Journal of Neuroscience Research 11:351–357 (1984)

Metabolic Fate of [^{14}C]-Glutamine in Mouse Cerebral Neurons in Primary Cultures

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The metabolic fate of L-[^{14}C]-glutamine was followed in cerebral cortical neurons in primary cultures, a GABAergic preparation. Part of the glutamine was converted to GABA (0.3 nmol/min per mg protein), which is consistent with the presence of glutaminase and glutamate decarboxylase activity in the cells and with findings by other authors in vivo or in brain slices. However, an even larger part (1.8 nmol/min per mg protein) was converted to CO_2 and succinate via an oxidative deamination to α-ketoglutarate. This is not consistent with the concept that transfer of glutamine from astrocytes to neurons should replenish neuronal GABA stores quantitatively after release of GABA and its partial accumulation into astrocytes, but it is well compatible with the recent demonstration of a net glutamine uptake by the brain.

Key words: carbon dioxide formation, GABA, glutamate, glutamine, neurons in primary cultures, oxidative metabolism

INTRODUCTION

Glutamine is present in the extracellular space of the central nervous system at a high concentration, i.e., probably about 0.2 mM [Hamberger et al, 1983]. In contrast to its metabolites glutamate and GABA, glutamine has no transmitter activity. It has, however, been suggested that the amino acid transmitters GABA and glutamate, which after their neuronal release are at least partly accumulated into astrocytes, may be converted to glutamine in the astrocytes, released from these cells, and accumulated into neurons, where they may replenish the released GABA or glutamate. At its extreme, this concept envisages a one-to-one relationship between the

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Received December 15, 1983; accepted February 6, 1984.

amounts of GABA or glutamate molecules released from neurons and accumulated into astrocytes and the amount of glutamine molecules returned to the neurons for conversion into GABA [Van den Berg and Garfinkel, 1971; Quastel, 1978]. We have previously questioned the concept of a *stoichiometric* relation between GABA transferred from neurons to astrocytes and glutamine transferred in the opposite direction on account of an equally intense glutamine uptake into astrocytes and into neurons [Hertz et al, 1980; Schousboe et al, 1983] and of a nonnegligible glutaminase activity combined with a relatively low actual rate of glutamine synthesis in the astrocytes [Schousboe et al, 1979; Yu et al, 1982; Schousboe and Hertz, 1983]. In the present study we have carried these investigations one step further by following the actual metabolic fate of [^{14}C]-labeled glutamine into glutamate and GABA as well as into CO_2, formed by oxidative metabolism, after its accumulation into cultured cerebral cortical neurons, a GABAergic preparation [Yu et al, 1982].

METHODS

Primary cultures of cortical neurons were prepared from the brain hemispheres of 15-day-old Swiss mouse embryos. After removal of the meninges and of the olfactory bulbs, basal ganglia, and hippocampal formations, the remaining neopallium—i.e., the portion dorsal and lateral to the lateral ventricle—was cut into small cubes and trypsinized for 2 min using 0.2% trypsin (1:250, Grand Island Biological Company) in Puck's solution at room temperature. After inhibition of the trypsin by addition of modified [Hertz et al, 1982] Eagle's minimum essential tissue culture medium (MEM) containing 20% horse serum, the tissue was triturated with a pipette. The resulting cell suspension was centrifuged for 2 min at 900g. The pellet was resuspended in serum-free medium with an increased glucose concentration (30 mM instead of 7.5mM) and filtered through a Nitex®mesh (from L. and S.H. Thompson & Co. Ltd., Montreal, P.Q., Canada) with a pore size of 80 μm. A cell suspension corresponding to 1–1$\frac{1}{2}$ brain per dish was seeded in 60-mm Falcon plastic tissue culture dishes, which had been coated by exposure overnight to 12.5 μg/ml of D-polylysine in water. After 15 min of incubation at 37°C, unattached cells (mostly nonneuronal cells) were removed together with the medium which was replaced with similar, fresh, modified MEM with 5% horse serum. The cultures were incubated at 37°C in a 95/5% (v/v) mixture of atmospheric air and carbon dioxide (90% humidity). After 3 days of culturing, the cultures were exposed to 40 μM cytosine arabinoside for 24 hr to curtail astrocytic growth. Thereafter, the cultures were refed fresh modified MEM with serum but without the cytotoxic agent and used at the age of 14 days. Such cultures are highly enriched in well-differentiated GABAergic neurons [Yu et al, 1984].

In order to determine formation of glutamate and GABA, each culture (about 1 mg protein) was incubated for 5–60 min in 3 ml of tissue culture medium (containing 7.5 mM glucose) with 500 μM L-[U-^{14}C]-glutamine (from New England Nuclear Corp.) and 5% serum in an atmosphere of 5% carbon dioxide in air. After the incubation the cultures were rinsed five times with ice-cold buffered saline and subsequently another two times with Tris solution (1 mM, pH 7.4), scraped off the culture dishes in 70% ethanol, left in the solution at room temperature for at least 30 min, and centrifuged for 2 min at 11,000g. Protein was determined in the pellets using the conventional Lowry technique [Lowry et al, 1951]. The contents of gluta-

mate, GABA, and glutamine in the extracts or in diluted media were determined by high-pressure liquid chromatography (HPLC) after precolumn derivatization with O-pthaldialdehyde essentially as described by Lindroth and Mopper [1979], employing a Waters HPLC system (Waters Associates, Toronto, Ontario) consisting of one M-45 and one 6000A pump, a model 720 system controller, a U6K sample injector, a data module M730, a model M420 fluorescence detector, and a Resolve 5μ Bondapak C18 column. The mobile phase was 0.1 M potassium acetate (pH 5.7) and methanol. Initially the methanol concentration was increased from 25% to 70% in a single linear step (flow rate 0.8 ml/min during 15 min), then it was maintained at 70% for 10 min and finally reversed (over 5 min) to equilibrate the column with 25% methanol. Effluents corresponding to glutamate and glutamine were collected for subsequent determination of radioactivities.

For determination of $^{14}CO_2$ production rates from purified [1-^{14}C]-L-glutamine [Zielke et al, 1978], individual cultures incubated in serum-free tissue culture medium (to be able to determine protein content) were placed without lids in a gas-tight chamber [Yu et al, 1982]. At the beginning of the experimental period, the radioisotope, diluted in medium, was added to the cultures by injection, and at its end hyamine hydroxide (2 ml) was injected into a suspended beaker and 0.4 M perchloric acid (500 μl) into the culture dishes to acidify the medium. After quantitative trapping of CO_2 the radioactivity in the hyamine hydroxide was determined. From this value, the specific activity of glutamine in the media and the protein content in the cultures, the carbon dioxide production per mg protein was calculated.

RESULTS AND DISCUSSION

The pool sizes (contents) of glutamine, glutamate, and GABA as a function of time of exposure to 0.5 mM [U-^{14}C]-labeled glutamine are shown in Figure 1 (top) together with the increases in their specific activities (Fig. 1, bottom). It can be seen that the radioactivity in glutamine reaches a steady level (about 6×10^3 dpm/nmol) after about 30 min. This level is one-third lower than the specific activity in the incubation medium, which remained relatively constant at 9.3×10^3 dpm/nmol. This indicates that part of the glutamine pool in the cultured cells does not mix readily with exogenous glutamine. The activity in glutamate rises more slowly and shows no sign of approaching that of its precursor, glutamine, within the experimental period. This suggests that a minor part of the glutamate pool may not be readily accessible for glutamine, either because there is a metabolic compartmentation, (although not nearly as pronounced as that reported by Borg et al [1983]) or because glutamate at the same time is formed also from other sources. GABA, formed from glutamate, is labeled even more slowly, and it cannot be determined whether this amino acid ultimately will reach the same specific activity as glutamate.

The half-time for [^{14}C]-glutamine uptake is approximately 8 min (Fig. 1, bottom); i.e., the rate constant is 0.087 min^{-1}, and the pool size for readily exchangeable glutamine is two-thirds of a total pool of 40 nmol/mg protein (Fig. 1, top), i.e., 25–30 nmol/mg protein. Multiplication of these values yields an uptake rate of about 2.3 nmol/min per mg protein, i.e., a value comparable to that (2.0–3.9 nmol/min per mg protein), which previously has been directly established [Hertz et al, 1980; Yu et al, 1984]. The rate of glutamate formation cannot be calculated with similar accuracy because (1) there is no time period when the specific activity of the precursor

(glutamine) has reached a steady level and that of the product (glutamate) still increases at its initial velocity, and (2) it is unknown whether a small pool is in almost instantaneous equilibrium with glutamine or a larger pool is more slowly labeled. A *minimum* value can, however, be calculated for the time period 15–30 min when the glutamine specific activity is relatively constant and the specific activity in glutamate increases with 66 dpm/nmol per min (Fig. 1, bottom) or with a glutamate pool of 65 nmol/mg protein (Fig. 1, top), 4.3 × 10^3 dpm/mg protein per min. From an average specific activity of glutamine of 5.0 × 10^3 dpm/nmol during this period, it can be calculated that the formation of glutamate corresponds to 0.9 nmol/min per mg protein. A maximum value for net synthesis of glutamate from glutamine is the glutamine uptake rate of about 2.3 nmol/min per mg protein since the glutamine pool size remains relatively constant during the first 30 min (Fig. 1, top) and glutamine is presumably not formed in neurons [Norenberg and Martinez-Hernandez, 1979]. This is a maximum value because glutamine is also released from neurons [Ramaharobandro et al, 1982; Schousboe et al, 1983; Yu et al, 1984]. It is high compared to the glutaminase activity, which in the presence of phosphate is about 8 nmol/min per mg protein [Hertz et al, 1983]. The formation rate for GABA can be more accurately determined than that for glutamate, since (Fig. 1, bottom) GABA formation continues at its initial rate (16.7 dpm/nmol per min) after the specific activity of glutamate has become relatively constant (2.6 × 10^3 dpm/nmol). With a GABA pool size of 45

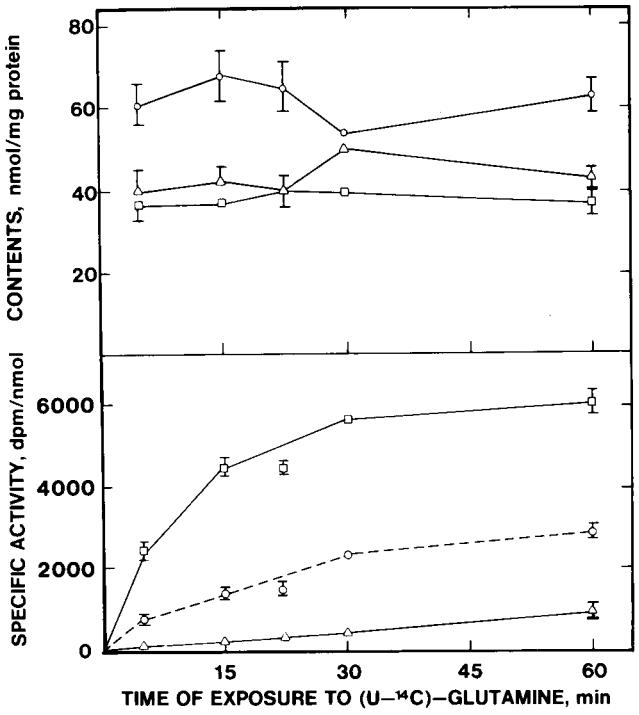

Fig. 1. Pool sizes (top) and specific radioactivities (bottom) of glutamine (□), glutamate (○), and GABA (△) in primary cultures of mouse cerebral cortical neurons as a function of time of incubation in a tissue culture medium with 500 μM L-[U-^{14}C]-glutamine. Results are average ± SEM of 4–6 experiments, except for the 30-min values, which are the average of two experiments.

nmol/mg protein (Fig. 1, top) this corresponds to a GABA formation rate of 0.3 nmol/min per mg protein, calculated as above and on the assumption of a uniformly labeled glutamate pool. This is compatible with a GAD activity of 10–15 nmol/min per mg protein [Hertz et al, 1983; Yu et al, 1984] and comparable to the GABA uptake into astrocytes [Schousboe and Hertz, 1983; Schousboe et al, 1983].

Production rates for $^{14}CO_2$ from L-[1-^{14}C]-glutamine were determined in the presence of 5 mM aminooxyacetic acid (AOAA), since radioactive CO_2 also is produced from [1-^{14}C]-glutamine when glutamate, formed from glutamine, is decarboxylated to GABA. AOAA effectively inhibits not only glutamate decarboxylase activity in the cultured cells (unpublished experiments) but also transamination between glutamate and α-ketoglutarate [Yu et al, 1982], so that in the presence of this compound the production of labeled CO_2 unequivocally represents decarboxylation of α-ketoglutarate formed from labeled glutamate by an oxidative deamination. Figure 2 shows that a substantial amount of labeled CO_2 is produced from [1-^{14}C]-labeled glutamine. The production is less during the first 15 min (when the glutamine and glutamate pools are still far from equilibration) and is thereafter rectilinear as a function of time and corresponds to 0.5 nmol/min per mg protein, calculated on the basis of the specific activity of the medium. This is a minimum value. However, since the specific activity of glutamate in the cultured cells is only 2.6×10^3 (Fig. 1, bottom) when that of glutamine in the medium is 9.3×10^3 dpm/nmol, this value should probably [Hertz et al, 1983] be multiplied by 3.6 (9.3/2.6) so that the actual CO_2 production rate would be 1.8 nmol/min per mg protein. This value is six times higher than the GABA production rate. It is compatible with a glutamate dehydrogenase activity of 25–30 nmol/min per mg protein [Hertz et al, 1983]. Moreover, the sum of CO_2 production (in the presence of AOAA) and GABA formation (2.1 nmol/min per mg protein) falls within the estimated range for glutamate production (0.9–2.3 nmol/min per mg protein).

The present work has thus confirmed that GABAergic neurons in primary cultures are able to accumulate glutamine avidly [Hertz et al, 1980; Schousboe et al, 1983; Yu et al, 1984], and it has demonstrated that they can convert this glutamine to

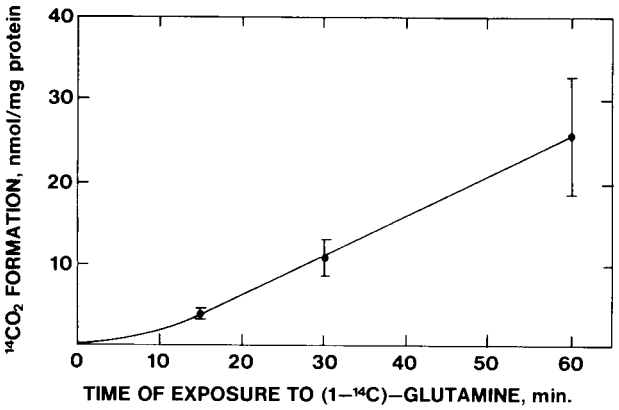

Fig. 2. Production of $^{14}CO_2$ (nmol/mg protein) in primary cultures of mouse cerebral cortical neurons incubated in a tissue culture medium with 500 μM L-[1-^{14}C]-glutamine. Results are average $\pm$ SEM of three experiments.

glutamate and from there to either GABA or, in an oxidative degradation via α-ketoglutarate, to succinyl coenzyme A (CoA) and CO_2. Such a formation of GABA from glutamine is in accordance with observations in the brain in vivo [Gauchy et al, 1980; Ward et al, 1983] and in brain slices [Reubi et al, 1978; Kemel et al, 1979]. However, the large formation of CO_2, which also has been observed in noncultured brain tissue [Tildon, 1983; Yu and Hertz, 1983; Tildon and Roeder, 1984] indicates that neuronal stores of GABA and glutamate cannot be quantitatively replenished by the release of glutamine, formed from glutamate or GABA *in the astrocytes.* The use of glutamine, both as a metabolic substrate and as a transmitter precursor, would therefore suggest that the availability of glutamine could be rate-limiting for both processes unless glutamine is accumulated from the blood into the central nervous system. In contrast to previous conclusions that this is not the case [e.g., Lund, 1971; Abdul-Ghani et al, 1978], such a *net* uptake has recently been demonstrated by Eriksson et al [1983]. This circumvents the need for a stoichiometrically operating cycle and probably explains the findings that glutamine at the same time can be metabolized to a considerable extent and be a major GABA precursor.

ACKNOWLEDGMENTS

The Medical Research Council of Canada (grant MT 5957 to L.H.), the Saskatchewan Health Research Board (grant to A.C.H.Y.), and the Novo Foundation (grant to A.S.) are thanked for financial support.

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