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Glutathione, but not glutamine, is detected in ^{13}C-NMR spectra of perchloric acid extracts from C6 glioma cells

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Glutamine, which is expected to be produced by C6 glioma cells, is not detected in both amino-acid analyses and ^{13}C-NMR spectra of perchloric acid extracts of cells incubated for 4 h with [1-^{13}C]glucose in the absence of extracellular glutamine. However, the resonances of a glutamate-linked product are observed in these spectra. The analysis of the pH dependence of chemical shifts from various glutamate-derived compounds shows that the observed resonances came from glutathione. Glutamine and glutathione signals are in close proximity on the frequency scale, leading to possible misinterpretation of the spectra.

C6 cell; Glutathione; Glutamine; ^{13}C-NMR spectroscopy

1. INTRODUCTION

Since the work of Martinez-Hernandez et al. [1] who demonstrated the glial localization of glutamine synthetase, glial cells and particularly astrocytes are assumed to produce glutamine in brain. It is generally acknowledged that this amino acid is released from astrocytes and taken up by neurons wherein it is converted into glutamate or γ-aminobutyric acid (GABA), two major neurotransmitter amino acids [2]. After neurotransmission, the amino acid is inactivated partly by recapture by astrocytes where it is recycled into glutamine or oxidized in the Krebs cycle [2]. Some recent papers [3–6] have reported the use of cultured cells in conjunction with ^{13}C- or ^{1}H-NMR techniques to study the respective role of glial cells and neurons in the compartmentation of brain glutamate metabolism. In these studies, primary cultures of astrocytes and neurons have been incubated with ^{13}C-enriched glucose [3,6], pyruvate [5] or acetate [6], resulting in the isotopic enrichment of intermediary metabolites, particularly glutamate and glutamine.

The metabolism of brain tumor cell lines, such as the C6 glioma or the NIE-115 neuroblastoma line, has also been investigated with the same approach [3,5]. These studies showed some metabolic differences between normal and tumor cells. Since it is known that tumor cells require glutamine in the medium to grow [7], we have investigated the ability of C6 cells incubated in a glutamine-free medium to synthesize glutamine from glucose. Here, we report that glutamine is not detected in C6 cells, either by amino acid analysis or by ^{13}C-NMR of extracts of cells incubated with [1-^{13}C]glucose.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; GSH, glutathione; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein.

2. EXPERIMENTAL

2.1. *Materials*

Dulbecco's modified Eagle's media (DMEM) with or without glucose, glutamine and pyruvate were supplied by Gibco (USA). [1-^{13}C]Glucose was purchased from the CEA (France). Glutamate, glutamine, glutathione and pyroglutamate were from Sigma (France).

2.2. *Cell culture and incubation with [1-^{13}C]glucose*

C6 cell culture and incubation with [1-^{13}C]glucose were performed as previously described [3]. Cells used in this study were taken between the 15th and 30th passage. Approximately $3 \cdot 10^8$ cells were washed twice with phosphate-buffered saline (PBS) and incubated at 37°C in DMEM deprived of glutamine and pyruvate, but containing 5.5 mM 99% enriched [1-^{13}C]glucose. After a 4-h (or 48-h as indicated in the text) incubation period, the medium was removed and the cells were washed twice with PBS. Cell perchloric acid extracts were prepared as described in Portais et al. [3]. The neutralized extracts were passed through a Chelex-100 column (H^+ form) and freeze-dried. The resulting lyophilized powders were dissolved in D_2O containing 0.1 M phosphate buffer (pH 7.4) before NMR analyses. The amino acid content of the extracts were determined with an Applied Biosystem amino acid analyzer.

2.3. *NMR spectroscopy*

NMR measurements were carried out on a Bruker AM 400 spectrometer with a 5 mm broad-band probe-head. ^{13}C-NMR spectra were acquired at 100.6 MHz, using a 63.5° flip angle, 0.5 s interpulse delay and 16K memory size. A proton decoupling (WALTZ-16, 1W) was applied during both the acquisition and the relaxation delay. Chemical shifts were expressed as ppm relative to the resonance at 63.3 ppm at

pH 7.4 of ethylene glycol used as reference. The chemical shifts of pyroglutamate were determined by analyzing a solution containing 50 mM ethylene glycol and 50 mM pyroglutamate, in D_2O buffered at pH 7.4 with 100 mM phosphate. To determine the pH dependences of glutamate, glutamine and glutathione resonances, two standard solutions were prepared in D_2O; they contained either 50 mM glutamate and 100 mM glutathione or 50 mM glutamate and 100 mM glutamine, respectively. Both solutions contained 50 mM ethylene glycol as a reference. Prior to the ^{13}C-NMR analyses, aliquots of these solutions were adjusted to pH 3, 5, 7.4, 9 and 11 (without correction for isotopic effect), by adding NaOH or HCl.

3. RESULTS

Fig. 1 (spectrum A) shows the 15–65 ppm region of a perchloric acid extract from C6 glioma cells incubated for 4 h in a medium containing 5.5 mM [1-^{13}C]glucose and deprived of glutamine and pyruvate. The peaks on this spectrum correspond to resonances of enriched carbons as the control spectrum, obtained under the same conditions from an extract of cells incubated with natural abundant glucose, which displays only very low signals apart from those of inositol carbons (spectrum not shown). As previously reported [3], the metabolism of [1-^{13}C]glucose leads to the enrichment of various metabolite carbons, particularly glutamate C2 (55.4 ppm), C3 (27.7 ppm) and C4 (34.2 ppm) which give the most prominent peaks in spectrum A. The ratios between the intensities of the three resonances at 54.9, 27.0 and 32.1 ppm (noted X, Y and Z in spectrum A) to that of glutamate C2, C3 and C4, respectively, are very similar, suggesting they corresponded to a metabolite closely related to glutamate metabolism. In previous studies, these resonances have been assigned to glutamine [3,5]. However, the amino acid analysis of the cell extracts demonstrated that they did not contain glutamine. Control experiments were carried out where a standard of glutamine was added to the cells after the incubation period but prior to the extraction. In that case, glutamine in the extract was correctly determined by amino acid analysis. Glutamine was also detected in extracts from C6 cells incubated in the routine DMEM, that contains 5.5 mM glucose and 4 mM glutamine. In this case, the intracellular glutamine content at steady-state was about 110 nmol/mg of protein. These results indicated that glutamine could not be lost during the extraction procedure and therefore, that the extracts from cells incubated with glucose in the absence of glutamine did not contain a detectable amount of glutamine.

Glutamine was added to a C6 cell extract obtained after cell incubation with [1-^{13}C]glucose prior to NMR analysis (Fig. 1, spectrum B). Although the resonances of glutamine C2 and C3 overlapped peaks X and Z, respectively, the resonance of glutamine C4 is located upfield from peak Y, demonstrating that the resonances X, Y and Z cannot be assigned to glutamine. We examined the possibility of the presence of pyroglutamate, a compound which can be easily synthesized from glutamate, but the chemical shifts of the pyroglutamate carbons (C2: 59.1 ppm, C3: 26.1 ppm and C4: 30.5 ppm at pH 7.4) were very different from the three unassigned resonances. The involvement of another compound, glutathione (GSH), which is also produced from glutamate, was then examined. Fig. 1C shows the spectrum obtained after addition of GSH to the C6 cell extract to which glutamine has been previously added. The three resonances arising from the γ-glutamyl moiety of GSH overlapped the peaks X, Y and Z, suggesting that GSH was a good candidate for the assignment of these peaks. Other resonances from GSH, corresponding to the glycyl- and cysteinyl-moieties, were also observed in spectrum C. These resonances were not present in spectrum A, indicating that they corresponded to carbons remaining unenriched after cell incubation with [1- ^{13}C]glucose.

Table I reports the chemical shifts of carbons 2, 3 and 4 of glutamate, glutamine, GSH and pyroglutamate at pH 7.4. At this pH, glutamine and GSH C2 or C3 have very similar chemical shifts, precluding any distinction between these two compounds. In contrast, the chemical shifts of C4 were slightly but sufficiently different to allow a distinction.

We have assessed the pH dependences of glutamate,

Table I

Chemical shifts of carbons 2, 3 and 4 of glutamate, glutamine and γ-glutamyl moiety of glutathione at pH 7.4

	Chemical shift (ppm)		
	C2	C3	C4
Glutamate	55.4	27.7	34.2
Glutamine	54.9	27.1	31.7
Glutathione (γ-glutamyl)	54.9	27.0	32.1
Pyroglutamate	59.1	26.1	30.5

The chemical shifts were determined from spectra of perchloric acid extract of C6 glioma cells, after addition of glutamine and glutathione. Pyroglutamate chemical shifts were determined by analyzing a standard solution prepared in the same way as a perchloric acid extract. Chemical shifts are expressed relative to the resonance of ethylene glycol at 63.3 ppm (absolute reference: trimethylsilane).

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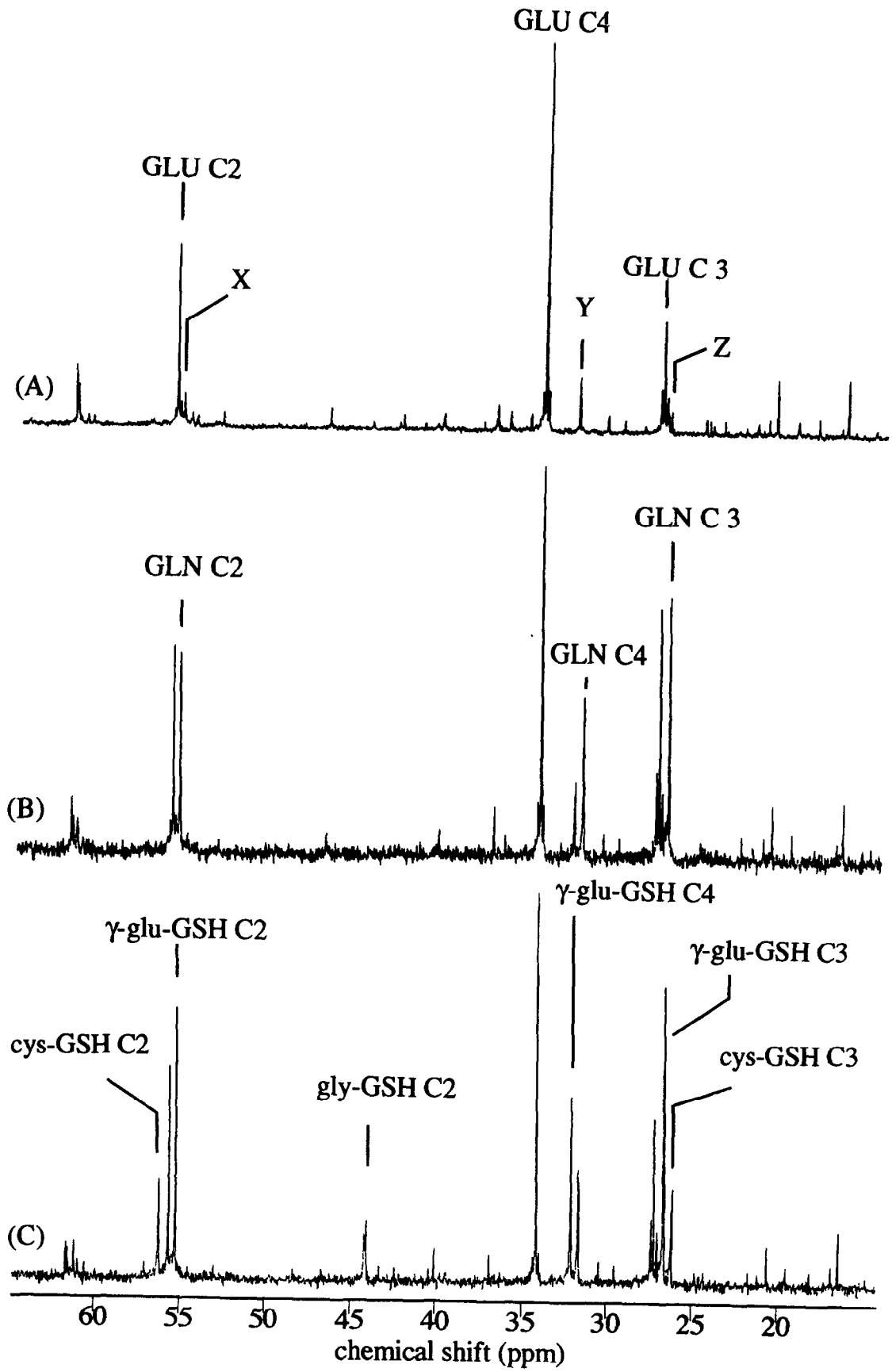
Fig. 1. Carbon-13 NMR spectra of C6 cell extract, before and after addition of glutamine and glutathione. This figure displays the 15–65 ppm region of spectra of: (A) extract from C6 cells incubated for 4 h in a medium containing [1-^{13}C]glucose but no glutamine; (B) same extract after addition of glutamine; (C) same extract after addition of glutathione. Each spectrum corresponds to an overnight data accumulation. The resonances of carbons 2 (C2), 3 (C3) and 4 (C4) of glutamate (GLU), glutamine (GLN) and γ-glutamyl-glutathione (γ-glu-GSH), as well as those of the cysteinyl-(cys-GSH) and glycyl-(gly-GSH) moieties of glutathione, are shown in spectra B and C, respectively correspond to the unassigned resonances in spectrum A.


GLU C4
GLU C2
X
GLU C 3
Y
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(A)
GLN C 3
GLN C2
GLN C4
(B)
γ-glu-GSH C4
γ-glu-GSH C2
γ-glu-GSH C3
cys-GSH C2
cys-GSH C3
gly-GSH C2
(C)
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chemical shift (ppm)

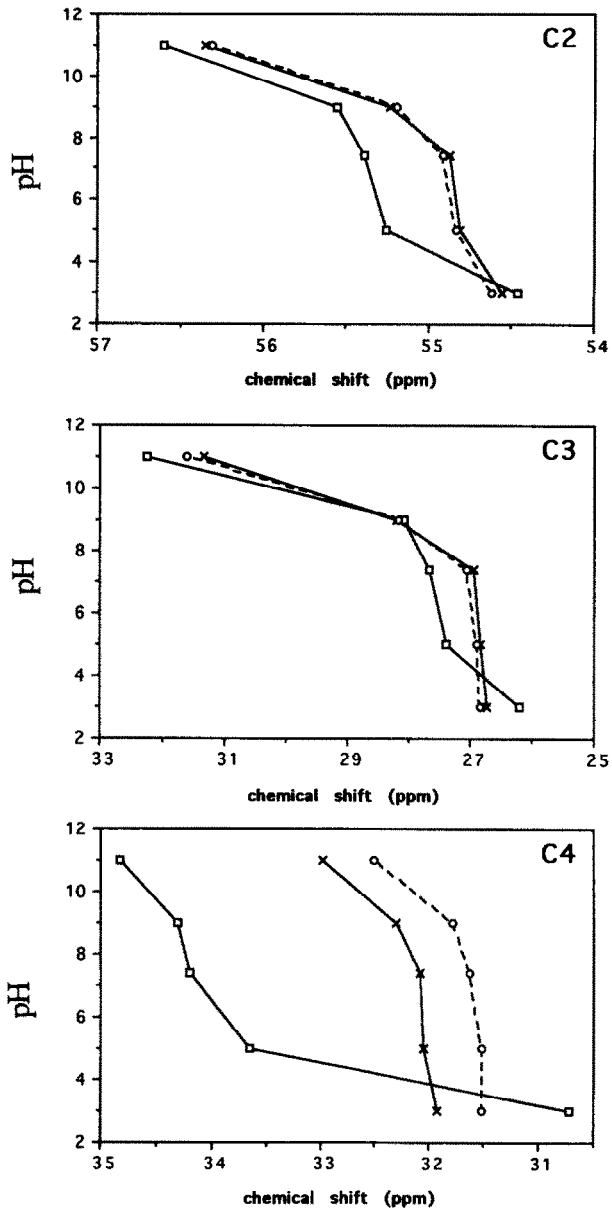

Fig. 2. pH dependences of glutamate, glutamine and glutathione resonance chemical shifts. The chemical shifts of the resonances from carbons 2 (C2), 3 (C3) and 4 (C4) of glutamate (–□–), glutamine (–○–) and glutathione (--×--), were determined by ^{13}C-NMR analyses of standard solutions at different pH, using ethylene glycol as internal reference.

glutamine and γ-glutamyl-GSH resonances, by analyzing standard solutions of these compounds adjusted to pH 3, 5, 7.4, 9 and 11. The plots of the chemical shifts versus pH values for the C2, C3 and C4 positions of glutamate, glutamine and γ-glutamyl-GSH are given in Fig. 2. The results indicated that the chemical shift of each resonance was pH dependent. However, the chemical shifts for glutamine and γ-glutamyl-GSH C2 or C3 remained very similar, whereas the difference between the chemical shifts of C4 was nearly constant, whatever the pH value (on the pH range studied). The pH dependences of resonances X, Y and Z in spectra of C6 cell extracts were identical to that of GSH C2, C4 and C3, indicating that they can be effectively assigned to GSH.

Fig. 3 shows the spectrum of an extract prepared after a 48-h cell incubation with 5.5 mM [1-^{13}C]glucose. As compared to spectrum A in Fig. 1, the resonances of the GSH carbons were more intense, and coupling patterns linked to the presence of neighbouring ^{13}C-enriched carbons clearly detected. They were very similar to those of glutamate carbons (as shown in the expanded region of the spectrum containing the C4 resonances, Fig. 3), demonstrating the close relationship between the two compounds in terms of structure and metabolism. Assuming a metabolic steady-state, the higher intensity ratio between the resonances of GSH and glutamate in spectra from cells incubated for 48 h with [1-^{13}C]glu-

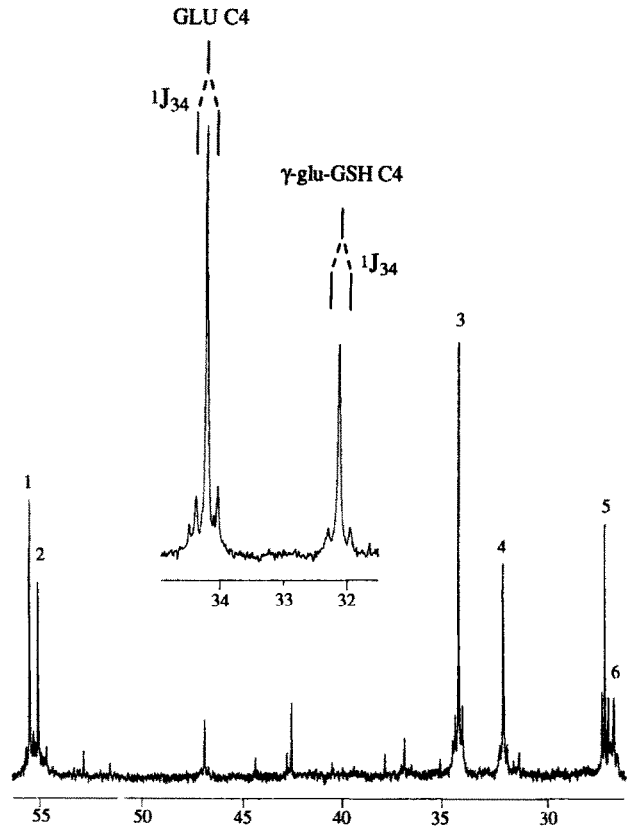

Fig. 3. Carbon-13 NMR spectrum of a perchloric acid extract of C6 cells incubated for 48 h in a medium containing 5.5 mM [1-^{13}C]glucose. The spectrum, corresponding to an overnight accumulation (16K zero filling and 1 Hz line broadening). The homonuclear ^{13}C3-^{13}C4 spin coupling (coupling constant $^1J_{34}$) patterns of the resonances from the C4 of glutamate (GLU) and γ-glutamyl-glutathione (γ-glu-GSH) are shown in an expanded view of the spectrum. The percentages of [3,4-^{13}C]glutamate and γ-glutamyl-glutathione obtained from this spectrum are 21.2 and 20.5, respectively. 1, glutamate C2 (55.4 ppm); 2, γ-glu-GSH C2 (54.9 ppm); 3, glutamate C4 (34.2 ppm); 4, γ-glu-GSH C4 (32.1 ppm); 5, glutamate C3 (27.7 ppm); 6, γ-glu-GSH C3 (27.0 ppm).

cose, as compared to 4 h incubations, suggested that the turnover of GSH was probably lower than that of glutamate.

Under the assumption that the lack of glutamine in cells could be the consequence of a limiting amount of ammonia in the incubation medium, cell perchloric acid extracts were prepared after incubation of C6 cells in the [1-^{13}C]glucose containing medium supplemented with 3 mM ammonium chloride. Additional experiments were performed with C6 cells cultured in the presence of 0.5 mM dibutyryl-cAMP, a differentiation promoting agent, that was controlled to induce morphological differentiation and more intense GFAP-positive immunoreactivity. In both cases, glutamine was never detected in the ^{13}C-NMR spectra of the corresponding perchloric acid extracts, whereas GSH was (spectra not shown).

4. DISCUSSION

The present work reports that the resonances of glutathione but not those of glutamine were observed in ^{13}C-NMR spectra of perchloric acid extracts from C6 glioma cells incubated with [1-^{13}C]glucose in the absence of extracellular glutamine. This conclusion was based on the fact that glutamine was not detected by amino acid analysis of the extracts, and on a close examination of the chemical shift of the C4 resonance of both compounds. Only the resonances of the γ-glutamyl moiety of GSH were observed with relative enrichment and homonuclear spin coupling patterns for C2, C3 and C4 similar to those of the homologous glutamate carbons, indicating that the observed glutathione was neosynthesized from enriched glutamate.

Glutathione is known to be present in C6 cells at a rather high level (around 40 nmol/mg of protein) [8], and in primary cultured astrocytes [9]. Its production mainly depends on the extracellular content in cystine and glutamate, and is favoured when cysteine is high and glutamate low [8]. As the medium used in this study initially contained 0.2 mM cystine but no glutamate, it was therefore not surprising to detect glutathione in the ^{13}C-NMR spectra. However, in a previous report, we have assigned the corresponding resonances to glutamine [3]. The same misinterpretation has probably been done by others ([5] in Fig. 3b of their work, the resonance noted as Gln C4 is likely to be GSH C4). Such a confusion was the consequence of the similarity of glutamine and glutathione carbon chemical shifts, and of the fact that C6 glial cells were assumed to produce glutamine. However, it is noteworthy that the chemical shifts of C4 are sufficiently different to allow unambiguous assignment, whatever the pH of the extract (Table I, Fig. 2). The assignment can be erroneous when only one compound is detected and the pH not strictly controlled.

Glutathione is known to play various roles in the synthesis and transport of proteins and DNA, enzyme activity, metabolism, and protection of cells [10]. In general, glutathione is assumed to have antioxidant properties. It has recently been reported that a glutathione depletion is involved in the cytotoxic effect of glutamate in brain [11]. It has also been shown that glutathione has specific binding sites in brain [12], and that it is released upon K^+ depolarization [13], suggesting that it could act as a neuropeptide. Eventually, some authors have suggested that GSH could be an intermediary in the transfer of glutamate from astrocytes to neurons [14]. Thus, there is an increasing need to further investigate the role of GSH in brain. The present work indicates that ^{13}C-NMR studies of cultured cells offer an additional possibility in the field. Then it was interesting to question whether glutathione resonances were present in spectra obtained from primary cultured astrocytes. Although they have not been assigned, it is likely that glutathione resonances, in addition to those of glutamine, are also present in ^{13}C-NMR spectra of extracts from primary cultures of both cerebellar [3] and cortical [5,6] astrocytes and in spectra of extracts from neuron plus astrocyte co-cultures (resonance noted as unassigned in Fig. 3 of [6]). Glutathione resonances also seem to be present in spectra from neuron and neuroblastoma extracts [5].

An important result of the present study is the absence of glutamine resonances in the ^{13}C-NMR spectra of C6 cells. As it was verified that glutamine could not be degraded during the analytical procedure, this absence appears to be linked either to the lack of glutamine formation from glucose or to a very low cellular content in glutamine. As it has been shown that glutamine synthetase is active in C6 cells [15], this result suggests that the activity of the enzyme is very low under our conditions of cell incubation with [1-^{13}C]glucose, i.e. in a medium deprived of glutamine. A simple explanation for this result could be that ammonium ions were limiting for the glutamine synthetase reaction. However, in experiments where C6 cells were incubated with [1-^{13}C]glucose in the presence of 3 mM ammonium chloride, no more glutamine was produced. Another explanation could be that the cells were not sufficiently differentiated. In preliminary experiments where C6 cells were cultured for 4 days in the presence of 0.5 mM dibutyryl-cAMP before incubation with [1-^{13}C]glucose, no glutamine was observed in the ^{13}C-NMR spectra although two differentiation criteria (morphology and GFAP immunoreactivity) had evolved positively. Thus, although C6 cells have been used for years as a model of glial cells, they cannot be considered as astrocytes, that produce glutamine, but as immature glia that possess astrocytic properties [15]. In this sense, they constitute a model for tumor cells. In our study, the weak glutamine production, if any, appears to be in accordance with the tumor nature of C6 cells, because it is known that tumor cells exhibit a requirement for glu-

tamine in the medium [7]. But, until now, the metabolism of glutamine in brain tumor cells has been poorly documented, in vitro as well as in vivo. If such a requirement for glutamine occurs in the case of tumors growing in the brain (glioma being the major form of brain tumors), there would be a competition between neurons and tumor cells for the capture of the glutamine produced by non-tumorous astrocytes. As a function of tumor growth, the part of glutamine available for neurons would decrease, leading to a considerable perturbation of the glutamate–glutamine cycle, and thus of brain function. Inoculation of C6 cells in the rat brain constitutes an interesting way to study both the metabolism of tumor cells in situ and the impact of brain tumors on surrounding cells [16]. Further characterization of the metabolism of these cells in vitro, and more particularly concerning the metabolism of glutamine, should also be helpful in interpreting in vivo data.

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