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Cerebral metabolism of acetate and glucose studied by ^{13}C-n.m.r. spectroscopy

A technique for investigating metabolic compartmentation in the brain

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The time courses of incorporation of ^{13}C from ^{13}C-labelled glucose or acetate into cerebral amino acids (glutamate, glutamine and 4-aminobutyrate) and lactate were monitored by using ^{13}C-n.m.r. spectroscopy. When [1-^{13}C]glucose was used as precursor the C-2 of 4-aminobutyrate was more highly labelled than the analogous C-4 of glutamate, whereas no label was observed in glutamine. A similar pattern was observed with [2-^{13}C]glucose: the C-1 of 4-aminobutyrate was more highly labelled than the analogous C-5 of glutamate. Again, no labelling of glutamine was detected. In contrast, [2-^{13}C]acetate labelled the C-4 of glutamine and the C-2 of 4-aminobutyrate more highly than the C-4 of glutamate; [1-^{13}C]acetate also labelled the C-1 and C-5 positions of glutamine more than the analogous positions of glutamate. These results are consistent with earlier patterns reported from the use of ^{14}C-labelled precursors that led to the concept of compartmentation of neuronal and glial metabolism and now provide the possibility of distinguishing differential effects of metabolic perturbations on the two pools simultaneously. An unexpected observation was that citrate is more highly labelled from acetate than from glucose.

INTRODUCTION

There is increasing need to understand the quantitative metabolic relationships between neurotransmitter amino acids and their precursors, and in the roles played by neuronal and glial compartments in their production.

Over 20 years ago Waelsch and his colleagues demonstrated, using ^{15}N- and ^{14}C-labelled precursors, that under certain circumstances glutamine could be more highly labelled than its metabolic precursor, glutamate (Berl *et al.*, 1961, 1962, 1968). These and subsequent studies (Nicklas *et al.*, 1969; Van den Berg *et al.*, 1969; Van den Berg & Garfinkel, 1971; Van den Berg, 1973; Berl, 1973; Van den Berg *et al.*, 1975) led to the concept of two separate metabolic pools or compartments for glutamate and glutamine: a small high-turnover 'glial' pool with emphasis on rapid conversion of glutamate into glutamine, and a large more slowly-turning-over 'neuronal' pool. The ideas on the cellular sites of these pools have been supported by histochemical and biochemical studies on the relevant enzymes and transport carriers: i.e. enrichment of glutaminase in neurons and especially synaptosomes, and of glutamate transport and glutamine synthetase in glial cells (Quastel 1975; Martinez-Hernandez *et al.*, 1977; Muir *et al.*, 1986; Nicholls *et al.*, 1987; see McIlwain & Bachelard, 1985). These earlier studies clearly demonstrated that the glutamine of the small high-turnover pool is labelled in preference to glutamate if acetate is the precursor, whereas the reverse is the case when glucose is the precursor.

It is of great interest to know the precise metabolic sources of transmitter glutamate, as opposed to non-transmitter 'metabolic' glutamate, and of 4-aminobutyrate.

One great advantage of ^{13}C-n.m.r. spectroscopy is that the enormous resolving power of the technique allows for simultaneous identification and quantification of individual C atoms on the same molecule, in addition to distinguishing between molecules (London, 1988). However, it is very insensitive, so that ^{13}C-enriched precursors must be used.

Thus acetate (labelled in positions 1 or 2) labels the C atoms primarily of glutamine, and glucose (also labelled in positions 1 or 2) labels the C atoms primarily of glutamate (Morris *et al.*, 1986, 1987). By observing labelling patterns one can follow precisely the time course of precursor–product relationships and characterize, for example, which precursor pool is involved in production of 4-aminobutyrate.

Preliminary results on the use of ^{13}C-labelled glucose (Morris *et al.*, 1986) and of ^{13}C-labelled acetate (Morris *et al.*, 1987) have been reported as abstracts to scientific meetings.

MATERIALS AND METHODS

Chemicals

^{13}C-labelled precursors were obtained from Omicron Biochemical Inc., Ithaca, NY, U.S.A. $^{2}H_2O$ was from Norsk Hydro, Oslo, Norway, and amino acids were from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

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Tissue preparation

Male Hartley guinea pigs (300–350 g) were stunned and exsanguinated, and the brains were quickly removed. The two cerebral hemispheres were separated and the white matter was removed. Each cortex was halved with a scalpel and cut at right-angles to give slices of 0.35 mm thickness. The slices were immediately suspended in the superfusion buffer, containing NaCl (124 mM), KCl (5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.2 mM), $NaHCO_3$ (26 mM) and glucose (10 mM), and gassed with O_2/CO_2 (19:1) at 37 °C. The accumulated slices (approx. 4 g fresh wt.) were washed several times with gassed medium at 37 °C. They were then incubated in the same medium at 37 °C for 20 min to ensure restoration of metabolic viability (McIlwain & Bachelard, 1985). After this metabolic recovery period the slices were placed in the superfusion apparatus (see Bachelard *et al.*, 1985) for direct ^{13}C-n.m.r. spectroscopy of the metabolizing tissues. At this stage the ^{13}C-labelled precursors were added to the superfusion medium.

In order to obtain higher resolution of the resonances and therefore more accurate quantification, ^{13}C-n.m.r. spectroscopy was performed on tissue extracts. In these experiments the slices were incubated with the above medium containing [1-^{13}C]- or [2-^{13}C]-acetate (5 mM in the presence of 5 mM unlabelled glucose) or [1-^{13}C]- or [2-^{13}C]-glucose (4 mM). After incubation for the periods of time described in the Results section, tissues were removed by rapid filtration, washed briefly with ice-cold unlabelled medium and homogenized in 0.6 M-$HClO_4$ at 0 °C. After centrifugation (and washing the pellet with $HClO_4$), the pellets were kept for protein determination and the supernatants were neutralized at 0 °C with 0.6 M-$KHCO_3$. The supernatants, after separation from the insoluble $KClO_4$ by centrifugation, were evaporated to dryness in a rotary film evaporator and the residues were dissolved in ^2H_2O to provide the tissue extracts. Rotary film evaporation was employed because in preliminary experiments losses by 'spitting' occurred on freeze-drying.

Protein was determined in the pellets, after dissolving them in 1 M-NaOH in a boiling-water bath, by the method of Miller (1959).

The tissue extracts were used for ^{13}C-n.m.r. spectroscopy without further treatment, and samples were taken for chemical analysis of amino acids and lactate.

Lactate was measured by using enzymic spectrophotometric methods (Lowry & Passonneau, 1972); amino acids were measured with an LKB 4400 amino acid analyser.

^{13}C-n.m.r. spectroscopy

These studies were performed on a Bruker AM 400 wide-bore spectrometer, operating at 100.62 MHz.

Superfused tissues. The superfusion system was set up as previously described (Bachelard *et al.*, 1985). ^{1}H-decoupled ^{13}C-n.m.r. spectra were recorded in 5 min blocks of 300 scans, with the use of a 25 mm ^{13}C/^{31}P dual probe. Flip angles of 90° were used. After periods of 30 min, during which naturally abundant ^{13}C-n.m.r. spectra were accumulated, the superfusion medium was replaced by one containing the ^{13}C-labelled substrate. The incorporation of ^{13}C into different metabolites was monitored for periods up to 100 min, after which control media were used to monitor the loss of label for a further 20–100 min. ^{31}P-n.m.r. spectra (not shown) were interleaved to confirm the metabolic viability of the tissues.

Tissue extracts. Scans (1000–4000 field-induction) were acquired with the use of 90° pulses repeated at 4 s intervals. Spectra were broad-band decoupled at 10 W, only during acquisition in order to avoid differential nuclear Overhauser effects and to minimize sample heating. Before Fourier transformation, field-induction decays were multiplied by an exponential function giving a line-broadening of 4–8 Hz to enhance signal-to-noise ratios. Chemical shifts are given relative to an internal dioxan standard set at 67.4 p.p.m., corresponding to tetramethylsilane = 0 p.p.m. ^{13}C spin-relaxation times (T_1 values) were measured in tissue extracts in ^2H_2O and also in pure solutions of unlabelled amino acids in ^2H_2O.

Measurement of relaxation times. Inversion recovery (180°–τ–90° acquire) and saturation recovery (saturation pulses–τ–90° acquire) techniques (see Shaw, 1984) were used to measure the spin–lattice relaxation times of the methylene and carboxylate carbon atoms of relevant amino acids dissolved (100 mM) either in ^2H_2O or in tissue extracts. For inversion recovery measurements, 128 field-induction decays were accumulated for each of 12 τ values chosen to span the range 0–60 s. The recovery interval between repetitions of the inversion recovery sequence was 100 s. For saturation recovery, 512 field-induction decays were accumulated with 15 τ values spanning the range 0–60 s. In both cases T_1 relaxation times were determined from the spectra by using an exponential fitting routine supplied by Bruker Spectrospin. The S.D. values supplied by this routine are given in the results (Table 1).

Calculation of percentage enrichment. Comparisons of quantification of resonances by area measurement (triangulation) with cutting and weighing showed no significant difference, so area measurement was routinely used. Calculation of percentage ^{13}C enrichment in individual carbon atoms was based on the 1.1% natural abundance of the ^{13}C of the four carbon atoms of the internal dioxan standard. Corrections were made for the naturally abundant ^{13}C present in each resonance and for the relaxation times where necessary. The pool size of the metabolite based on amino acid analysis or enzymic assay (Table 2) was then used to calculate percentage enrichment.

The total amount of ^{13}C in the resonance of a particular metabolite was calculated as in eqn. (1):

$$[^{13}C]^t_m = \frac{[^{13}C]_D \cdot (\text{area})_m \cdot (Sf)_m}{(\text{area})_D \cdot (Sf)_D} \quad (1)$$

where (area) represents the measured area of the ^{13}C resonance, (Sf) represents the saturation factor, from the T_1 value, where necessary, m represents the metabolite and D represents the dioxan standard. From this, the percentage enrichment of the ^{13}C in each resonance was calculated as in eqn. (2):

$$\text{Percentage enrichment} = \frac{([^{13}C]^t_m - [^{13}C]^a_m) \cdot 100}{[m]} \quad (2)$$

where $[^{13}C]^t_m$ is the total amount of ^{13}C, from eqn. (1),

Table 1. T_1 relaxation times of the ^{13}C of selected amino acids

Rows (a), pure amino acid in ^2H_2O; rows (b), amino acid in tissue extract. The T_1 of dioxan ^{13}C atoms was 6.94 s. Techniques for the measurement of T_1 values (inversion recovery or saturation recovery) are given in the Materials and methods section. The fitting routine supplied by Bruker Spectrospin gave S.D. values of 0.005–0.04 s for methylene groups and 0.02–0.04 s for carboxylate groups.

Amino acid		T_1 (s) C-1	C-2	C-3	C-4	C-5
Glutamate	(a)	13.47	1.67	1.03	1.37	14.11
	(b)	7.12	2.09	1.28	1.54	9.28
Glutamine	(a)	15.16	1.71	1.11	1.48	19.98
	(b)	6.17	1.76	1.05	1.35	10.90
4-Aminobutyrate	(a)	20.00	2.17	2.23	2.34	
	(b)	11.47	2.52	2.60	2.81	

$[^{13}C]^a_m$ is the naturally abundant ^{13}C in the metabolite and [m] is the pool size of the metabolite (Table 2).

Measured T_1 relaxation times for carboxylate groups were shorter (6–12 s) in tissue extracts than in pure solution (14–20 s), whereas the T_1 values for methylene groups were similar in both at 1–3 s (Table 1). Resonances of the methylene groups therefore required no correction for their relatively short T_1 values, in contrast with the carboxylate groups, where considerable correction factors had to be applied. With constituents of small pool size and with low amplitudes in the spectra, the necessary combination of correction factors resulted in reproducibility of the calculated percentage enrichment of approx. 20%. However, reproducibility of the major methylene residues was within 5%.

RESULTS

The labelling patterns of intermediates, from the different precursors used, are summarized in Scheme 1.

Superfusion studies

Although real-time experiments were not successful in demonstrating the metabolism of ^{13}C-labelled acetate, resonances of some of the individual ^{13}C atoms labelled from [1-^{13}C]glucose were clearly discernible. The ^{13}C resonance of the C-4 of glutamate could be detected within 5 min of the addition of [1-^{13}C]glucose to the superfusing medium. Fig. 1 shows the spectra obtained from the accumulation of 20 min blocks, in which the C-1 positions of α- and β-glucose, C-2 of glutamate + glutamine, C-3 of glutamate + glutamine, C-4 of glutamate and C-3 of lactate are apparent. The resolution of the spectra did not enable distinction between glutamate and glutamine labelled in the C-2 or C-3 positions. However, the absence of glutamine resonances in the more highly resolved spectra from tissue extracts (below) made it reasonable to attribute these to glutamate.

Real-time superfusion experiments with [2-^{13}C]glucose as precursor also did not produce detectable resonances; we believe this to be due to the long T_1 relaxation times of the carboxylate groups (Table 1) that would be labelled.

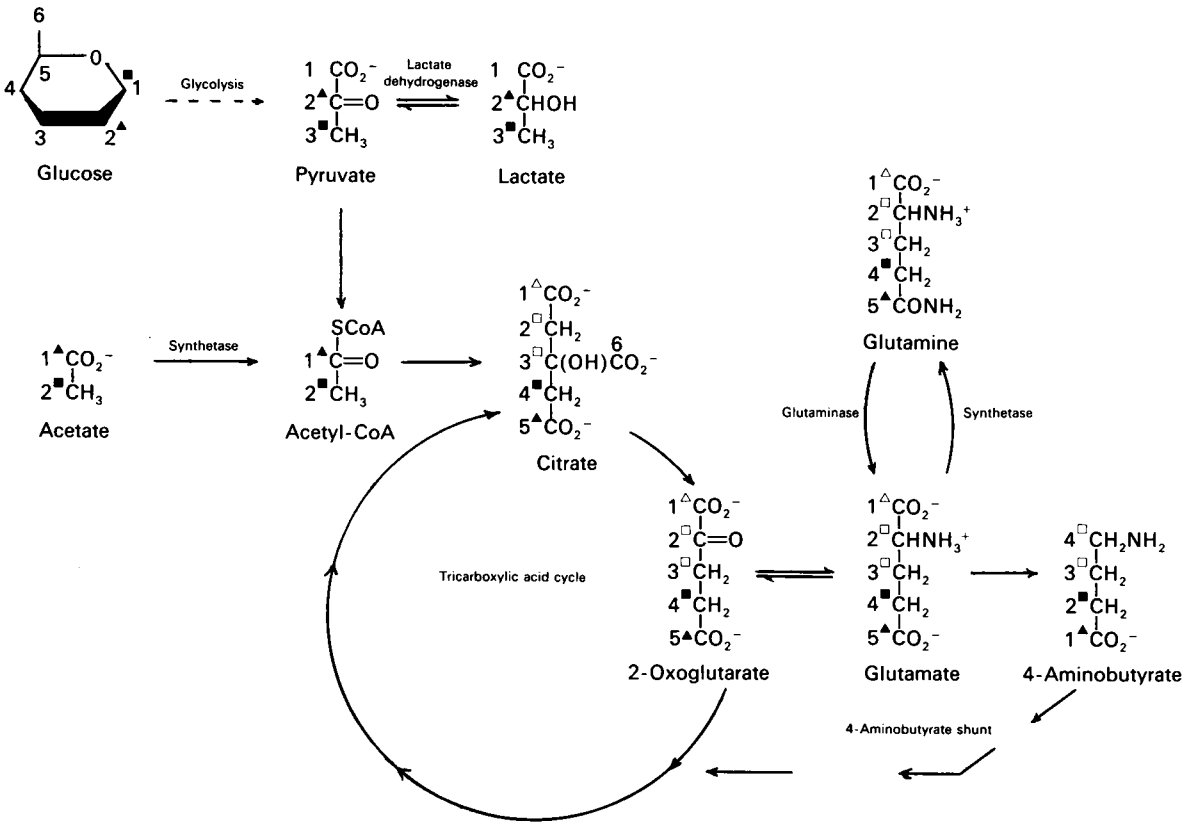
Tissue extracts

Fig. 2 shows the labelling patterns when (*a*) [1-^{13}C]glucose and (*b*) [2-^{13}C]glucose were used as precursors. In Fig. 2(*a*) the spectrum is dominated by the resonance of the C-4 of glutamate, which could be detected within 5 min, with no labelling of glutamine discernible. Of interest is the labelling of the C-2 of 4-aminobutyrate (which is metabolically derived from the C-4 of glutamate; see Scheme 1). Since the pool size of 4-aminobutyrate is only some 15% of that of glutamate (Table 2), this means that the percentage enrichment of the 4-aminobutyrate is considerably higher than that of the glutamate. The spectrum also shows that the C-2 of glutamate has less than 50% of the enrichment of the C-4 of glutamate. The ^{13}C present in the C-3 position of glutamate (Fig. 2*a*) appears to be less than that of the C-

Table 2. Pool sizes of relevant metabolites

Values from the present experiments are given as means ± S.D. The ranges of values *in vivo* are quoted from McIlwain & Bachelard (1985).

Time of incubation (min)	No. of determinations	Pool size (μmol/100 mg of protein) Glutamate	Glutamine	4-Aminobutyrate	Aspartate	Lactate
15	4	10.1 ± 1.8	2.8 ± 1.1	1.5 ± 0.3	1.9 ± 0.3	1.5 ± 0.1
45	5	8.6 ± 1.4	2.3 ± 0.6	1.2 ± 0.2	1.9 ± 0.1	1.46 ± 0.8
Range *in vivo*		7.8–12.5	2.1–5.6	0.8–2.3	1.5–2.7	0.8–2.0



Scheme. 1. Labelling patterns of the carbon atoms of intermediates from various ^{13}C-labelled precursors

Key: ■, initial labelling with [1-^{13}C]glucose or [2-^{13}C]acetate; □, subsequent labelling with [1-^{13}C]glucose or [2-^{13}C]acetate; ▲, initial labelling with [2-^{13}C]glucose or [1-^{13}C]acetate; △, subsequent labelling with [2-^{13}C]glucose or [1-^{13}C]acetate.

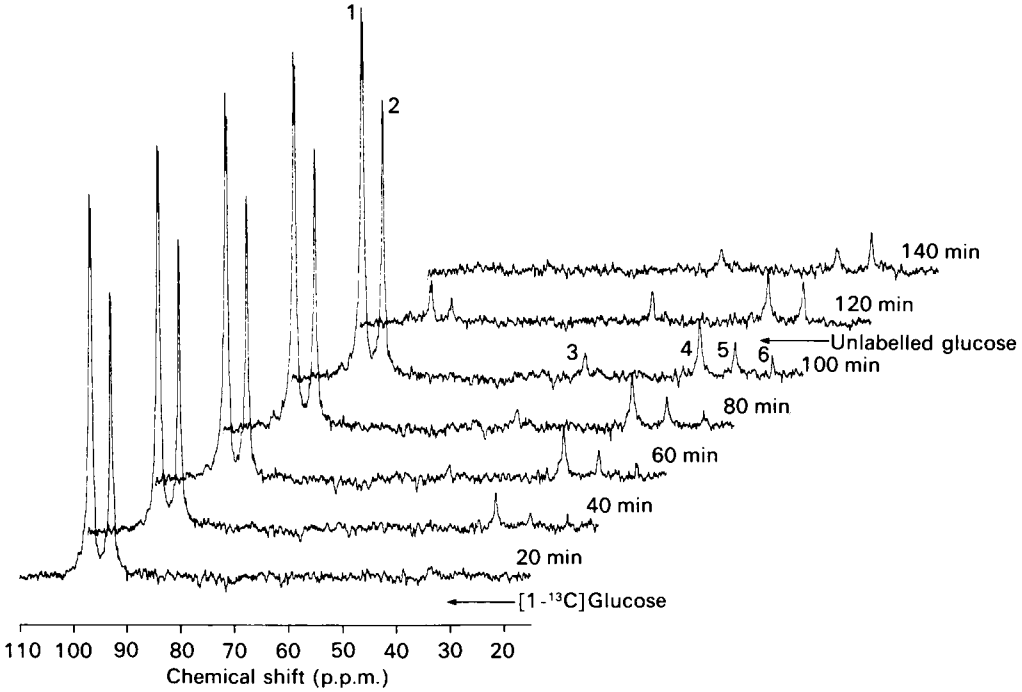


Fig. 1. ^{13}C-n.m.r. spectra of superfused guinea-pig brain slices

Each spectrum is the sum of four 5 min blocks in the presence of 5 mM-[1-^{13}C]glucose, after subtraction of the control spectrum obtained with unlabelled glucose to correct for natural abundance (see the Materials and methods section). After 80 min the superfusing medium was replaced by control medium (unlabelled glucose) in order to monitor loss of label. 1 and 2, β- and α-Glucose (essentially present in the medium); 3, glutamate + glutamine C-2; 4, glutamate C-4; 5, glutamate + glutamine C-3; 6, lactate C-3.

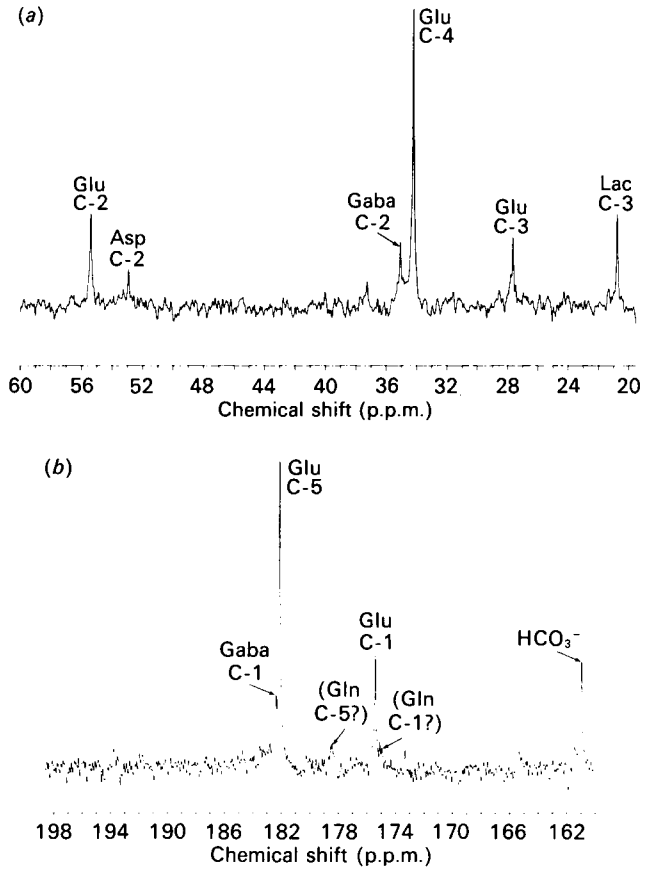


Fig. 2. ^{13}C-n.m.r. spectra of guinea-pig brain slice extracts labelled with [^{13}C]glucose

Slices were incubated in the presence of 4 mM-[1-^{13}C]-glucose (*a*) or [2-^{13}C]glucose (*b*) for 45 min, and tissue extracts were prepared as described in the Materials and methods section. Abbreviations: Gaba, 4-aminobutyrate; Lac, lactate. Assignments (?) are tentative.

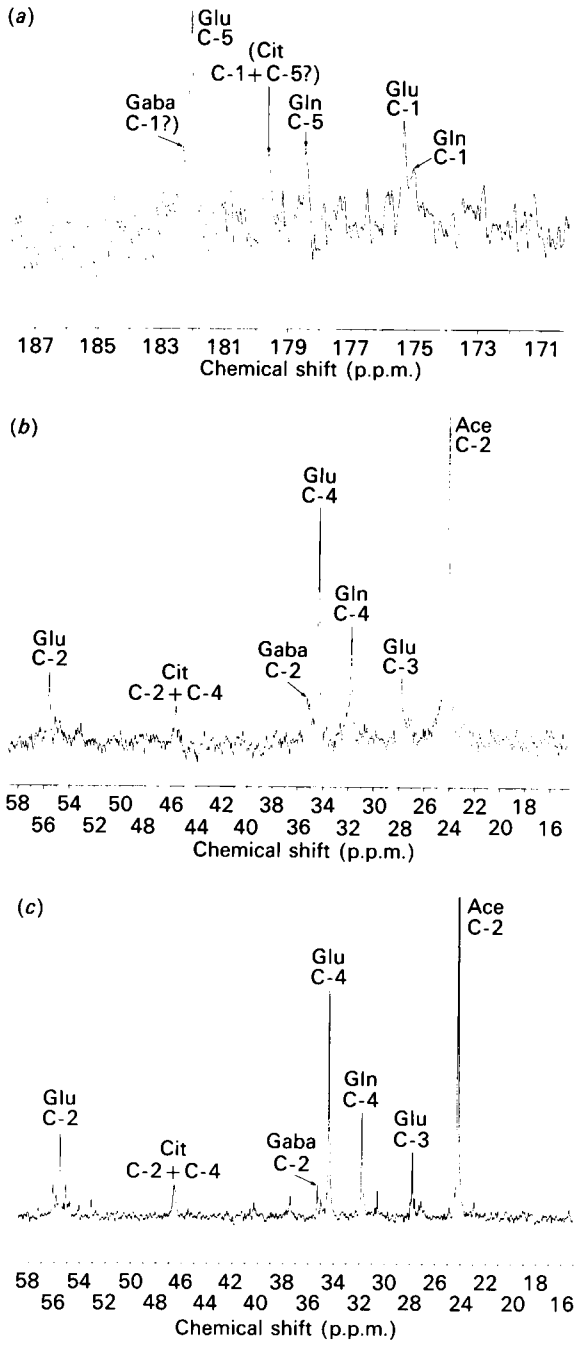


Fig. 3. ^{13}C-n.m.r. spectra of guinea-pig brain slice extracts labelled with [^{13}C]acetate

Slices were incubated in the presence of 5 mM unlabelled glucose and 5 mM-[1-^{13}C]acetate (*a*) or [2-^{13}C]acetate (*b*) for 60 min, and tissue extracts were prepared as described in the Materials and methods section. The extract of (*b*) was re-examined after addition of 4 mM-EDTA to give spectrum (*c*) (see the text). Abbreviations: Gaba, 4-aminobutyrate; Ace, acetate; Cit, citrate.

2 of glutamate. However, close examination of the C-3 resonance shows a triplet, due to C-3-to-C-4 spin–spin coupling. If the satellite resonances are taken into account, there is no discrepancy between these resonances. As would be expected, through symmetrical labelling via succinate, these two resonances should show the same intensity. The time course of these labelling patterns is described below.

With [2-^{13}C]glucose (Fig. 2*b*) detectable labelling is restricted to the carboxylate groups of these amino acids (see Scheme 1). The labelling pattern is similar to that of Fig. 2(*a*) in that the dominant resonance is the C-5 of glutamate, with barely detectable C-5 of glutamine. Labelling of the C-1 of glutamate is less than that of the C-5; the labelling of the C-1 of 4-aminobutyrate (metabolically related to the C-5 of glutamate; see Scheme 1) is such that its calculated percentage enrichment is higher than that of glutamate (see below).

Fig. 3 shows the labelling patterns of tissue extracts incubated with (*a*) [1-^{13}C]acetate and (*b*) [2-^{13}C]acetate. Here it can be seen that [1-^{13}C]acetate labels mainly the C-5 positions of glutamate and glutamine. Both are more highly labelled than the corresponding C-1 positions. Although the analogous positions of glutamate appear more prominently in the spectrum, calculation of the percentage enrichment confirmed that they were less highly labelled than glutamine (see Fig. 4 below). In this spectrum we could not detect any resonance attributable solely to 4-aminobutyrate; the shoulder on the glutamate C-5 peak corresponds to 4-aminobutyrate but may include acetate. It therefore is tentatively assigned in Figs. 3(*a*) and 4. With [2-^{13}C]acetate (Fig. 3*b*) the C-4

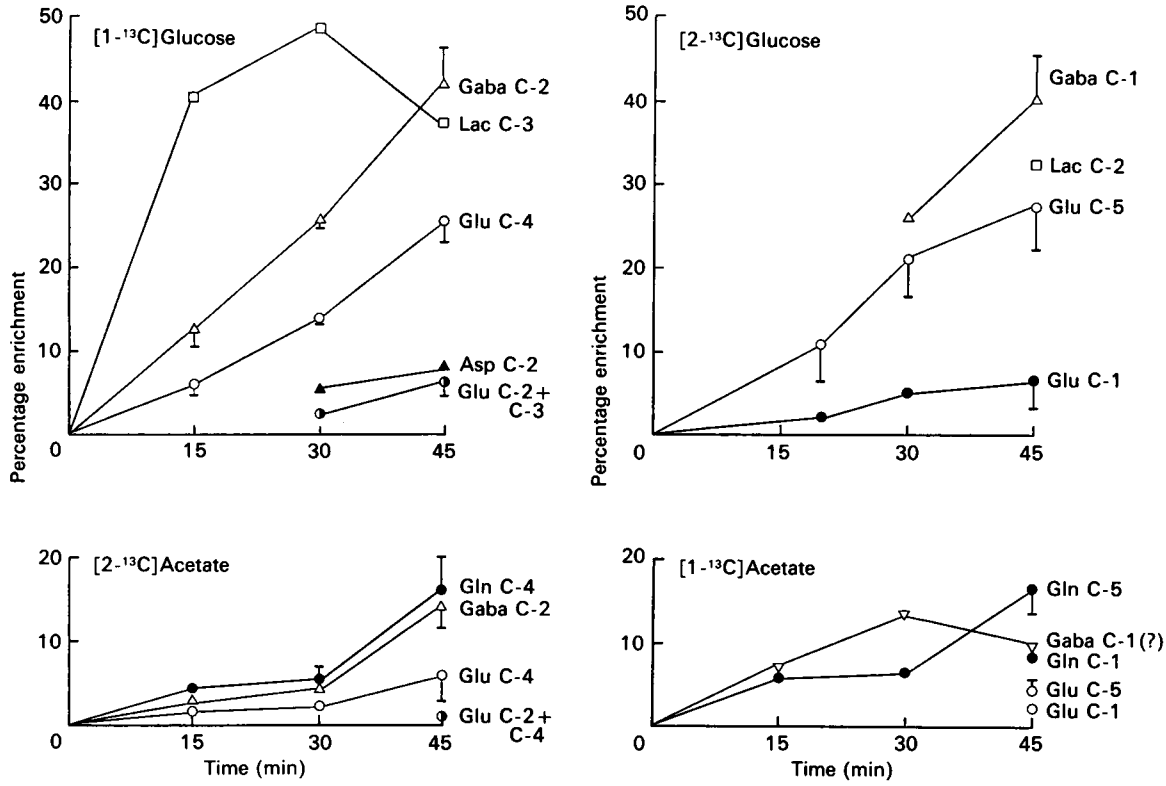


Fig. 4. Time courses of the labelling of intermediates of guinea-pig brain slice extracts from ^{13}C-labelled precursors

The percentage enrichments were calculated as described in the text. The bars represent the ranges of means obtained from two or three experiments. Abbreviations: Gaba, 4-aminobutyrate; Lac, lactate.

positions of glutamine and glutamate dominated, with small resonances attributed to the C-2 of 4-aminobutyrate. When pool sizes were taken into account, the percentage enrichments of the glutamine (C-4) and 4-aminobutyrate (C-2) were about the same, and both higher than that of the C-4 of glutamate. As discussed above (for Fig. 2*a*), the resonances of the C-2 and C-3 positions of glutamate are of equal intensity if the triplet of the C-3 resonance is taken into account.

The differences in these labelling patterns are seen more clearly in the time courses of Fig. 4, where the percentage enrichments have been calculated as described in the Materials and methods section. As noted in the Materials and methods section, the calculation of the percentage enrichments of carboxylate groups tends to be less precise than the calculation of those of the methylene groups. The general pattern that emerges is that when glucose is used as precursor the percentage enrichment of 4-aminobutyrate is higher than that of glutamate, with none clearly detectable in glutamine. In contrast [^{13}C]acetate labels 4-aminobutyrate and glutamine with much less enrichment of the glutamate. In some experiments the time course was monitored for longer than 45 min (results not shown). With [1-^{13}C]-glucose at 100 min the percentage enrichments were, in descending order: C-2 of 4-aminobutyrate; C-3 of lactate; C-4 of glutamate; C-3 and C-4 of 4-aminobutyrate; C-4 of glutamate; C-2 and C-3 of glutamate. No resonance of glutamine was detected. With [1-^{13}C]acetate the order was: C-4 of glutamine = C-2 of 4-aminobutyrate; C-4 of glutamate.

An unexpected observation in the spectra of tissue extracts labelled with [2-^{13}C]acetate (Fig. 3*b*) was the resonance at 45.6 p.p.m. This could be due to citrate (C-2 and C-4 methylene groups); on titration of citrate with Mg^{2+} the chemical shift of the citrate resonance changes from approx. 45 p.p.m. with 2 mM-Mg^{2+} to 46.8 p.p.m. in the absence of Mg^{2+} (Cohen, 1983). Accordingly we re-ran the extract in the presence of 4 mM-EDTA: the resonance then showed a chemical shift of 46.6 p.p.m. (Fig. 3*c*), thus tending to confirm the resonance as citrate. Comparison of Fig. 2(*a*) ([1-^{13}C]glucose) with Fig. 3(*b*) ([2-^{13}C]acetate) indicates that the citrate is considerably more enriched from acetate than from glucose. Also, comparison of Fig. 3(*a*) ([1-^{13}C]acetate) with Fig. 2(*b*) ([2-^{13}C]glucose) shows a definite resonance at approx. 179.6 p.p.m. present with acetate but not glucose as precursor; this peak could be tentatively assigned to the C-1 and C-5 carboxylate groups of citrate.

DISCUSSION

The results illustrate the resolving power of ^{13}C-n.m.r. spectroscopy in distinguishing between individual carbon atoms of appropriate metabolites. The limitations in sensitivity are reflected by our inability to detect less immediately labelled atoms of intermediates of large pool size (e.g. the C-2 and C-3 atoms of glutamate) until the longer time intervals (Fig. 4). Also, labelling of the carboxylate group of 4-aminobutyrate (of relatively small pool size) could not be quantified until longer time

intervals (Fig. 4). However, the feasibility of observing these precursor–product relationships has been clearly demonstrated.

In these experiments, whereas labelling of lactate from [1-^{13}C]glucose was close to the theoretical maximum of 50% within 15 min, labelling of the glutamate only reached some 25–30% in 45 min. The source of the unlabelled glutamate could be unlabelled glutamine (since no labelled glutamine was detected) or some other unknown pool. In contrast, the calculated enrichment of 4-aminobutyrate also approached 50% (Fig. 4).

When [1-^{13}C]glucose or [2-^{13}C]glucose was used, 4-aminobutyrate was more highly labelled than glutamate, thus suggesting that only that part of the glutamate pool is derived from glucose is acting as precursor. Also, when [2-^{13}C]acetate was used, 4-aminobutyrate was labelled to the same extent as the glutamine (Fig. 4). This confirms previous suggestions that glutamine is a significant precursor for 4-aminobutyrate (Reubi *et al.*, 1978; Paulsen *et al.*, 1988). We were unable to quantify labelling of 4-aminobutyrate from [1-^{13}C]acetate, presumably owing to the combination of its relatively small pool size with the long relaxation time of its carboxylate group (Table 1).

Quantification of methylene groups (C-2, C-3 and C-4 of glutamate or glutamine and C-2 of 4-aminobutyrate) was generally better than that of the carboxylate groups, owing to the much longer relaxation times of the latter (Table 1). Therefore use of [1-^{13}C]glucose or [2-^{13}C]-acetate as precursor seems preferable to use of [2-^{13}C]glucose or [1-^{13}C]acetate in distinguishing between metabolic compartments. Preliminary studies have confirmed that use of these two precursors ([1-^{13}C]glucose with [2-^{13}C]acetate) gives labelling patterns that provide the possibility of distinguishing differential effects of metabolic perturbations on the two pools simultaneously.

The unexpected observation, that citrate is more highly labelled from acetate (Fig. 3) than from glucose (Fig. 2), is of interest. Previous studies have shown that fluorocitrate, and its metabolic precursor fluoroacetate, are selectively taken up into glial cells and are specifically gliotoxic (Clarke *et al.*, 1970; Szerb & Issekutz, 1987; Paulsen *et al.*, 1987, 1988). There is a possibility that our results on citrate labelling may reflect a higher turnover of the tricarboxylic acid cycle in glial cells than in neurons.

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