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**Nuclear magnetic resonance spectroscopy as a tool to study carbohydrate metabolism**

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Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical tool that has been widely applied to metabolic studies both *in vitro* and *in vivo*. The NMR phenomenon relies on the magnetic properties of nuclei, which, unless they have even atomic and mass numbers (for example, the common isotopes of C, O and S,  $^{12}\text{C}$ ,  $^{16}\text{O}$  and  $^{32}\text{S}$ ), have a net spin by virtue of the spins of their constituent protons and neutrons. As nuclei are positively charged, there will be a magnetic moment associated with the spin. This combination of spin angular momentum and magnetic moment causes the nuclei to precess in a magnetic field in much the same way that a gyroscope precesses in the earth's gravitational field. The NMR experiment measures this precessional frequency. It is proportional to the strength of the magnetic field at the nucleus itself and, thus, is influenced by the local electronic environment; it is this feature that gives the technique its remarkable chemical specificity. Chemical shifts are normally expressed in parts per million (ppm) relative to some suitable reference. Thus:

$$\delta = \frac{(f - f_{\text{ref}}) \times 10^6}{f_{\text{ref}}}$$

where  $\delta$  is the chemical shift,  $f$  the observed frequency and  $f_{\text{ref}}$  the reference frequency. For  $^{31}\text{P}$  NMR, the normal choice of reference (for which  $\delta$  is set to 0) is phosphocreatine, whereas for  $^1\text{H}$  and  $^{13}\text{C}$  NMR tetramethylsilane (TMS) is standard. The strength of the NMR signal (the area under a peak in the NMR spectrum) is proportional to the number of spins contributing to it. Quantification is possible, therefore, but it is rarely straightforward for *in vivo* experiments.

Several stable nuclei are potentially of interest for metabolic studies, including  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{31}\text{P}$ . The 100%-abundant stable isotope of F,  $^{19}\text{F}$ , with a sensitivity second only to  $^1\text{H}$ , has also been used in pharmacokinetic studies and is a popular choice for use

as a label in tracer experiments. Early *in vivo* applications, following the pioneering study of Hoult *et al.* (1974) on isolated skeletal muscle, concentrated largely on  $^{31}\text{P}$ , the 100%-abundant stable but magnetically active isotope of P. Such measurements afford an excellent indication of bioenergetic status (for example, see Gadian, 1982) and are also contributing to our understanding of how respiration is controlled to meet energy demand (Radda, 1986). The  $^{31}\text{P}$  NMR spectrum is an excellent indicator of ischaemic compromise and has been used clinically in studies of birth asphyxia (Cady *et al.* 1983) and stroke (Bottomley & Smith, 1986), but its main clinical application remains to muscle bioenergetics.

Although all glycolytic intermediates are phosphorylated, resonances from these metabolites are rarely seen under normal circumstances due to their low abundance. NMR suffers from relatively poor sensitivity and the detection threshold is typically a concentration of about 100  $\mu\text{M}$ . The intermediates can accumulate of course if there is a lesion in the glycolytic pathway; for example, fructose-6-phosphate accumulates in the case of phosphofructokinase (*EC* 2.7.1.4) deficiency (Chance *et al.* 1982). We have also recently demonstrated that in severe hypoxia the brain is unable to regenerate sufficient NAD (from NADH) by conversion of pyruvate to lactate; instead dihydroxyacetone phosphate is converted to glycerol-3-phosphate which accumulates (Ben-Yoseph *et al.* 1993). The latter compound appears, therefore, to be a useful marker of severe hypoxia.

Although sugar phosphates are rarely seen in  $^{31}\text{P}$  spectra of living systems, other phosphomonoesters are commonly observed; phosphoryl choline and phosphoryl ethanolamine, intermediates on the pathway of lipid synthesis, occur in relatively high concentrations in many tissues under conditions of active growth, for example in neonatal brain, regenerating liver and most tumours. Phosphodiester, such as glycerophosphoryl choline and glycerophosphoryl ethanolamine are associated with phospholipid breakdown, and are also commonly observed.

$^1\text{H}$  NMR, was once thought to be inappropriate for *in vivo* studies because of the ubiquitous presence of the proton. However, the widespread availability of high-field instrumentation and the development of effective water suppression techniques has resulted in a dramatic change of position and  $^1\text{H}$  NMR is now the commonest form of human magnetic resonance spectroscopy (MRS). Most studies are conducted on the head, and interest has centred on the well-resolved resonances from lactate and from *N*-acetyl aspartate, a putative neuronal marker. Little work has been conducted using  $^1\text{H}$  MRS on carbohydrate metabolism; resonances from glucose can be readily identified in  $^1\text{H}$  spectra recorded from the brains of diabetic subjects (Bruhn *et al.* 1991) but they are normally barely discernible in control subjects.

The wide chemical shift range (>200 ppm) of the  $^{13}\text{C}$  nucleus permits metabolites to be identified directly and, in many cases, unambiguously from their  $^{13}\text{C}$  NMR spectra. The low natural abundance (1.1%) of this stable C isotope means that only metabolites such as triacylglycerols or glycogen, that are highly concentrated in certain tissues, can be directly observed at natural abundance *in vivo* (Alger *et al.* 1981; Stevens *et al.* 1982). Nevertheless, pioneering  $^{13}\text{C}$  NMR studies by Shulman *et al.* (1990) at Yale have contributed significantly to our understanding of glycogen metabolism in man. In particular, they have demonstrated unequivocally that muscle glycogen synthesis is the principal pathway of glucose disposal in normal and diabetic subjects. A pilot  $^{13}\text{C}$  NMR study on the post-exercise repletion of liver and muscle glycogen in human subjects is described later (p. 337) and illustrates both the power and the limitations of this approach.

Most applications of  $^{13}\text{C}$  NMR involve the administration of specific highly enriched substrates, such as  $[1-^{13}\text{C}]\text{glucose}$ . The great advantage of this approach is that the metabolic time-course of substrate utilization can be followed and used to determine fluxes through pathways as well as to elucidate the pathways themselves. In recent years, these techniques, which were originally applied to studies of cell suspensions, e.g. yeast (den Hollander *et al.* 1979), or hepatocytes (Cohen *et al.* 1979), have been extended to the study of brain metabolism in isolated tissue (Morris *et al.* 1986; Badar-Goffer *et al.* 1990), experimental animals (Behar *et al.* 1986) and man (Gruetter *et al.* 1992a,b). Some new approaches in this area are discussed.

#### GLYCOGEN-REPLETION STUDIES

Natural-abundance  $^{13}\text{C}$  NMR spectra were recorded without proton decoupling, from the liver and thigh muscle of healthy volunteers. Subjects were placed inside the room-temperature bore of a 3 T whole-body superconducting magnet, supplied by Oxford Magnet Technology. Spectra were recorded at 32 MHz using a magnetic resonance system constructed at the University of Nottingham with funding from the British Technology Group. For the liver, a circular surface coil of radius 60 mm was constructed from a single turn of 6 mm diameter Cu pipe. This was mounted on a plastic spacer and taped to the skin of the volunteer. For the muscle measurements, an elliptical surface coil of major axis 120 mm and minor axis 70 mm was constructed from the same Cu pipe and attached to a thermoplastic cast, previously moulded to the upper leg of the subject.  $^{13}\text{C}$  NMR spectra were recorded by accumulating (signal averaging) blocks of 10 000 free induction decays, each excited by a single square radiofrequency pulse of 92  $\mu\text{s}$  duration. This pulse length corresponded to a flip angle of approximately  $180^\circ$  at the coil centre. The block acquisition time was 5.5 min.

A typical spectrum recorded from the liver of a resting subject, before exercise, is shown in Fig. 1. The  $^{13}\text{C}$  resonances appear mostly as multiplets (doublets, triplets and quartets) because no proton decoupling was applied during acquisition, and under these circumstances the  $^{13}\text{C}$  resonance frequency is sensitive to the spin orientations of any attached protons. The more prominent resonances arise from mobile lipids. One smaller signal originates from a small vial, containing a solution of sodium acetate labelled in the C-1 position (carboxyl group), attached to the reverse of the coil at roughly the same height as the depth of tissue from which maximum signal was expected; this served as a reference standard. A second small signal, seen as a clear doublet at approximately 100 ppm (relative to TMS = 0), is the natural abundance signal from the C-1 position of glycogen. Resonances from the other glycogen carbons (C-2–C-6) are hidden under the lipid peaks (mostly the triacylglycerol quartet).

After recording resting spectra from liver and muscle, the subject was exercised to voluntary exhaustion on an electrically-braked cycle at 75% of maximal  $\text{O}_2$  uptake ( $V_{\text{O}_2\text{max}}$ ); this was measured under medical supervision before the study. Further  $^{13}\text{C}$  spectra were recorded immediately post exercise and the subject then consumed a drink containing 192 g glucose (unlabelled). Subsequently,  $^{13}\text{C}$  time-courses were recorded in an interleaved fashion from liver and thigh muscle. Representative spectra from the liver time-course are shown in Fig. 2. Figs 3 and 4 respectively show the glycogen contents of liver and muscle, determined from the integral of the C-1 doublet peak area and ex-

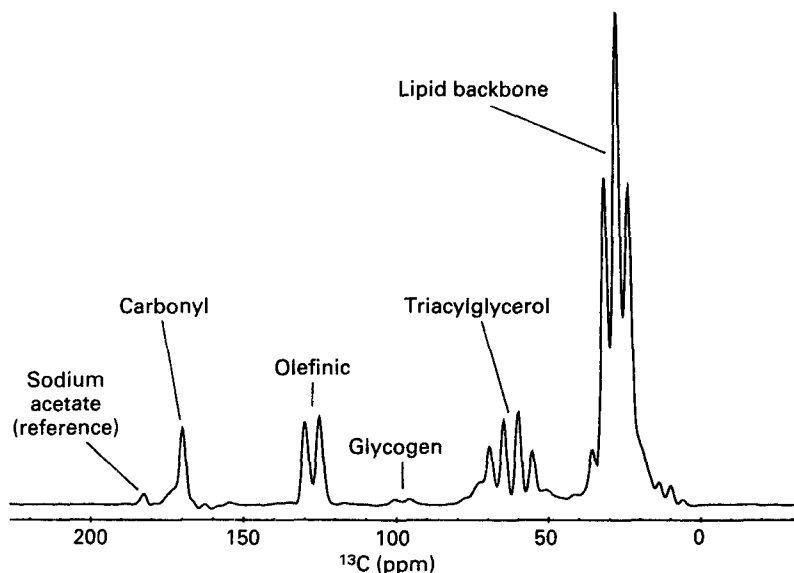


Fig. 1. Natural abundance  $^{13}\text{C}$  nuclear magnetic resonance spectrum (10 000 scans, without proton decoupling) of human liver, recorded at 3 T, using a single-turn surface coil of 60 mm radius.

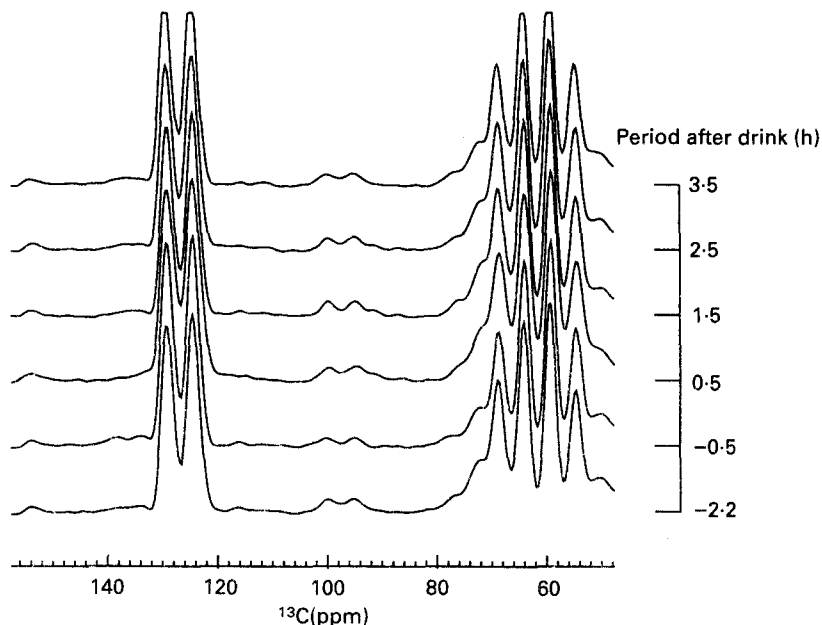


Fig. 2.  $^{13}\text{C}$  nuclear magnetic resonance time-course of human liver following exercise (for details see p. 337).

pressed as a percentage of the lipid peak. We believe that the measurements represent total glycogen (rather than a more mobile subfraction) on the basis of previous  $^{13}\text{C}$  studies of glycogen in rat liver (Stevens *et al.* 1982). Muscle glycogen, severely depleted following exercise, recovers monotonically with a time constant of some hours. In

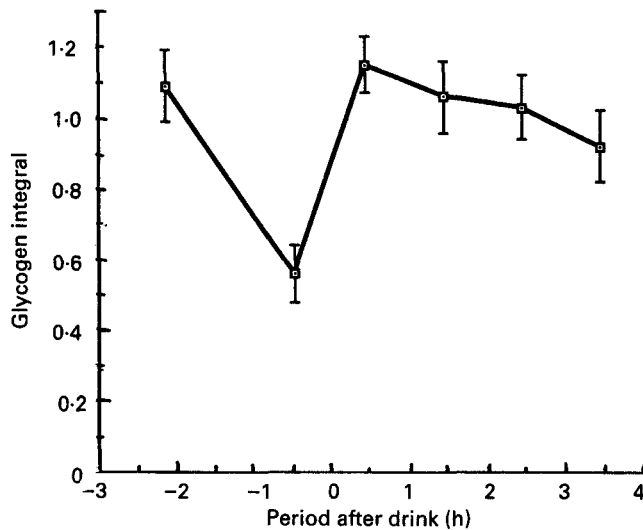


Fig. 3. Time-course of liver glycogen repletion following exercise. Measurements are areas of the C-1 glycogen doublet expressed as a percentage of the area of the lipid resonance. Values are means; vertical bars represent standard deviations.

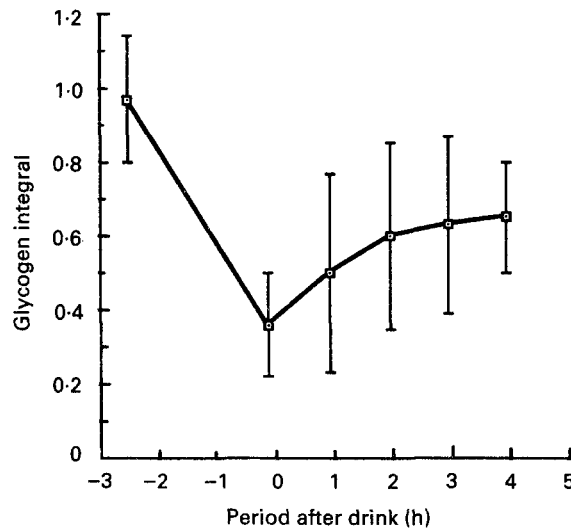


Fig. 4. Time-course of muscle glycogen repletion following exercise. Measurements are areas of the C-1 glycogen doublet expressed as a percentage of the area of the lipid resonance. Values are means; vertical bars represent standard deviations.

contrast, the liver glycogen rises rapidly after the glucose intake and thereafter appears to decline slowly.

These results indicate the power of natural-abundance  $^{13}\text{C}$  NMR for the study of human glycogen metabolism. They also illustrate some of the difficulties. In particular,

there is a dynamic range problem; the glycogen peaks are swamped by the much larger lipid resonances. If necessary, this problem could be circumvented by using selective pulses to specifically suppress the lipid resonances, or alternatively to avoid exciting them. As only 1.1% of the glycogen-C atoms are  $^{13}\text{C}$ , there is also a significant sensitivity problem. It can be improved by broadband proton decoupling which collapses the  $^{13}\text{C}$  multiplet structure (the C-1 glycogen doublet becomes a singlet with the same total area). It is then important to avoid excessive heating; composite pulse decoupling sequences, such as WALTZ16, rather than continuous broadband irradiation, are generally necessary to keep within the safety guidelines on radiofrequency power absorption. A further way to improve sensitivity is to use an indirect observation technique. This will be discussed (p. 341).

### $^{13}\text{C}$ NEUROSPECTROSCOPY

As the brain has no substantial carbohydrate store, natural-abundance *in vivo*  $^{13}\text{C}$  experiments are out of the question. The substrate for *in vivo* brain studies is usually  $[1-^{13}\text{C}]$ glucose. The  $^{13}\text{C}$  label from this source 'flows' through glycolysis to pyruvate and thence to lactate, or else enters the tricarboxylic acid (TCA) cycle, accumulating in the C-4 of glutamate, and redistributing to the C-2 and C-3 positions in subsequent turns of the TCA cycle, as well as to other key cerebral metabolites. The rate of appearance of label in C-3 of lactate and C-4 of glutamate gives a measure of the cerebral glycolytic and TCA cycle fluxes respectively. Shulman's group have estimated the latter as  $1.4 \mu\text{mol/g}$  per min in the rat (Fitzpatrick *et al.* 1990).

Under hypoxic conditions, we find in our studies on superfused cerebral cortical slices that glycolysis is accelerated and the TCA cycle suppressed, whereas under depolarizing conditions, both are stimulated (Badar-Goffer *et al.* 1992). Less predictably, in severe hypoxia, we also observe significant accumulation of alanine and glycerol-3-phosphate, reflecting, we believe, the inability of lactate dehydrogenase (*EC* 1.1.1.27) to prevent the accumulation of NADH under these conditions (for discussion of  $^{31}\text{P}$  NMR, see p. 336).

Cerebral metabolism is compartmented and acetate is thought to be metabolized exclusively by glial cells.  $^{13}\text{C}$  label, supplied as  $[2-^{13}\text{C}]$ acetate is incorporated into glutamine and citrate, which we take to be indicative of glial metabolism (Badar-Goffer *et al.* 1990). Under depolarizing conditions,  $[1-^{13}\text{C}]$ glucose labels these two metabolites much more strongly than under control conditions, suggesting a selective stimulation of glial metabolism. By using the combined precursors  $[1-^{13}\text{C}]$ glucose and  $[1,2-^{13}\text{C}]$ acetate, we have shown in preliminary experiments that it is possible to study neuronal and glial metabolism simultaneously. Essentially, the latter substrate is always incorporated into both the C-4 and C-5 positions of glutamate whereas the former is (initially) incorporated only into the C-4 position. These labelling patterns can be distinguished in the C-4 multiplet, enabling the origin of the labelled material to be discerned.

For  $^{13}\text{C}$  studies of the human brain, large quantities of labelled material (up to 60 g) are required (Gruetter *et al.* 1992a), making these investigations extremely expensive – up to US \$3000 per patient study. In addition, the spatial resolution is poor, often corresponding to regions of interest in excess of 100 ml. A most promising alternative

approach is to make use of the inherently higher NMR sensitivity of protons to detect the  $^{13}\text{C}$  atoms to which they are chemically bonded.

#### INDIRECT DETECTION

NMR sensitivity is proportional to the third power of the magnetogyric ratio (an inherent property of the nucleus), giving a potential gain in sensitivity of  $4^3$  or 64 on going from direct  $^{13}\text{C}$  NMR to indirect detection via  $^1\text{H}$  NMR. Unfortunately, the noise also depends on the NMR operating frequency, a fact often ignored when estimating the potential benefits of inverse detection but, assuming that the sample itself is the dominant source of noise, the improvement in signal:noise we can reasonably expect is still a factor of about 16.

Much technical effort is being expended in developing indirect detection techniques. The basis of all these methods is the scalar (J) coupling between protons and  $^{13}\text{C}$  nuclei, which has a magnitude of typically 126 Hz and is clearly visible in the multiplets of Fig. 1. To pick out just those protons that are coupled to  $^{13}\text{C}$  nuclei, proton observe C edit (POCE) experiments, originally used to observe cell suspensions, have been refined. In the basic experiment, a non-selective  $^{13}\text{C}$  inversion ( $180^\circ$ ) pulse is applied in alternate scans which are accumulated separately. Subtraction of the spectra yields a  $^1\text{H}$  difference spectrum in which only those protons that are bonded to  $^{13}\text{C}$  atoms appear (Bendall *et al.* 1981; Freeman *et al.* 1981). Although in theory this experiment should eliminate the troublesome water peak, the selectivity is far from perfect and additional steps must be taken to suppress it. Further improvements to the editing technique as well as to the spatial selection and particularly to the shimming (Gruetter & Boesch, 1992) have resulted in improved resolution that permits the glutamate C-3 and C-4 resonances to be resolved in human brain spectra (Chen *et al.* 1992).

#### FUTURE DEVELOPMENTS

Localized  $^{13}\text{C}$  measurements of glycogen metabolism in liver and muscle are possible on a routine basis. Cerebral metabolism can also be studied with sufficient spectral resolution to distinguish C-2, C-3 and C-4 glutamate and glutamine resonances (Gruetter *et al.* 1992b) and to measure cerebral glucose levels (Gruetter *et al.* 1992a). Localized  $^1\text{H}$  spectroscopy can be used to observe cerebral glucose and its metabolites directly at higher spatial resolution (see for example Bruhn *et al.* 1991).

A promising new approach to indirect detection experiment is the gradient enhanced heteronuclear multiple quantum coherence technique, in which gradient pulses are used not only for selective volume excitation but also for coherence selection (van Zijl *et al.* 1992). This method provides extremely efficient elimination of the water peak, permitting resonances that lie close to it, such as C-1 of glucose and C-2 of glutamate to be observed. We can expect to see a continuing refinement of indirect detection methods for the observation of  $^{13}\text{C}$ -labelled metabolites. We can also expect that, as the anticipated sensitivity advantages of this method are realized, it will be possible to reduce the selected volume from 100 ml, certainly to  $<10$  ml and perhaps eventually to  $<1$  ml. Inevitably, there will be considerable effort in correlating indirect  $^{13}\text{C}$  NMR results with those from other nuclei, especially  $^{31}\text{P}$  measurements of bioenergetic status. All these

developments will be aided by the use of the higher-field clinical magnets (3 and 4 T) that are now becoming more widely available.

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