Compartmentation of cerebral glutamate *in situ* as detected by $^{1}H/^{13}C$ n.m.r.

Risto A. KAUPPINEN,*§ Tiina-R. M. PIRTTILÄ,* Seppo O. K. AURIOLA† and Stephen R. WILLIAMS‡

*NMR Research Group, Department of Biochemistry and Biotechnology, A.I. Virtanen Institute, University of Kuopio, Kuopio, Finland,
†Department of Pharmaceutical Chemistry, University of Kuopio, Kuopio, Finland, and ‡Department of Biophysics, Institute of Child Health, London WC1N 1EH, U.K.

Incorporation of ^{13}C label from either [1-^{13}C]glucose to glutamate C-4 and lactate C-3 or from [2-^{13}C]acetate to glutamate C-4 was monitored *in situ* in a superfused brain slice preparation by using ^{1}H-detected/^{13}C-edited ($^{1}H/^{13}C$) n.m.r. spectroscopy. The fractional enrichments of both metabolites were determined by this means in both brain slices and acid extracts of the preparations in order to assess their ^{1}H-n.m.r. detectabilities. The $^{1}H/^{13}C$ satellite resonances from glutamate C-4 and lactate C-3 in brain tissue were followed from 4 min onwards in the presence of 5 mM [1-^{13}C]glucose. Fractional enrichment of glutamate C-4 in the slice preparations was higher than in their acid extracts throughout the incubation of 100 min; at 30 min the enrichment was 15.9 ± 0.6 % in the slice preparations and 10.6 ± 0.9 % in extracts and at 100 min 24.5 ± 1.7 % compared with 19.7 ± 0.4 % respectively. In contrast, lactate C-3 reached a steady-state fractional enrichment of approx. 43 % by 15 min and there was no difference between the values determined in the slice preparations and the acid extracts. There was a significant difference between the glutamate C-4 fractional enrichments in the brain slices (7.4 ± 0.6 %) and extracts (5.1 ± 0.3 %) after 60 min of incubation with [2-^{13}C]acetate. Thus ^{13}C label from both glucose and exogenous acetate enters a pool of glutamate that is more amenable to ^{1}H n.m.r. detection than total acid-extracted brain biochemical glutamate, whereas lactate is labelled with full ^{1}H n.m.r. visibility. The results are discussed in the light of the biochemical factors that affect glutamate ^{1}H-n.m.r. susceptibility and thus its n.m.r. visibility.

INTRODUCTION

L-Glutamic acid is the major excitatory neurotransmitter in the mammalian brain (Fonnum, 1984; Nicholls, 1989; Maycox et al., 1990; McMahon and Nicholls, 1991). It plays a predominant role in integrated brain function, and a growing body of evidence suggests that it is involved in the development of irreversible injury in a number of disorders causing neuronal degeneration (Rothman and Olney, 1987; Choi, 1990). Thus the metabolism, release and uptake of glutamate have been targets of extensive neurochemical research. The development of water-suppressed ^{1}H n.m.r. has made it possible to measure glutamate non-destructively *in vivo*. Previous studies have indicated that ^{1}H n.m.r. does not detect all of the biochemically assayed brain glutamate (de Graaf and Bovee, 1990; Kauppinen and Williams, 1991; Pirttilä et al., 1993). It has been concluded that as much as 30 % of cerebrocortical glutamate does not contribute to the C-4 peak of glutamate at 2.35 p.p.m. in animal brain (Kauppinen and Williams, 1991; Pirttilä et al., 1993). Data from clinical ^{1}H-n.m.r. spectroscopy have indicated that glutamate in human brain quantified by this means may be less than that determined by biochemical methods (Michaelis et al., 1993). This peak is well resolved in the brain ^{1}H-n.m.r. spectrum collected either at high field (> 7 T) or at low field (approx. 1.5 T) using short echo times (approx. 20 ms) (Frahm et al., 1990). The recent demonstration that tricarboxylic acid-cycle activity can be estimated non-invasively in brain from ^{1}H-n.m.r. measurements of glutamate enrichment with ^{13}C label (Fitzpatrick et al., 1990) emphasizes the importance of understanding the n.m.r. visibility of glutamate.

It has been suggested that the partial ^{1}H-n.m.r. invisibility of glutamate may be caused by its distribution between neurons and glia (de Graaf and Bovee, 1990). We have previously used the superfused brain slice preparation as a model of cerebral cortex in order to study glutamate metabolism non-destructively by n.m.r. (Kauppinen and Williams, 1991; Pirttilä et al., 1993). Our data showed that, under controlled bioenergetic conditions, 75 % of brain glutamate is ^{1}H-n.m.r. visible (Pirttilä et al., 1993). Both severe energy failure and challenge with a K^{+}/H^{+} ionophore, nigericin, without complete collapse of ATP caused all of the glutamate to become detectable by ^{1}H n.m.r. (Pirttilä et al., 1993). These observations lend support to the idea that ^{1}H-n.m.r. invisibility of glutamate may be due to subcellular compartmentation. In order to explore this hypothesis, we have studied n.m.r. detectability of newly synthesized [^{13}C]glutamate from either exogenous glucose or acetate in a superfused brain slice preparation by using ^{1}H n.m.r. specifically designed to detect the fractional enrichment of ^{13}C-labelled metabolites.

EXPERIMENTAL

Preparation of cerebral brain slices, incubations and n.m.r. methods

Transverse cortical brain slices (thickness 0.35 mm) from male guinea pigs (250–400 g, Dunkin–Hartley strain) were prepared and superfused as described previously (Cox et al., 1983). The cerebral slices were initially incubated in Krebs–Henseleit buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 5 mM D-glucose equilibrated with O_2/CO_2 (95 %/5 %, v/v) for 30–45 min at 37 °C. Medium phosphate was omitted in order to monitor and utilize the intracellular P_i as an indicator of pH_i (Taylor et al., 1983) using ^{31}P n.m.r. In labelling experiments, 5 mM D-glucose in the superfusion medium was replaced by either 5 mM D-[1-^{13}C]glucose or 5 mM [2-^{13}C]acetate and 2.5 mM D-glucose.

^{31}P-n.m.r. spectra were recorded using a Bruker 15 mm ^{1}H/broad-band (tuned to ^{31}P at 162.0 MHz) n.m.r. probe in a 9.4 T vertical magnet interfaced with a Bruker AM400 spectrometer as previously described (Pirttilä et al., 1993). ^{1}H-n.m.r. spectra [spectral width 5 kHz, 4096 data points, interpulse interval 1.7 s, with optimum excitation adjusted at 2.02 p.p.m. (Hore, 1983;

Abbreviations used: NAA, *N*-acetylaspartate; PCA, perchloric acid; pH_i, intracellular pH; TE, spin-echo time; T_2, spin–spin relaxation decay.

§ To whom correspondence should be addressed.

Williams et al., 1988) and spin–echo time, TE = 120 ms] were recorded using the following spin-echo sequence incorporating solvent suppression pulses:

$$(1\text{–}\bar{3}\text{–}3\text{–}\bar{1})\text{–}(TE/2)\text{–}(2\text{–}\bar{6}\text{–}6\text{–}\bar{2})\text{–}(TE/2)\text{–acquire} \quad (1)$$

^{1}H-n.m.r. spectra were processed and peak areas determined using a line-fitting routine (Glinfit, Abacous Spectrospin Co.) as previously described (Pirttilä et al., 1993). Chemical shifts are expressed relative to a value of 2.02 p.p.m. for the methyl resonance of *N*-acetylaspartate (NAA).

The detection of protons bonded to ^{13}C carbons was accomplished by exploiting the spin–spin coupling between directly bonded ^{1}H and ^{13}C nuclei, based on the method described by Rothman and co-workers (Rothman et al., 1985). In these experiments the broad-band channel was returned to the ^{13}C frequency (100.6 MHz), and used to deliver composite ^{13}C inversion pulses with phase cycling as previously described (Tycko et al., 1985). These pulses were incorporated into the binomial spin–echo sequence above on alternate scans, 3.9 ms after the excitation pulse train as follows:

$$^1\text{H} \quad (1\text{–}\bar{3}\text{–}3\text{–}\bar{1})\text{---}60\text{ ms}\text{---}\ (2\text{–}\bar{6}\text{–}6\text{–}\bar{2})\text{---}60\text{ ms}\text{---acquire} \quad (2)$$

$$^{13}\text{C} \quad 3.9\text{ ms}\text{--}180^\circ\ \text{----------acquire} \quad (3)$$

The 3.9 ms delay equals $1/(2J)$ for the ^{1}H/^{13}C coupling ($J_{(HC)}$ = 127 Hz) and allows the ^{1}H/^{13}C doublet to precess into antiphase so that, in the presence of inversion pulse, the signal from ^{13}C-bonded protons is inverted relative to that of ^{12}C-bonded protons. Subtraction of the two subspectra yields the ^{13}C satellite resonances of protons bonded to ^{13}C. Decoupling at the ^{13}C frequency was not used during acquisition. The subtraction artifact obtained by the present method in phantoms was less than 1 % and thus could be ignored.

Fractional enrichment of glutamate C-4 in both slices and extracts (see below) were calculated by adding peak areas of satellites at 2.51 and 2.19 p.p.m. and dividing this by the sum of satellite areas + peak area of ^{1}H/^{12}C C-4 at 2.35 p.p.m. In the computations of lactate C-3, fractional enrichment satellite area at 1.49 p.p.m. was multiplied by two and this was divided by the sum of satellite areas + peak area of the lactate CH_3 at 1.33 p.p.m.

Extraction of brain tissues and their n.m.r. analyses

Brain corticies (1 vol.) were homogenized in 0.9 M ice-cold perchloric acid (PCA) (3 vol.) using a Potter–Elvehjem homogenizer as previously described (Kauppinen and Williams, 1991). The concentrations of metabolites are expressed against PCA-precipitated protein. The same pulse sequence as for the cortical slice preparations in the same 15 mm n.m.r. probe was used for inverse detection of ^{1}H/^{13}C satellites in the extracts. An interpulse interval of 4.1 s was used in these experiments. The intensity of satellite resonances relative to the central ^{1}H/^{12}C was, as expected, independent of the interpulse interval; the fractional enrichments of glutamate C-4 in extracts were calculated to be 12.9, 12.6, 12.6 and 12.9 % in spectra recorded using intervals of 1.5, 5, 10 and 25 s respectively. High-resolution ^{1}H/^{13}C correlation n.m.r. spectra via zero and double quantum coherence without decoupling during acquisition (spectral width 4 kHz, 16384 data points, interpulse interval of 7.1 s, optimized for $J_{(HC)}$ = 127 Hz) were collected using a dedicated 5 mm inverse ^{13}C probe (Bruker, Rheinestetten, Germany) (see Bax and Subramanian, 1986).

Some metabolites were quantified in absolute terms by ^{1}H n.m.r. Fully relaxed spectra were acquired using 90° repeated every 7.1 s (spectral width 4 kHz, 16384 data points) with low-power presaturation of the residual ^{2}H signal. Chemical shifts of peaks are referenced to 3-[^{2}H](trimethylsilyl)propionic acid at 0 p.p.m., which was also used as a concentration standard.

Proton-decoupled ^{13}C-n.m.r. spectra (interpulse interval 6.5 s, spectral width 11.6 kHz, 32768 data points) were acquired with a continuous broad-band irradiation accomplished by composite pulses at the ^{1}H frequency for both decoupling and nuclear Overhauser enhancement. Chemical shift was referenced to either C-1 of α-D-glucose at 93.01 p.p.m. or C-2 of acetate at 24.13 p.p.m.

M.s. analysis of [^{13}C]glutamate

Stable isotope enrichment of glutamate ($M+1$) in neutralized PCA extracts was determined using g.c.–m.s. as described previously (Kapetanovic et al., 1990). Briefly, the residues were derivatized with 200 μl of acetonitrile/*N*-methyl-*N*(*t*-butyldimethylsilyl)trifluoroacetamide (3:1, v/v) at 80 °C for 30 min. The derivatives of amino acids were separated on a capillary column (DB-5 25 m, internal diameter 0.20 mm, pore size 0.11 μm; J&W Scientific, Folsom, CA, U.S.A.) in an HP 5890 gas chromatograph, and the individual compounds were analysed in a VG Trio-2 quadrupole mass spectrometer (VG Masslab; Manchester, U.K.) in an electron ionization mode. Gas-chromatography oven-temperature profile was as follows: hold 0.5 min at 50 °C, program 20 °C/min to 180 °C, program 18 °C/min to 270 °C respectively, ionization chamber set at 200 °C. This provided an adequate separation of glutamate (retention time of 12.7 min) from other acid-extractable metabolites. Selected ion recording was used for determination of ion abundances. The prominent and characteristic $[M-57]^+$ ions were monitored for glutamate (m/z 432) and for its $M+1$ isotope (m/z 433). Stable isotope enrichment was calculated by making first-order corrections (for natural abundance).

Reagents

[1-^{13}C]Glucose (99 % atomic enrichment) and [2-^{13}C]acetate (sodium salt, 99 % atomic enrichment) were purchased from Sigma, Poole, Dorset, U.K. Silyl derivatization reagents for m.s. were from Aldrich. All other standard chemicals were of analytical grade and obtained from Sigma or E. Merck.

RESULTS

Typical ^{1}H-n.m.r. spectra from a preparation superfused in the presence of D-[^{12}C]glucose (Figure 1a) or D-[1-^{13}C]glucose (Figures 1b and 1c) and from an extract (Figures 1e and 1f) are shown. When D-[1-^{13}C]glucose was used as a substrate for 56 min, the intensity of the peak from protons bonded to ^{12}C-4 of glutamate resonating at 2.35 p.p.m. was severely reduced relative to NAA and a peak at 2.50 p.p.m. was increased (Figure 1b) compared with the peak intensities in the presence of D-[1-^{12}C]glucose (Figure 1a). The ^{13}C-edited spectrum in Figure 1(d) reveals the satellite resonances from the protons bonded to [^{13}C]glutamate C-4 and [^{13}C]glutamate C-3 and [^{13}C]lactate C-3 in the slice preparations. Fractional enrichments in brain slices calculated from spectra using spin-echo delays of 5 and 60 ms were similar within experimental error (24.2 % compared with 23.7 % respectively, $n = 2$). This indicates that the spin–spin

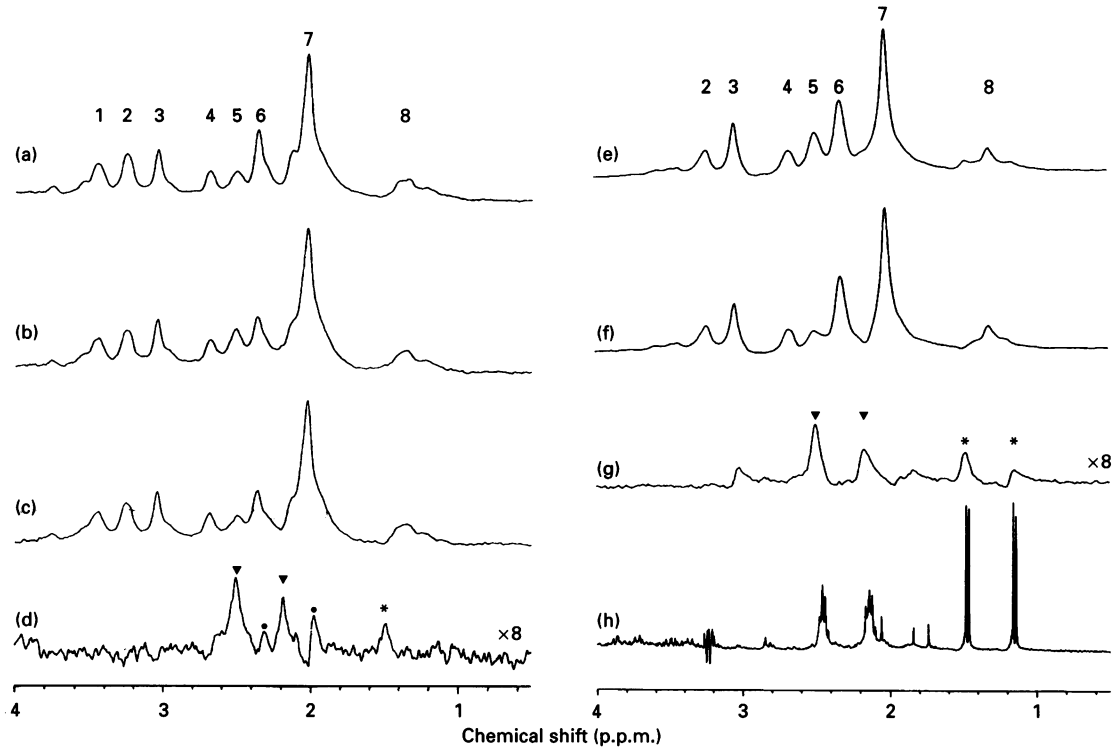

Figure 1 ^1H-n.m.r. and ^1H-detected/^{13}C-edited n.m.r. spectra of a superfused brain slice preparation and an acid extract

Cortical brain slices were superfused and ^1H-n.m.r. and ^1H-detected/^{13}C-edited n.m.r. spectra acquired as described in the Experimental section. In (**a**) superfusion medium contained 5 mM D-glucose as substrate and sequence (1) was used. Peaks are assigned as follows: 1, glucose; 2, glucose + choline-containing compounds; 3, creatine + phosphocreatine; 4, aspartate + NAA; 5, glutamine + NAA; 6, glutamate; 7, NAA; 8, lactate. The spectra in (**b**) and (**c**) were collected after 56 min superfusion with 5 mM [1-^{13}C]glucose, and the ^1H-detected/^{13}C-edited n.m.r. sequence (2) was used for the spectrum (**b**) and sequence (3) for the spectrum (**c**). (**a–c**) are sums of 128 scans each. (**d**) is the difference spectrum of (**b**) − (**c**) and it consists of 256 scans and is 8-fold magnified relative to (**b**). The spectra (**e**) and (**f**) (512 scans each) are from an extract of a preparation after 100 min superfusion in the presence of 5 mM [1-^{13}C]glucose; they were collected using sequence (2) and (3) respectively. (**g**) is the difference spectrum of (**e**) − (**f**) and is 8-fold magnified relative to (**e**). The spectrum (**h**) is a high-resolution $^1H/^{13}C$ edited spectrum of the same extract. Satellite resonances from glutamate C-4 (▼), glutamate C-3 (●) and lactate C-3 (✱) are indicated.

relaxation times (T_2) of ^{12}C- and ^{13}C-bonded protons were not significantly different. Therefore the fractional enrichments of metabolites in the brain slices do not require correction for the spin–spin relaxation.

The edited spectrum of an extract (Figure 1g) shows the satellites of glutamate C-4 and lactate C-3 with similar relative intensities to those detected in the slice preparation (Figure 1d). A high-resolution $^1H/^{13}C$ correlation spectrum of the same extract (Figure 1h), recorded using a sequence that excites equally the chemical-shift range, demonstrates that there were no overlapping peaks at either glutamate or lactate frequency regions.

The time courses of the evolution of the glutamate and lactate satellites in a typical brain slice preparation superfused in the presence of D-[1-^{13}C]glucose are shown in Figure 2. The satellites of both glutamate C-4 and lactate C-3 in the brain slices were detectable after 4 min incubation with labelled glucose (Figure 2). Owing to non-uniform excitation of the $1\text{–}\bar{3}\text{–}3\text{–}\bar{1}$ pulse train, the downfield satellite (at 1.17 p.p.m.) is barely visible. In the subsequent quantification, only the peak at 1.49 p.p.m. was used for estimation of lactate C-3 enrichment. It should be noted that we did not detect consistently satellites of glutamate C-3 (Figure 1d), presumably because of its low fractional enrichment relative to C-4 (see below). The increase in the fractional ^{13}C enrichment of glutamate C-4 (Figure 3a) and the decrease in the intensity of [^{12}C]glutamate C-4 at 2.35 p.p.m. relative to NAA (Figure 3b) showed reciprocal behaviour. As glycolysis produces 1 mol of ^{13}C-labelled pyruvate per mol of [1-^{13}C]glucose utilized, C-4 of glutamate can reach a theoretical maximal fractional enrichment of 50%. The fractional enrichment of the C-4 in the brain slices attained a steady state of $24.5 \pm 1.7\%$ by 100 min (Figure 3a) which matched well the reduction of ^{12}C-4 relative to NAA by $23.8 \pm 3.9\%$ (Figure 3b). Lactate C-3 reached a steady-state enrichment of $43 \pm 4\%$ after 15 min of superfusion under the same conditions. These maximum fractional enrichments of glutamate C-4 and lactate C-3 are in line with previous observations (Badar-Goffer et al., 1990, 1992). There were no resolvable satellites at 2.29 and 1.97 p.p.m. at approx. 40 min which could correspond to glutamate C-3 (Figure 2).

The fractional enrichment from glucose of glutamate C-4 determined in the acid-extracted brain slices was significantly less than that measured in cerebral cortex throughout the observation period of 100 min. At 4 min of superfusion with [1-^{13}C]glucose, the enrichment of glutamate C-4 in the brain slices was $5.2 \pm 0.3\%$, which was as much as the labelling of C-4 in the tissue extracts ($4.0 \pm 0.7\%$) at 10 min (Figure 3a). At 30 min of incubation, the fractional enrichment of glutamate C-4 in the extracts was $10.6 \pm 0.94\%$ and at 100 min $19.7 \pm 0.37\%$, corresponding to 58.2% and 80.4% respectively of that determined in the slice preparations. Thus the labelling of the acid-extracted (total biochemical) pool of glutamate was less at all time points than that determined in slices. Lactate C-3 labelling in the brain slices did not differ from that determined in their acid extracts at any time point analysed (Figure 3c). Neither the phosphocreatine/β-ATP ratios nor the pH_i changed during the superfusions (Table 1), indicating that the intracellular free ADP

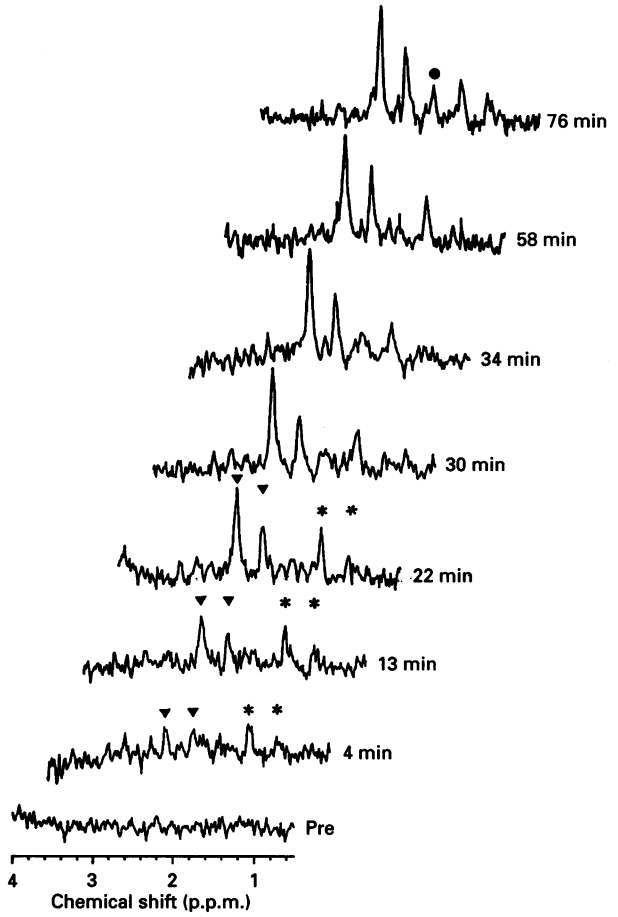

Figure 2 Time course of ^{1}H-detected/^{13}C-edited n.m.r. spectra from a brain slice preparation after superfusion in the presence of 5 mM [1-^{13}C]glucose

Conditions as in Figure 1(b)–(d). ^{1}H-n.m.r. and ^{1}H-detected/^{13}C-edited n.m.r. spectra (128 scans each) were collected in the presence of 5 mM D-glucose (pre) and 5 mM [1-^{13}C]glucose at time points (mid-time of the acquisitions) indicated. Peak assignments were as in Figure 1(d). Satellite resonances from glutamate C-4 (▼), glutamate C-3 (●) and lactate C-3 (✱) are shown.

concentration was also constant. Glutamate/NAA and NAA concentrations in the extracts also did not change during the course of the experiments (Table 1).

Glutamate labelling in tissue acid extracts ($M+1$) was also determined by m.s. These data are shown together with glutamate C-4 enrichment assayed by n.m.r. in Figure 4(a). The labelling of glutamate determined by m.s. was approx. 30% greater at all time points analysed than that found in C-4 by n.m.r. The proton-decoupled ^{13}C-n.m.r. spectrum of acid extracts from preparations superfused in the presence of [1-^{13}C]glucose revealed label in glutamate (C-2, C-4 and C-3), lactate (C-3), acetate and γ-aminobutyrate (C-2, C-3). This is in accord with published data on ^{13}C labelling both in brain slices (Badar-Goffer et al., 1990, 1992) and *in vivo* (Rothman et al., 1985; Fitzpatrick et al., 1990). From these extract spectra, we calculate that the glutamate C-4 peak contained 71±4 and 68±4% of the total glutamate ^{13}C label at 10 and 100 min incubation times respectively. The glutamate C-3 contained 15±3% and 17±1% and the C-2 14±1% and 16±3% of the total label at 10 and 100 min. On the basis of these data we ascribe the difference between m.s. and n.m.r. results (Figure 4a) to the labelling of glutamate C-2 and C-3. The conclusion is consistent with earlier work on ^{13}C-labelling of brain slices (Badar-Goffer et al., 1990).

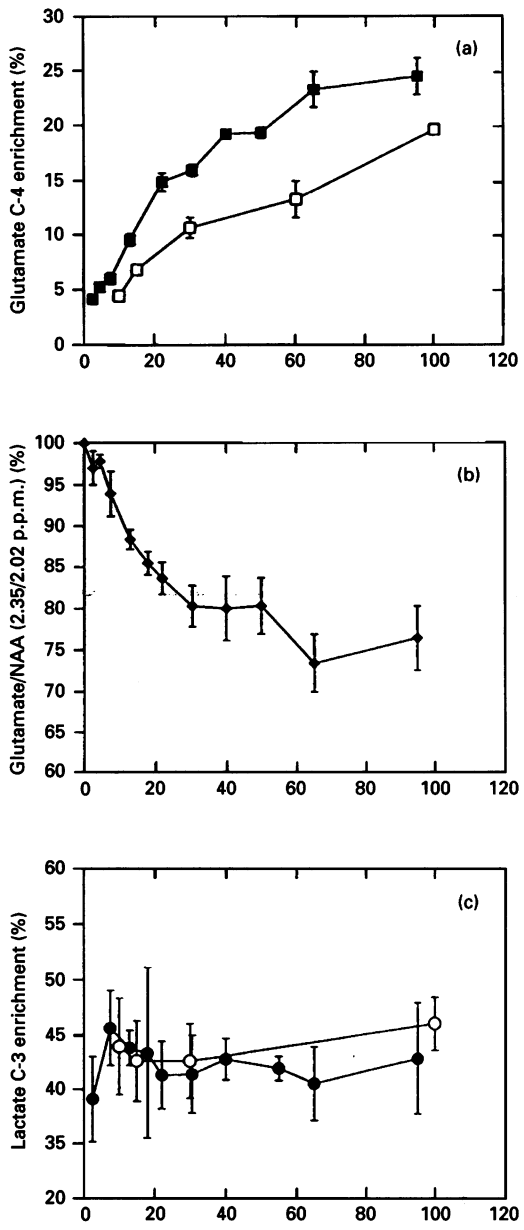

Figure 3 Glutamate C-4 enrichment (a), glutamate/NAA ratios (b) and lactate C-3 enrichment (c)

Fractional enrichments of glutamate C-4 (**a**) and lactate C-3 (**c**) were determined in both the slice preparations (filled symbols) and extracts (open symbols) using n.m.r. as described in the Experimental section. Glutamate/NAA peak area ratios were calculated from the ^{1}H-detected/^{13}C-edited n.m.r. subspectra without ^{13}C composite pulses [sequence (2)]. Values are means±S.E.M. for four to seven independent experiments.

[2-^{13}C]Acetate is converted directly into acetyl-CoA and labels glutamate in the C-4 position on the first turn of the tricarboxylic acid cycle. The proportion of C-4 of glutamate labelled from acetate was much smaller than from glucose and we could not follow the fractional enrichment with the same temporal resolution as from [1-^{13}C]glucose. In addition, the strong satellites from the substrate itself at 2.07 and 1.76 p.p.m. masked any peaks from labelled metabolites. After 30 min of superfusion with [2-^{13}C]acetate (together with 2.5 mM D-glucose) followed by a washout of the substrate for 5 min, satellites of glutamate C-4 were resolvable (Figure 5c). The ^{13}C-edited spectrum of an acid

Table 1 Phosphocreatine/β-ATP ratios and pH_i in brain slices and glutamate/NAA ratios and NAA concentration in their acid extracts

Phosphocreatine/β-ATP and pH_i were determined in the slice preparations in the presence of 5 mM D-glucose (control) and after 60–70 min of incubation in the presence of either 5 mM [1-^{13}C]glucose ([1-^{13}C]glucose) or 5 mM [2-^{13}C]acetate and 2.5 mM D-glucose ([2-^{13}C]acetate). NAA (in μmol/g dry weight of acid-extracted protein) and glutamate/NAA ratios were determined in the acid extracts of the slices at 10 min (control) or 100 min of superfusion with 5 mM [1-^{13}C]glucose or after 60 min of superfusion with [2-^{13}C]acetate and 2.5 mM D-glucose. Values as means ± S.E.M. for four to seven independent experiments.

Parameter	Control	[1-^{13}C]Glucose	[2-^{13}C]Acetate
Phosphocreatine/β-ATP	1.54 ± 0.14	1.54 ± 0.19	1.31 ± 0.06
pH_i	7.33 ± 0.03	7.25 ± 0.05	7.24 ± 0.08
NAA	11.4 ± 0.6	12.8 ± 0.6	11.4 ± 0.8
Glu/NAA	0.74 ± 0.03	0.70 ± 0.04	0.75 ± 0.06

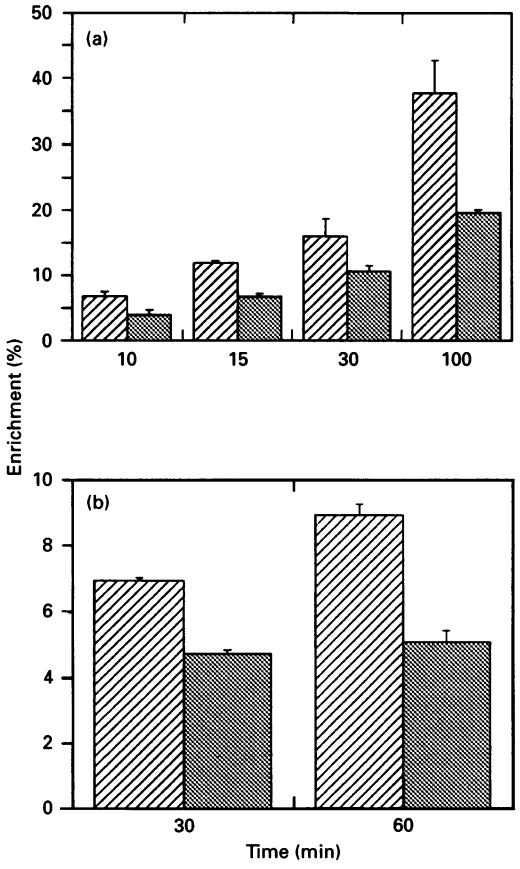

Figure 4 ^{13}C enrichment of glutamate in the acid-extracted brain slices

Cortical brain slices were superfused in the presence of 5 mM [1-^{13}C]glucose (**a**) or 5 mM [2-^{13}C]acetate (**b**) as indicated. ^{13}C enrichment of glutamate ($M+1$) was determined by m.s. (▨). These values are compared with glutamate C-4 enrichment determined by ^{1}H-detected/^{13}C-edited n.m.r. (▩, Figure 3a). Values are means ± S.E.M. for four to six independent experiments.

extract shows that there was no overlap from glutamate C-4 (satellites ought to appear at 2.62 and 2.30 p.p.m.).

Enrichments of C-4 of glutamate in the slice preparations and their extracts were 5.7 ± 0.5 % and 4.8 ± 0.1 % ($n = 5$) respectively. Thus acetate appears to label the total glutamate pool with uniform efficiency. However, after 60 min of superfusion, glutamate C-4 enrichment in slices was 7.4 ± 0.6 % which was greater

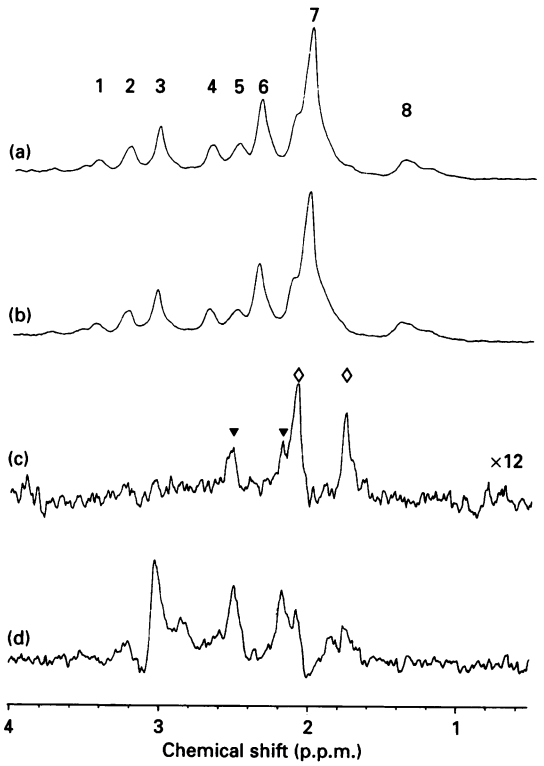

Figure 5 ^{1}H-detected/^{13}C-edited n.m.r. spectra of a brain slice preparation superfused in the presence of [2-^{13}C]acetate and its acid extract

Brain slices were superfused in the presence of 5 mM [2-^{13}C]acetate and 2.5 mM D-glucose for 30 min followed by a washout of 5 min, and the ^{1}H-detected/^{13}C-edited n.m.r. spectra were collected using sequence (2) for (**a**) and sequence (3) for (**b**) (128 scans each). (**c**) Difference spectrum of (**b**)–(**c**) consisting of 256 scans; it is 12-fold magnified relative to (**a**). The spectrum (**d**) is from the extract of the same preparation [sum of 512 scans, scaled independently of (**c**)]. The peaks are assigned and labelled as in Figure 1. ◇, Satellites from acetate. Note that acetate satellites have lower intensities relative to glutamate C-4 in the extract than in slices because of longer washout of exogenous [2-^{13}C]acetate in the latter case.

than that (5.1 ± 0.34 %, $P < 0.01$, Student's t test) determined in the extracts. Glutamate enrichment ($M+1$) measured by m.s. was 6.9 ± 0.1 and 8.9 ± 0.3 % at 30 and 60 min respectively (Figure 4b). Proton-decoupled ^{13}C n.m.r. of the extracts revealed that 72 ± 6 % of glutamate label was in C-4, 8 ± 4 % in C-3 and 20 ± 3 % in C-2. Phosphocreatine/β-ATP and pH_i in the slice preparations and glutamate/NAA ratios and NAA concentrations in their extracts were unchanged after 60 min of superfusion with acetate-containing medium (Table 1).

DISCUSSION

The present results demonstrate the superiority of indirect detection of ^{13}C label by ^{1}H n.m.r. for following labelling as it happens, non-destructively, and also show that the technique gives data from tissue acid extracts which is in agreement with a previous report (Badar-Goffer et al., 1990). We have addressed the ^{1}H-n.m.r. detectability of both newly synthesized glutamate and lactate, the concentrations of which were sufficiently high to allow their fractional enrichments to be compared in both brain slices and extracts. It is evident that there are significant differences between the ^{1}H-n.m.r. detectability of these two metabolites. Our data provide evidence concerning the metabolic compartmentation of brain glutamate and the influence of this on the compound's ^{1}H-n.m.r. visibility, which will need to be taken into account when interpreting labelling patterns *in vivo*.

The use of spin echo improves water suppression and simplifies cerebral ^{1}H-n.m.r. spectra by reducing the contribution of short T_2 components from macromolecular species (Behar and Ogino, 1991; Kauppinen et al., 1992). However, signal amplitudes vary as a function of echo time as a result of: (i) intrinsic (spin–spin) relaxation processes; (ii) molecular diffusion in the presence of intracellular magnetic-field gradients (Brindle et al., 1979); and (iii) homonuclear spin–spin coupling. Factors (i) and (ii) may vary in different cellular and subcellular compartments, and contribute to differential n.m.r. visibility for given compounds. Our data for lactate indicate that the n.m.r. visibility of newly synthesized [3-^{13}C]lactate is the same as that of [^{12}C]lactate. Furthermore, on the basis of the very similar lactate/NAA ratio in slices and extracts, we conclude that spin-echo ^{1}H n.m.r. detects 100% of intracellular lactate. The T_2 value of lactate CH_3 under control conditions is approx. 100 ms, which is similar to that of NAA at 2.02 p.p.m. (Kauppinen and Williams, 1991). We have previously shown that these two compounds have similar T_2 values and saturation factors in superfused slices so that no correction to the ratio in slices is necessary (Kauppinen and Williams, 1991). A similar conclusion was drawn in a quantitative ^{1}H-n.m.r. study of brain *in vivo* where NAA was also shown to be fully detectable by ^{1}H n.m.r. (Williams et al., 1988). Therefore it appears that intracellular magnetic-field gradients and molecular diffusion affect the signal intensities of these metabolites equally in slice preparations. There is no evidence for subcellular compartmentation of either NAA or lactate, although it should be remembered that NAA is considered to be solely a neuronal metabolite (Urenjak et al., 1992).

The issue of glutamate ^{1}H-n.m.r. detectability is more complex than that of lactate or NAA, for reasons relating to both n.m.r. and biochemistry. Strong spin–spin coupling of the non-equivalent C-4 protons to each other and to the C-3 protons makes it impossible to determine glutamate T_2 in slices using the sequences we have employed. We have assumed that glutamate has the same T_2 as lactate, NAA and creatine, all of which we have measured and shown to be not significantly different (Kauppinen and Williams, 1991). If our assumption were wrong, then the quantification relative to NAA would be wrong (i.e. our estimate of glutamate invisibility would be in error), but the fractional enrichment calculations would still be correct (we have shown that the T_2 of ^{12}C- and ^{13}C-bonded protons cannot be very different in brain slices). As the differences in fractional enrichment between slices and extracts can only be explained on the basis of an unlabelled n.m.r.-invisible pool of glutamate, our original assumption seems justified. The possibility that acid extraction results in 'release ' of peptide-bonded glutamate is thought to be unlikely. This conclusion is supported by previous reports which demonstrated that the concentration of a dipeptide, *N*-acetylaspartylglutamate, in neural extracts is similar whether organic solvents (Miyake et al., 1981) or PCA (Holowenko et al., 1992) are used in the extraction. Furthermore, by using the Carr–Purcell–Meiboom–Gill sequence to suppress spin–spin coupling and diffusion effects, we have previously shown (Williams et al., 1985) in severely hypoxic perfused slices that glutamate and NAA do indeed have similar intrinsic T_2 values (Pirttilä et al., 1993).

It is well established that the metabolism of glutamate is compartmentalized at both intercellular (Berl et al., 1962; Van den Berg et al., 1969; Brainard et al., 1989; Cerdan et al., 1990) and subcellular (Naito and Ueda, 1983; Maycox et al., 1990; McMahon and Nicholls, 1991) levels in the brain. Experimental data suggest that the so-called small pool corresponds to glial cells and the large pool to neuronal cells. The half-life of glutamate in the small pool has been calculated to be approx. 1 min (Berl et al., 1970), whereas the turnover of the amino acid in the large pool is substantially slower (Van den Berg and Garfinkel, 1971; Fitzpatrick et al., 1990). On the basis of labelling studies and enzyme distribution between individual cell types, it has been suggested that glucose is primarily metabolized in neuronal cells to glutamate, whereas exogenous acetate is converted in the first place into glutamate in glial cells which is subsequently transported to neurons as glutamine (Shank et al., 1985; Badar-Goffer et al., 1990; Cerdan et al., 1990). As well as cellular compartmentation, glutamate is also distributed subcellularly in cytosol, mitochondria and transmitter vesicles (Naito and Ueda, 1983; Maycox et al., 1990; McMahon and Nicholls, 1991).

Labelling of glutamate C-4 from either [1-^{13}C]glucose or [2-^{13}C]acetate takes place in mitochondria. In both neuronal and glial cells, mitochondria are expected to have efficient exchange mechanisms and transporters to expel glutamate (see LaNoue and Schoolwerth, 1979). If the labelled glutamate in the mitochondrial compartment were not detected by ^{1}H n.m.r., then the fractional enrichment would be greater in tissue extracts than in slice preparations even if mitochondrial transport is very efficient, as, at best, this would equilibrate label in the two pools. Our data show the exact opposite; it appears that there is a pool of non-labelled glutamate that is n.m.r.-invisible. Therefore mitochondrial glutamate must be amenable to ^{1}H-n.m.r. detection. As the fractional enrichment from glucose of the total glutamate pool relative to the enrichment of the ^{1}H-n.m.r.-visible pool increases with time of incubation, we conclude that [1-^{13}C]glucose initially labels the visible pool, which subsequently slowly exchanges label into the invisible pool.

In the early phase of acetate superfusions, there is no significant difference between the fractional enrichments of the total pool and the ^{1}H-n.m.r.-visible pool. This implies that acetate labels glutamate C-4 at the same rate in both the n.m.r.-visible and -invisible pools. Previous evidence points to metabolism of acetate primarily in glial cells, whereas glucose is metabolized in both cellular pools (Badar-Goffer et al., 1990). Therefore glutamate synthesized in glia may be partially n.m.r. invisible. However, because of the low level of fractional enrichment attained after 30 min exposure to [2-^{13}C]acetate, it is dangerous to place too much emphasis on this result. Given the errors in our measurements, the fractional enrichment for total glutamate would have to be less than 60% that of n.m.r.-visible glutamate for it to attain significance. At later times, when the absolute enrichment is greater, there is a significance difference between the enrichments of the total and n.m.r.-visible pools. In fact, the surest conclusion from these data is that glutamate synthesized in glia is largely (if not totally) n.m.r. visible. Therefore the invisible pool of glutamate cannot be explained on the basis of the known intercellular compartmentation, as this would require the small (glial) pool to be invisible, which the acetate data clearly exclude.

Cerebral cortex must contain a substantial pool of transmitter glutamate, as some 30% of all cortical nerve terminals use glutamate as a neurotransmitter (McMahon and Nicholls, 1991). It has been estimated that about one-quarter of total brain glutamate is in a neurotransmitter pool (Fonnum, 1988). In isolated nerve terminals, transmitter glutamate which is located in vesicles, equilibrates with cytoplasmic glutamate very slowly (Wilkinson and Nicholls, 1989). In isolated transmitter vesicles, the pH is substantially lower than in the cytoplasm because of the nature of the amino-acid-transport mechanism (Naito and Ueda, 1983). Microenvironmental differences may affect relaxation properties and/or resonance frequency so that the pool in a different compartment becomes undetectable (Pirttilä et al., 1993). Our earlier data showed that, after extreme energy failure

(aglycaemic hypoxia), glutamate became 100% n.m.r. visible (Kauppinen and Williams, 1991). This would be consistent with release of glutamate from an n.m.r.-invisible pool into a fully detectable subcellular compartment.

To summarize, newly synthesized glutamate is probably almost completely visible in both neurons (labelled from glucose) and glia (labelled from glucose and acetate). Mitochondrial glutamate either must be fully visible or its steady-state concentration must be so low that its contribution to the intact slice n.m.r. spectrum is negligible. In either case, the mitochondrion cannot be the site of glutamate invisibility. We propose that the most likely explanation for glutamate n.m.r. invisibility is subcellular sequestration which may include neurotransmitter and some other pools. It is conceivable that the milieu in these compartments may alter the n.m.r. properties of glutamate via relaxation, susceptibility, metal-ion or protein-binding effects, in such a way as to render it n.m.r. invisible. Further work is required to substantiate this hypothesis. It is important to understand the factors affecting glutamate visibility, not only for its own sake, but also because it will affect the interpretation of labelling patterns and kinetics determined by $^{1}H/^{13}C$ n.m.r. (Fitzpatrick et al., 1990; Rothman et al., 1992). The increasing use of these methods to estimate metabolic rates *in vivo* in animals and man emphasizes the relevance of this work.

This work was supported by grants from the Finnish Academy of Sciences and from European Science Foundation (ETP travel fellowship of advanced technology in Neuroscience).

REFERENCES

Badar-Goffer, R. S., Bachelard, H. S. and Morris, P. G. (1990) Biochem. J. **266**, 133–139
Badar-Goffer, R. S., Ben-Yoseph, O., Bachelard, H. S. and Morris, P. G. (1992) Biochem. J. **282**, 225–230
Bax, A. and Subramanian, S. (1986) J. Magn. Reson. **67**, 565–569
Behar, K. L. and Ogino, T. (1991) Magn. Reson. Med. **10**, 285–303
Berl, S., Takagaki, G., Clarke, D. D. and Waelsch, H. (1962) J. Biol. Chem. **237**, 2562–2570
Berl, S., Nicklas, W. J. and Clarke, D. D. (1970) J. Neurochem. **17**, 1009–1015
Brainard, J. R., Kyner, E. and Rosenberg, G. A. (1989) J. Neurochem. **53**, 1285–1292
Brindle, K. M., Brown, F. F., Campbell, A. D., Grathwohl, C. and Kuchel, P. W. (1979) Biochem. J. **180**, 37–44
Cerdan, S., Kunnecke, B. and Seelig, J. (1990) J. Biol. Chem. **265**, 12916–12926
Choi, D. W. (1990) Cerebrovasc. Brain Metab. Rev. **2**, 105–147
Cox, D. G. W., Morris, P. G., Feeney, J. and Bachelard, H. S. (1983) Biochem. J. **212**, 365–370
de Graaf, A. A. and Bovee, W. M. M. (1990) Magn. Reson. Med. **15**, 305–319
Fitzpatrick, S. M., Hetherington, H. P., Behar, K. L. and Shulman, R. G. (1990) J. Cerebr. Blood Flow Metab. **10**, 170–179
Fonnum, F. (1984) J. Neurochem. **42**, 1–11
Fonnum, F. (1988) in Glutamate, Glutamine and GABA in the Mammals (Kvamme, E., ed.), vol. 2, p. 57, CRC Press, Boca Raton, FL
Frahm, J., Michaelis, T., Merboldt, K. D., Bruhn, H., Gyngell, M. L. and Hänicke, W. (1990) J. Magn. Reson. **90**, 469–473
Holowenko, D., Peeling, J. and Sutherland, G. (1992) NMR Biomed. **5**, 43–47
Hore, P. J. (1983) J. Magn. Reson. **55**, 283–300
Kapetanovic, I. M., Yonekawa, W. D. and Kupferberg, H. J. (1990) J. Chromatogr. **500**, 387–394
Kauppinen, R. A. and Williams, S. R. (1991) J. Neurochem. **57**, 1136–1144
Kauppinen, R. A., Nissinen, T., Kärkkäinen, A.-M., Pirttilä, T. R. M., Palvimo, J., Kokko, H. and Williams, S. R. (1992) J. Biol. Chem. **267**, 9905–9910
LaNoue, K. F. and Schoolwerth, A. C. (1979) Annu. Rev. Biochem. **48**, 871–922
Maycox, P. R., Deckwerth, T. and Jahn, R. (1990) Trends Neurosci. **13**, 83–87
McMahon, H. T. and Nicholls, D. G. (1991) Biochim. Biophys. Acta **1059**, 243–264
Michaelis, T., Merboldt, K.-D., Bruhn, H., Hänicke, W. and Frahm, J. (1993) Radiology **187**, 219–227
Miyake, M., Kakimoto, Y. and Sorimachi, M. (1981) J. Neurochem. **36**, 804–810
Naito, S. and Ueda, T. (1983) J. Biol. Chem. **258**, 696–699
Nicholls, D. G. (1989) J. Neurochem. **52**, 331–341
Pirttilä, T.-R. M., Hakumäki, J. H. and Kauppinen, R. A. (1993) J. Neurochem. **60**, 1274–1282
Rothman, S. M. and Olney, J. W. (1987) Trends Neurosci. **10**, 299–302
Rothman, D. L., Behar, K. L., Hetherington, H. P., den Hollander, J. A., Bendall, M. R., Petroff, O. A. C. and Shulman, R. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1633–1637
Rothman, D. L., Novotny, E. J., Shulman, G. I., Howseman, A. M., Petroff, O. A. C., Mason, G., Nixon, T., Hanstock, C. C., Prichard, J. W. and Shulman, R. G. (1992) Proc. Natl. Acad. Sci U.S.A. **89**, 9603–9606
Shank, R. P., Bennett, G. S., Freytag, S. O. and Campbell, G. L. (1985) Brain Res **325**, 364–367
Taylor, D. J., Bore, P. J., Styles, P., Gadian, D. G. and Radda, G. K. (1983) Mol. Biol. Med. **1**, 77–94
Tycho, R., Cho, H. M., Schneider, E. and Pines, A. (1985) J. Magn. Reson. **61**, 90–101
Urenjak, J., Williams, S. R., Gadian, D. G. and Noble, M. R. (1992) J. Neurochem. **59**, 55–61
Van den Berg, C. J. and Garfinkel, D. (1971) Biochem. J. **123**, 211–218
Van den Berg, C. J., Krzalic, L. J., Mela, P. and Waelsch, H. (1969) Biochem. J. **113**, 281–290
Wilkinson, R. J. and Nicholls, D. G. (1989) Neurochem. Int. **15**, 191–197
Williams, S. R., Gadian, D. G., Proctor, E., Spraque, D. B., Talbot, D. F., Young, I. R. and Brown, F. F. (1985) J. Magn. Reson. **63**, 406–412
Williams, S. R., Proctor, E., Allen, K., Gadian, D. G. and Crockard, H. A. (1988) Magn. Reson. Med. **7**, 425–431

Received 17 May 1993/25 August 1993; accepted 8 September 1993