary account of these results has been given (16).

METHODS AND MATERIALS

Preparation of Liver Samples

Male Wistar rats weighing 250–300 g were used. The animals were anesthetized by pentobarbital (50 μl, 50 mg/ml given ip) and the liver was perfused in a perfusion system previously described (17). Perfusion medium was Krebs–Henseleit buffer (18) equilibrated with O_2/CO_2 (95/5%) at 37°C with a flow rate of 12 ml/min/100 g body wt. After 5–10 min of perfusion the medium was abruptly switched to ice-cold (0–4°C) medium, also

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[3] Abbreviations used: CP, cross polarization; MAS, magic angle spinning; NMR, nuclear magnetic resonance; PCA, perchloric acid.

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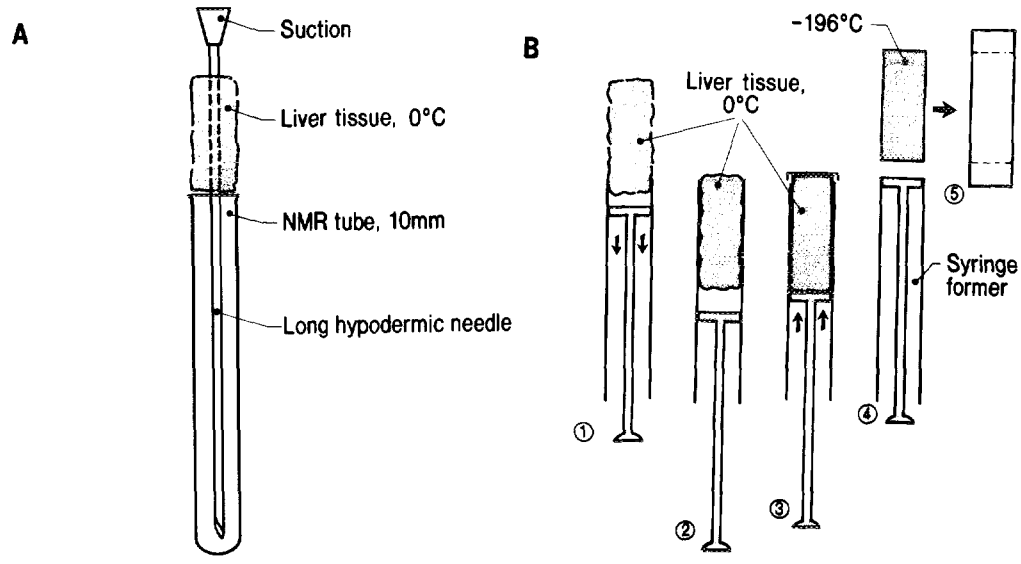

FIG. 1. Preparation of liver biopsies for liquid- and solid-state NMR spectroscopy. The rat liver was perfused with ice-cold (0–4°C) Krebs-Henseleit buffer, pH 7.4, for 10 min. While superfusing the liver with ice-cold perfusate a roughly cylindrical tissue biopsy was trimmed out with a scalpella and sucked into a standard 10-mm NMR tube as shown in (A). Another piece of tissue was trimmed out and sucked into a specially made aluminium syringe (5.8 mm inner diameter) as shown in (B). The syringe was loosely capped and the tissue was compressed so as to adapt to the shape of the former and subsequently frozen in liquid nitrogen. Dimensions of the frozen pellet were 5.8 mm diameter and 13 mm length, which made the pellet fit exactly into the sample holder of the MAS probe.

equilibrated with O_2/CO_2 (95/5%). After 4 min of cold perfusion an appropriately sized, roughly cylindrical tissue sample was cut out from the liver with a scalpel while superfusing with ice-cold perfusate. For high-resolution liquid state NMR spectroscopy the liver sample was sucked into a standard 10-mm NMR tube (see Fig. 1A), while for CP-MAS solid-state spectroscopy the sample was compressed in a cold former and then frozen as a cylindrical pellet which fitted exactly into the CP-MAS sample holder (see Fig. 1B). The frozen samples were sent on dry ice to Fort Collins, Colorado, for the CP-MAS analysis.

Preservation of Metabolic State after Cold Perfusion

In order to check the efficiency of the perfusion cooling of the rat liver in terms of maintaining *in vivo* metabolic state during and after cooling, biopsies of the liver were freeze-clamped (19) after 0, 20, 40, and 60 min of cold ischemia. The freeze-clamped biopsies were extracted with PCA (20). ATP, ADP, and glucose was measured by standard metabolite assays (21), P_i by a colorimetric assay (22), and glycogen according to (23).

Liquid-State NMR

Natural abundance ^{13}C NMR spectroscopy on the cooled, but otherwise untreated, liver biopsies was performed in a Bruker AM-500 spectrometer; 10% D_2O was added to the perfusate during the last 30 s of perfusion in order to provide an appropriate spin-lock signal. The perfusion-cooled liver sample was placed in standard 10-mm NMR tubes as explained above (see Fig. 1A). The NMR tubes were kept on ice until installation in the NMR instrument, which was thermostated to 2°C. The time between preparation of the cold liver biopsy and the NMR experiment was typically 20–25 min. Spectrometer conditions for these experiments were as follows: Spectrometer frequency was 125.76 MHz, sweep width 25 KHz, pulse width 13 μs corresponding to a 35° pulse and interpulse delay was 0.8 s. Broad band decoupling was applied during acquisition only. Data were collected in 16 K points, zerofilled to 32 K prior to Fourier transformation. The spectra represent the average of 25-2800 FIDs and are displayed with 2 Hz line broadening. The ^{13}C chemical shift assignments were performed indirectly via the spectrometer lock frequency and given relative to the ^{13}C-signal of dioxane (67.4 ppm). The reference experiment was carried out immediately after the measurements under the same temperature and instrumental conditions. Under these conditions the D_2O lock frequency was not significantly changed. This approach was taken to avoid any external disturbance of the liver sample by the reference compound.

Solid-State NMR

The solid-state experiments were performed on a Chemagnetics CMX300 NMR spectrometer equipped with a 7T superconducting magnet and variable temper-

TABLE 1

Changes of Metabolite Concentrations in Rat Liver during Cold Ischemia

Ischemic interval at 0–4°C	ATP	ADP	P_i	$\frac{ATP \times 10^3}{ADP \cdot P_i}$	Glu	Gly
0	3.81 (±0.38)	0.561 (±0.015)	2.15 (±0.44)	3292 (±877)	0.522 (±0.972)	275 (±21)
20	4.06 (±0.14)	0.653 (±0.097)	2.54 (±0.26)	2501 (±355)	1.14** (±0.16)	281 (±28)
40	3.83 (±0.21)	0.903* (±0.16)	3.20* (±0.41)	1379* (±353)	1.59** (±0.23)	278 (±10)
60	3.32* (±0.087)	1.13** (±0.14)	3.77** (±0.43)	800* (±164)	2.09** (±0.30)	±259* (±11)

Note. The livers were perfused with ice-cold (0–4°C) Krebs–Henseleit buffer (18), pH 7.4, for 10 min. Ischemia was initiated by stopping circulation and temperature was maintained by superfusing the organ with the ice-cold perfusate. Biopsies were freeze-clamped after the intervals of cold ischemia indicated. Biopsies were extracted in PCA (20) and metabolites were analyzed in the extracts by standard enzymatic assays (21). Results are means of four experiments ± SD, expressed as micromoles per gram wet weight. Statistical difference with respect to control (0 min of ischemia) was evaluated by paired data t test.

* and ** indicate $P < 0.05$ and 0.01, respectively.

ature $^1H/^{13}C$ CP–MAS probe, tuned to 299.1 and 75.4 MHz, respectively. The frozen, cylindrical liver sample enclosed in the NMR-sample holder (See Fig. 1B) was brought into the precooled probe at approximately −100°C. Measurements were performed at −40, −100, and −150°C. Fifteen minutes of temperature equilibration was allowed prior to FID collection. Spectrometer conditions were a contact time of 1 ms and a repetition delay of 2 s. Decoupling intensity was 60 kHz. MAS rate was 4.55 kHz. Between 2500 and 3000 FIDs were collected for each spectrum. Peak area quantitation and chemical shift assignment of the spectra was performed applying the Chemagnetics spectrometer software. The chemical shift reference was established by using hexamethylbenzene as a secondary substitution standard, with the methyl signal assigned to 17.4 ppm less shielded than external tetramethylsilane. The chemical shift stability relies on the inherent stability of the relatively low field magnet, as evidenced by the fact that there is no detectable field drift in over a week with respect to line width in solid-state spectra.

RESULTS

The capability of perfusion cooling (see Methods and Materials) to preserve the metabolic state of the liver during a subsequent ischemic interval was evaluated by measurements of ATP, ADP, P_i, glucose, and glycogen as shown in Table 1. During the first 20 min of cold ischemia there were no significant changes observed except that glucose had increased from 0.5 to 1 mM corresponding to hydrolysis of approximately 0.5% of the glycogen present in these experiments. After 40 min, however, ADP and P_i increased by some 50%, while ATP and glycogen did not change significantly. These changes progressed further from 40 to 60 min, but even then ATP had decreased only by 13% and glycogen by 6%. Thus, applying the described protocol of perfusion cooling liver ATP and glycogen may be evaluated quite accurately in a biopsy kept at 0–4°C for as long as 40–60 min. In a recent study the rate of change of ATP was measured under similar conditions of ischemia, but at 37°C, and a 50% decrease was found after 1 and 5 min in the fasted and fed liver, respectively (24).

Figure 2 shows a typical example of ^{13}C-natural abundance spectra of biopsies from perfusion cooled rat livers. Two extreme metabolic conditions are shown: In the lower panel the liver from a 48-h-fasted animal is shown, while in the upper panel the animal was refed for 24 h after a 48-h fast. Glycogen is expected to be fully NMR visible (8) with the C_1-resonance approximately at 100 ppm, located in a part of the NMR spectrum where no other signals are seen in biological material (1–8). In the fasted state no glycogen is present, as seen in Fig. 2 (lower panel). Refeeding after starvation will cause a physiological overshoot of liver glycogen with concentrations as high as 6–800 mM. Figure 2 (upper panel) indeed demonstrates this glycogen build-up with a large, rather broad peak at 100.5 ppm, representing C_1 of the glucose residues (1,3,8,9). In addition, the resonances of carbons 2–5 and 6 may be identified in the fed state, superimposed on the relatively small signals from free glucose, as inferred by the α- and β-C_1 resonances at 93.0 and 96.8 ppm, respectively. Experiments with purified rabbit liver glycogen (Boehringer, 106089) showed, in agreement with previously published data (8,9), that the C_1-glycogen signal was not significantly different from the sum of the α- and β-glucose signals obtained with identical instrument settings after complete hydrolysis of glycogen. The glycogen hydrolysis was accom-

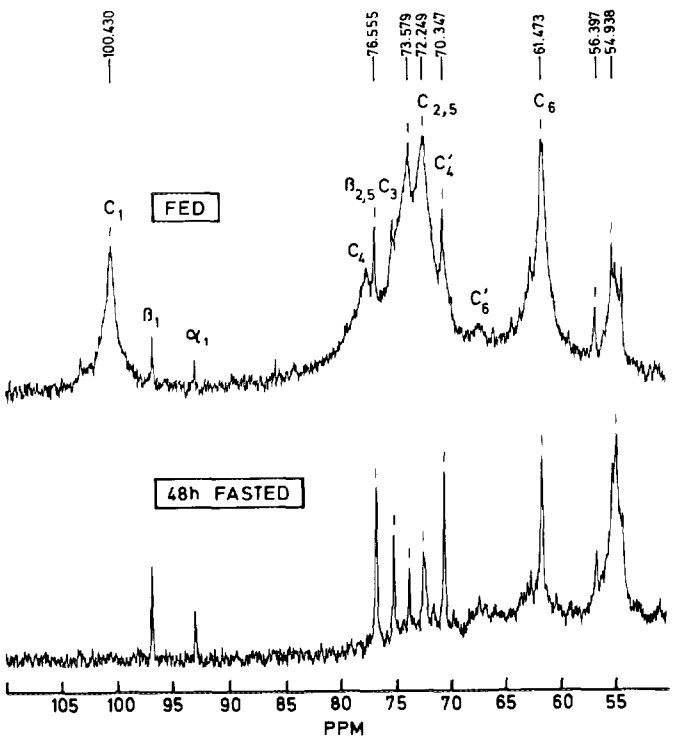

FIG. 2. Liquid-state natural abundance ^{13}C NMR spectra of rat liver biopsy. The liver biopsies were obtained as explained in the legend to Fig. 1A. The upper spectrum represents the fed state, while the lower spectrum represents a liver after 48 h of starvation. In the spectrum of the fed state the resonances C_1–C_6 of the glucose moieties of glycogen can be identified as indicated in the figure. In addition, the α- and β-anomer of free glucose are seen. The spectra show the average of 2750 FIDs, with broad band decoupling applied during acquisition only. The spectra are displayed with 2 Hz line broadening.

plished by adding approximately 20 U (1 mg) of amyloglycosidase (Boehringer, 10857) in the powder form directly in the NMR tube followed by 1 h incubation at 60°C. Thus, measuring glycogen at 2°C did not significantly change the NMR visibility of glycogen from the value of 100% visibility previously found at 37°C (8,9).

Solid-State Spectra of Rat Liver

Figure 3 shows the ^{13}C CP–MAS solid-state spectra of rapidly frozen rat liver (see Methods and Materials). Four nutritional states are shown, ranging from 24-h fasting with no glycogen (Fig. 3A) to the fasted/refed state with supercompensated glycogen levels (Fig. 3D). In all spectra the C_1-resonance of glycogen is clearly visible, except in the biopsy from the 24-h-fasted animal (Fig. 3A). Note, however, that in spite of appropriate bilevel proton decoupling (see Methods and Materials) the C_1-glycogen peak presents itself as a double peak at 102.1 and 96.7 ppm, a splitting which was not observed in the liquid-state spectrum of the liver (compare Fig. 2). While the resonance at 117.2 ppm can be accounted

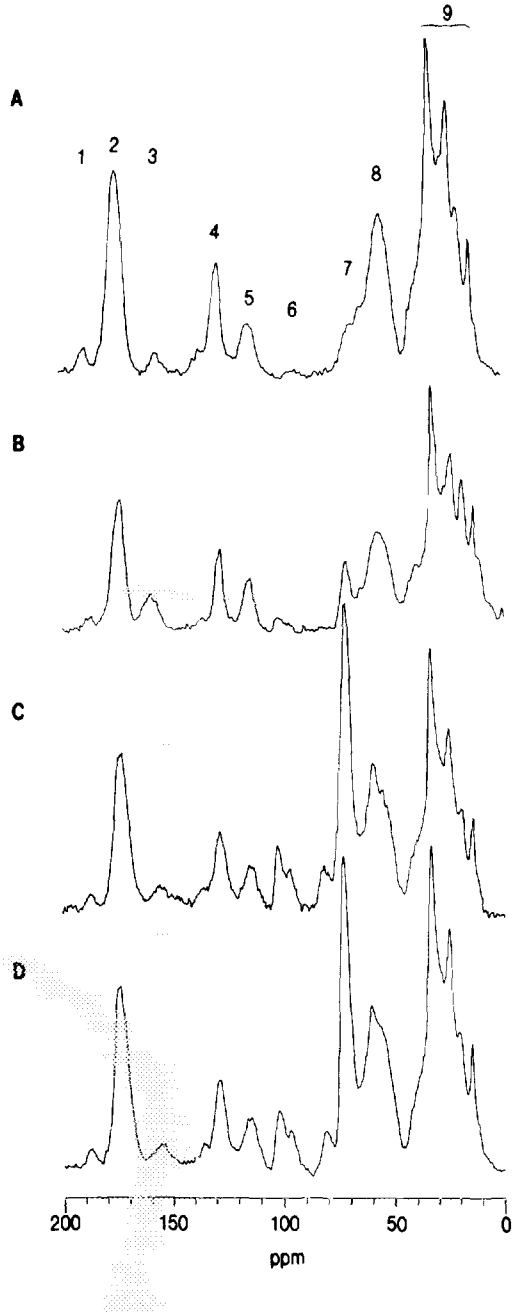

FIG. 3. ^{13}C-CP/MAS solid state NMR spectroscopy of frozen rat liver biopsy. The liver biopsy was obtained as explained in the legend to Fig. 1B. Biopsies were from rat livers in four different metabolic states: (A) From a 24-h-starved animal, (B) from an 8-h-starved animal, (C) from a fed animal, and (D) from a 24-h-starved/24-h-refed animal. Each spectrum is the average of approximately 2500 scans obtained at −150°C with cross polarization via spinlock with bilevel decoupling, displayed with 35 Hz line broadening. Spinning frequency was 4.55 kHz corresponding to 60.4 ppm. Thus, the peaks **1** and **5** may be assigned as spinning side bands, while **2** and **3** are carboxyl-carbon of esterified and free fatty acids, respectively; **4** represents unsaturated carbon of fatty acids. Peak **6** is the C_1-carbon of glycogen, while **7** and **8** represent the merged C_{3-5} and C_6 resonances of glycogen, respectively (cf. Fig. 2). Peak **9** represents the various saturated carbons of fatty acids (25,27).

TABLE 2

Glycogen Concentration in Rat Liver Determined by Natural Abundance ^{13}C Solid-State CP–MAS Spectroscopy and by Chemical Measurements

	Chemical		CP–MAS
Nutritional state	Glucose (mM)	Glycogen (mM)	Glycogen (arbitrary units)
Fasted 24 h	1.1	4.7	(<20)
Fasted 8 h	1.4	116	100
Fasted/refed normal chow	0.81	454	390
Fasted/refed high CHO diet	2.0	593	420

Note. Liver biopsies were obtained after perfusion cooling as described in the legend to Fig. 1. Following solid-state CP–MAS spectroscopy, the frozen pellet was removed from the MAS sample holder and extracted with ice-cold PCA (20) and glycogen and glucose concentrations were measured in the neutralized extracts as described in (21) and (23), respectively. Correlation between the two sets of glycogen measurements was tested by least squares linear regression, $r^2 = 0.96$.

for as a spinning side band of the 174.8 ppm carbonyl peak caused by the 4.55 kHz spinning rate, this is not the case for either of the two peaks at 102.1 and 96.7 ppm. The 5.4 ppm splitting of the C_1-carbon of glycogen may also be seen in a solid-state CP–MAS spectrum of a frozen solution of 170 mM purified rabbit liver glycogen (Boehringer catalog No. 106089), although the lines appeared broader than previously demonstrated by a similar solid-state experiment of a frozen solution of polymeric α-(1–4) glucans (38) (results not shown). Thus, all samples examined which contained liver glycogen displayed the C_1 splitting of 5.41 ± 0.11 ($\bar{x} \pm$ SD, $n = 8$). The remaining part of the glycogen molecule may also be observed in the solid-state spectra (Fig. 3) with C_4 at 80 ppm, $C_{2,3,5}$ at 72.1 ppm, and C_6 at 59.0 ppm, merging partly with the resonance at 55.2 ppm which probably represent mainly the methyl groups of choline and phosphatidylcholine (25–27) which are clearly separated from glycogen in the liquid-state spectrum, (compare Fig. 2).

Table 2 shows the relative amount of glycogen, represented as the area of the double peak around 100 ppm as well as the concentration of glycogen measured chemically in the same samples. The NMR data and the chemical measurements show good correlation, $r^2 = 0.96$, although it should be noted that only four points were available for the correlation test. Precise quantitation with NMR technique alone could have been carried out by appropriate use of an internal standard, which was, however, not done in these experiments.

It may be observed that the relative peak area as expected depends upon the temperature as demonstrated in Fig. 4; for example, while the ratio between the CH_2 manifold at 30 ppm and glycogen C_1 is roughly 0.5 at −40°C, it has decreased some fivefold at −100 and −150°C, (see Fig. 4). Also, the relative size of the fatty acid olefinic resonance at 129.1 ppm and the carbonyl group at 174.8 ppm seems to change with temperature.

DISCUSSION

Cold Perfusion: Preservation of in Vivo Metabolite State

One goal of this study was to apply methods which faithfully preserves the *in vivo* metabolic state of the liver, allowing extended time for NMR examination of the particular metabolic state. Quick freezing has been used extensively for this purpose, usually applying freeze-clamping between precooled aluminium tongs in order to shorten the distance of heat diffusion distance and thereby exponentially speeding up the cooling process (19,20,28). Thus, the freezing time (from 37 to 0°C) of the central part of a tissue wafer of 2 mm thickness is approximately 1 s, increasing by the square of the distance to the cooled surface (20,28). Here we use an alternative means of quick cooling by applying the capillary network of the liver as the route for the cooling medium, thus providing very short heat diffusion distances, and, most importantly, applying cooling without interrupting the circulation and oxygen supply to the cells. We have used this method either to study samples

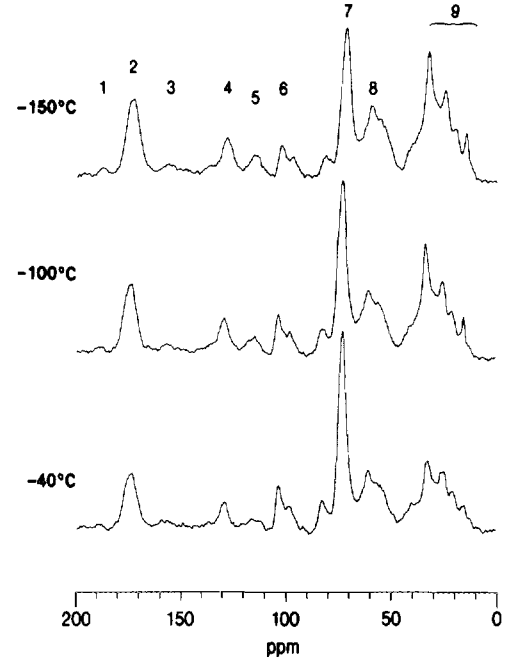

FIG. 4. Natural abundance ^{13}C CP–MAS solid-state NMR spectroscopy of frozen rat liver at −40, −100, and −150°C. Conditions and assignments are as described in the legend to Fig. 3. The glycogen concentration of this sample was 454 mM.

from the cooled, intact liver at 0–4°C with liquid NMR spectroscopy or to study the same samples at low temperature in the frozen state, applying solid-state CP–MAS spectroscopy. With the latter method the time available for the NMR examination is limited only by available instrument time. Recently Ikai *et al.* (29) alternatively substituted water with a cryoprotective solvent (glycerol) in order to allow liquid NMR studies at subzero temperatures. This elegant technique, however, has the drawback of a complicated sample preparation and furthermore, the cryoprotective agent may change the chemical conditions of the sample significantly. This would be a concern particularly in studies of NMR visibility of various compounds *in vivo*.

For the liquid-state measurements of the perfusion cooled samples it is important to know how long time the metabolic state of the sample remains close to *in vivo* conditions. The data presented in Table 1 suggest that at least 40 min at 0°C is safe in this respect; considering measurements of glycogen when present in large amounts in the liver samples, much longer examination times can be allowed. In fact, studies on isolated rat liver hepatocytes show that such cell preparations maintain their biosynthetic capacity when reincubated at 37°C after 24 h storage at 4°C (30). The increase of ADP (approx 0.4 mM) after 40 min of cold ischemia without a corresponding ATP decrease is unexpected; however, a similar increase has been observed during liver ischemia at 37°C (24). The increase of glucose from 0.5 to 1.6 mM after 40 min and 2.1 mM after 60 min most likely is due to glycogenolysis, but even 2 mM glucose represents only 0.8% of the glycogen present in the fed state.

Liquid- and Solid-State CP–MAS Spectroscopy of Liver

The solid-state CP–MAS spectroscopy in this study was performed on biopsies (approximately 320 mg wet wt) obtained from perfusion-cooled liver which was subsequently frozen. The spectra of Figs. 3 and 4 demonstrate that solid-state spectroscopy of these untreated liver biopsies is indeed possible and may offer a very useful means of evaluating the metabolism of the liver. Provided that rapid cooling can be arranged, CP–MAS spectroscopy of surgical biopsies from normal human tissue should be readily feasible, perhaps even peroperatively in analogy to the histological examination of quick-frozen biopsies which is common practice in conjunction with surgical treatment for malignancies.

Based upon the peak area, the NMR determination of the relative glycogen concentration correlate well with the chemical determination in the same samples (Table 2), in spite of the fact that no internal standard was used in these experiments to compensate for the small, unavoidable intersample differences such as sample size and spectrometer conditions. However, precise NMR quantitation of glycogen or other metabolites of the biopsy should be readily obtainable, for example by briefly warming the sample container to 0°C and injecting a known small amount of a suitable reference for spin counting (31) into the sample. After refreezing, the sample may be measured again, allowing precise quantitation.

The CP–MAS solid-state spectroscopy of the frozen liver has been carried out with appropriate proton decoupling. The resonances at 117.2 and 96.7 are unexpected, since in the liquid-state ^{13}C spectrum of the liver only the C_1-carbon of glycogen around 100 ppm should be present within the region 96–125 ppm (8,9), as shown in Fig. 2. While the 117.2 ppm signal can be accounted for as a spinning side band of the 174.8 ppm carbonyl peak (the symmetrical SSB is seen at 232.4 ppm) this is not the case for any part of the double peak at 102.1 and 96.7 ppm. For the following reasons we therefore conclude that both parts of this peak are genuine, representing C_1 of glycogen: (1) The spinning rate of 4.550 kHz was carefully chosen so as to avoid spinning side bands in the C_1 region. (2) The area of the total C_1 peak shows excellent correlation with chemical measurements of glycogen from the same liver sample (see Table 2). (3) The ratio of the two components of the double peak remains constant with varying amounts of glycogen. (4) The peak splitting remains constant, 5.41 ± 0.11 ppm, in all samples, including spectra of a frozen solution of purified liver glycogen.

One explanation for the splitting may be that two conformational states of glycogen are populated which cannot equilibrate in the frozen state. Alternatively, there may be two pools of glycogen with different structure, for example lysosomal and the nonlysosomal glycogen. However, the fact that the splitting is not seen in liquid state seems to exclude the latter possibility.

Previous solid-state CP–MAS studies on various starches and cyclodextrins have shown double or multiple resonances of C_1 and C_4 both in the powder state (32–37) and in frozen solution (38). This multiple resonance phenomenon very likely originates from conformationally sensitive sites of the molecules, since the rotation about the glycosidic bonds appear to be the major determinant of C_1 and C_4 chemical shift (32,33,37; for review see 39). Thus, based on these previous solid-state studies we tentatively conclude that the observed splitting of the C_1 of glycogen of the rat liver *in vivo* reflects different conformational states of the molecule, which equilibrates much faster than the time scale of the liquid-state NMR experiment, but which is inhibited in the frozen state of the present CP–MAS solid-state experiment.

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