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Rapid Uptake and Degradation of Glycine by Astroglial Cells in Culture: Synthesis and Release of Serine and Lactate

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ABSTRACT Free glycine is known to have vital functions in the mammalian brain, where it serves mainly as both neurotransmitter and neuromodulator. Despite its importance, little is known about the metabolic pathways of glycine synthesis and degradation in the central nervous system. In this study, the pathway of glycine metabolism in astroglia-rich primary cultures from rat brain was examined. The cells were allowed to degrade glycine in the presence of [U-^{14}C]glycine, [U-^{13}C]glycine or [^{15}N]glycine. The resulting intra- and extracellular metabolites were analyzed both by high-performance liquid chromatography and by ^{13}C/^{15}N nuclear magnetic resonance spectroscopy. Glycine was rapidly consumed in a process obeying first-order kinetics. The initial glycine consumption rate was 0.47 nmol per mg protein. The half-life of glycine radiolabel in the incubation medium was shorter than that of glycine mass. This suggests that glycine is produced from endogenous sources and released simultaneously with glycine uptake and metabolism. As the main metabolites of the glycine carbon skeleton in astroglia-rich primary cultures from rat brain, serine and lactate were released during glycine consumption. The main metabolite containing the glycine amino nitrogen was glutamine. To establish a metabolic pathway from glycine to serine in neural tissue, homogenates of rat brain and of neural primary cultures were assayed for their content of serine hydroxymethyltransferase (SHMT) and glycine cleavage system (GCS). SHMT activity was present in homogenates of rat brain as well as of astroglia-rich and neuron-rich primary cultures, whereas GCS activity was detectable only in homogenates of rat brain and astroglia-rich primary culture. Of the two known SHMT isoenzymes, only the mitochondrial form was found in rat brain homogenate. It is proposed that, in neural tissue, glycine is metabolized by the combined action of SHMT and the GCS. Owing to the absence of the GCS from neurons, astrocytes appear to be the only site of this part of glycine metabolism in brain. However, neurons are able to utilize as energy source the lactate formed by astroglial cells in this metabolic pathway. *GLIA 27:239–248, 1999.*

INTRODUCTION

Glycine, the simplest amino acid, is abundant in all mammalian body fluids and tissue proteins. Although not an essential amino acid, glycine is a key compound in the metabolism of one-carbon fragments, proteins, peptides, nucleotides, porphyrins, and bile acids. It also has vital functions in the mammalian brain, where it serves as both neurotransmitter and neuromodulator.

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The classical glycine neurotransmitter receptor is a ligand-gated chloride channel predominantly expressed in spinal chord and brainstem (for review see Rajendra et al., 1997) but probably also important in the forebrain (Engblom et al., 1996; Rampon et al., 1996) and retina (Greferath et al., 1994). Another high-affinity glycine binding site is located on the NMDA-type glutamate receptor, and occupation of this site by an agonistic ligand is required for channel opening. Glycine is a natural candidate for the endogenous ligand (Johnson and Ascher, 1987), though recent studies also see D-serine in this role (Kemp and Leeson, 1993). Glycine again is suspected to be metabolically related to D-serine (Iwama et al., 1997).

Considering the important functions of glycine in the mammalian brain, it is not surprising that disturbances of glycine metabolism lead to severe neurological disorders, such as nonketotic hyperglycinemia, an inherited disease caused by malfunction of the glycine cleavage system. Neurological symptoms associated with this disease are assumed to result from overactivation of N-methyl-D-aspartate (NMDA) receptors owing to high intracerebral glycine concentration (Kure et al., 1997). The considerable knowledge that has accumulated on glycine function in the CNS as well as on the origin and fate of glycine in peripheral tissues contrasts with the lack of detailed information on glycine metabolism in the CNS. It is still unknown whether biosynthesis of glycine is essential for the maintenance of glycine levels in the brain and spinal cord or whether neural cells depend on uptake and accumulation of supplied glycine. The same lack of knowledge exists with regard to the fate of extracellular glycine in the CNS.

As with all neurotransmitters, released glycine must be removed rapidly from the extracellular space for signal termination. Because no ectoenzymes are known to process glycine, the first step must be reuptake by cells participating in the synaptic event and by the surrounding astroglial cells. Two high-affinity glycine transporters are found in brain. Both isoforms show a distinct cellular distribution in the CNS. Glycinergic neurons express the transporter isoform GLYT2, permitting transmitter reuptake from the synaptic cleft (Luque et al., 1995). In contrast, GLYT1 is expressed by astrocytes preferentially but is not exclusively located around glycinergic neurons (Zafra et al., 1995). This suggests astrocytic involvement in the disposition of extracellular glycine. After the initial observation that astroglial cells in culture remove glycine from their culture medium (Bixel et al., 1993), the fate of [2-^{13}C]glycine was followed up by ^{13}C-nuclear magnetic resonance (NMR) spectroscopy (Dringen et al., 1998), where labelled creatine, serine, and glutathione were identified in the cell extracts. Additional arginine and methionine enlarged the amount of de novo synthesized [2-^{13}C]creatine and its precursor ^{13}C-labelled guanidinoacetate in cell extracts and in the media after 24 h of incubation. A major part of the glycine was utilized for the synthesis of glutathione in astroglial cells, which was found in the cell extract and in the incubation medium. The presence of labelled serine in the cell extracts and the incubation medium proves the capability of astroglial cells to synthesize serine from glycine and to release serine. In the present study we have investigated glycine metabolism in these cells using high-performance liquid chromatography (HPLC) and NMR techniques to gain further insight into the role of astrocytes in the homeostasis of cerebral glycine.

MATERIALS AND METHODS

Materials

Cells were cultured in 50-mm-diameter plastic dishes from Nunc (Wiesbaden, Germany). The general culture medium was 90% Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, purchased from Gibco (Eggenstein, Germany) and Boehringer (Mannheim, Germany), respectively. The incubation medium was DMEM without amino acids, inositol, and pyruvate, prepared from components obtained from E. Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany). Pediococcus lactic oxidase and tetrahydrofolic acid (THF) were purchased from Sigma (Deisenhofen, Germany). Glutamate:pyruvate aminotransferase and NAD^+ were acquired from Boehringer (Mannheim, Germany). Pyridoxal phosphate (PLP) was from Serva (Heidelberg, Germany). [U-^{14}C]serine and [U-^{14}C]glycine, specific radioactivities $>$150 mCi/mmol and $>$100 mCi/mmol, respectively, were obtained from Amersham (Braunschweig, Germany). [U-^{13}C]glycine and [^{15}N]glycine were purchased from Cambridge Isotope Laboratories (Wesel, Germany). HPLC-grade methanol and acetonitrile were obtained from Riedel de Haën (Seelze, Germany). All other chemicals, of the highest purity available, were from E. Merck (Darmstadt, Germany). The type 1225 vacuum filtration manifold was from Millipore (Eschborn, Germany). DEAE cellulose ion-exchange paper (DE-81) was purchased from Whatman (Maidstone, U.K.). The HPLC system was from Sykam (Gilching, Germany) and consisted of the solvent delivery system S1000, the low-pressure gradient mixer S3110, and a personal computer equipped with the Axxiom Multi-Channel Chromatography Data System. Detection was facilitated by an RF-535 fluorescence monitor from Shimadzu (Kyoto, Japan) or by a UV/vis detector from Linear Instruments (Reno, NV). The HPLC columns used were 120 mm $\times$ 4.6 mm Hypersil ODS, particle size 3 µm, 200 mm $\times$ 4 mm Inertsil ODS, particle size 5 µm, and 200 mm $\times$ 4 mm Grom-Sil OPA-2, particle size 5 µm, all supplied by Grom Analytik + HPLC (Herrenberg, Germany). Samples were applied either by a Hamilton syringe or by an intelligent sampler, model 851 AS, from Jasco Labor- und Datentechnik (Gross-Umstadt, Germany). Radioactivity of samples was measured in the liquid scintillation spectrometer LKB 1214 Rackbeta from LKB (Gräfeling, Germany), using Ultima Gold scintillation fluid from Packard (Groningen, The Netherlands).

Cell Culture

Astroglia-rich primary cultures derived from the brains of neonatal Wistar rats were prepared and cultivated as described (Hamprecht and Löffler, 1985). They contain primarily astroglial cells and are widely used for studying the metabolism of astroglial cells (for overview see Hamprecht and Dringen, 1995). The experiments described here were carried out using 16 to 18-day-old cultures. For the HPLC analysis of metabolites in the culture medium and for preparation of cell extracts for the analysis of intracellular metabolites by ^{13}C-NMR, cells were cultured in dishes respectively 50 mm and 84 mm in diameter. The incubation medium was removed, the cells were washed three times with ice-cold phosphate-buffered saline, and 1 ml 0.4 M perchloric acid (PCA) was added. The content of each dish was transferred to a microfuge tube and homogenized by sonication. The protein was removed by centrifugation in a desktop microfuge at top speed for 10 min. The supernatant was transferred to another tube and PCA was removed by addition of an equivalent amount of a solution containing 1 M KOH and 1 M $KHCO_3$. After centrifugation, the precipitate was discarded and the supernatant was lyophilized. For NMR experiments, the lyophilisate was taken up in D_2O. For HPLC experiments, it was dissolved in the appropriate HPLC eluent.

HPLC Techniques

Amino acid detection was facilitated by precolumn derivatisation with o-phthalodialdehyde (OPA) similar to the method of Jones and Gilligan (1983). The stock derivatisation reagent for amino acid analysis consisted of 10 mg/ml OPA and either 10 mg/ml mercaptopropionic acid or 10 mg/ml N-isobutyryl-L-cysteine in methanol for chromatography on a Hypersil ODS or on a Grom-Sil OPA-2 column, respectively. For standard precolumn derivatisation, 34 µl stock reagent solution was added to 1 ml 0.5 M borate buffer, pH 10, and equal amounts of this solution and of sample were mixed and allowed to react for 1 min prior to injection. The samples were diluted in borate buffer as needed. The stability of the OPA derivatives and the yield of the derivatisation reaction were investigated by derivatisation and chromatography of L-[U-^{14}C]serine and [U-^{14}C]glycine. In both cases, more than 97% of the radioactivity was eluted at the retention time of the respective amino acid. Both Hypersil ODS and Grom-Sil OPA-2 columns were eluted by binary gradients generated from buffer A [13 mM sodium phosphate, pH 7.2, 1.5% (v/v) tetrahydrofurane] and buffer B [50% (v/v) buffer A, 35% (v/v) methanol, 15% (v/v) acetonitrile]. The Inertsil column was eluted isocratically with buffer B.

Enzyme Assays

Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) was assayed according to a modification of the procedure described by Geller and Kotb (1989). A complete assay reaction mixture contained 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0, 3.0 mM dithiothreitol (DTT), 0.25 mM PLP, 2.5 mM ethylene diamine tetraacetate (EDTA), 2.0 mM THF, 0.4 mM L-serine containing L-[3-^{14}C]serine (approximately 900,000 cpm/ml), and homogenate. Weighed amounts of solid THF were added to the assay mixture immediately before starting the incubation with L-serine/L-[3-^{14}C]serine and were brought into solution by brief sonication. The reaction was carried out at 37°C. At appropriate times, the reaction was stopped by streaking a 25 µl-aliquot of the assay mixture onto a circle of DE-81 filter paper 2.3 cm in diameter that was placed in a vacuum filtration manifold. After washing four to five times with 15 ml distilled water, the filter was transferred to a scintillation vial containing 10 ml scintillation fluid for liquid scintillation counting. In order to ascertain that the DE-81 binding assay measured N^5,N^{10}-methylene-THF, C1 exchange with formaldehyde and reversal of the reaction by addition of glycine were demonstrated as described (Geller and Kotb, 1989). The glycine cleavage system (GCS; EC 1.4.4.2) was assayed by a procedure similar to the SHMT assay. The final reaction mixture contained 50 mM Tris buffer , pH 8.0, 3.0 mM DTT, 0.25 mM PLP, 2.5 mM EDTA, 2.0 mM THF, 0.5 mM NAD^+, 4 mM glycine containing [U-^{14}C]glycine (approximately 107 million cpm/ml) and homogenate. Solid THF was added to the reaction mixture prior to starting the incubation by addition of substrate and was brought into solution by sonication. The assay procedure was further continued as described for SHMT.

NMR Spectroscopy

Culture media containing 400 µM [U-^{13}C]glycine (experiment I) or 200 µM [U-^{13}C]glycine, and 200 µM [^{15}N]glycine (experiment II) that had been exposed to cells for 24 h were lyophilized. The residual material was dissolved in 0.4 ml D_2O, the solution was centrifuged and the pH was standardized to pH 7 with deuterated sodium hydroxide (NaOD) and deuterium chloride (DCl). ^{13}C-NMR spectra of media were recorded on a Bruker DRX-600 (32,000 accumulations; 64k data points), calibrated to β-glucose-C1 (96.8 ppm, flip angle 70°; 1.9 s repetition time). The measured integrals were corrected for partial saturation and nuclear Overhauser enhancement by calibration with spectra from model solutions (with defined concentrations of the metabolites, 400 mM KCl, and 5 mM $MgCl_2$ to obtain an ionic strength similar to that in the cell extract samples) measured with the same acquisition parameters.

^{15}N-NMR spectra were recorded with 64k data points, 64k accumulations, 2.45 s repetition time and 90° flip angle. Two-dimensional heteronuclear single quantum coherence (2D-HSQC) spectra have been acquired for spectral assignment using a 5 mm H,C,N inverse triple resonance probe with shielded gradients (Willker et al., 1997).

Each experiment was carried out at least twice on independent cultures with comparable results. The results shown in the figures are those of one representative experiment. The data points represent the mean values of three independent measurements. The error bars indicate the corresponding standard deviations.

RESULTS

Astroglia-rich primary cultures from rat brain grown in culture dishes 50 mm in diameter eliminated 1 µmol glycine from 3 ml culture medium with a half-life of 20.6 h (Fig. 1A). The process obeyed first-order kinetics for up to 66 h when followed by either glycine radioactivity or glycine concentration after application of [U-^{14}C]glycine (Fig. 1A,B, insets). Based on elimination of glycine radiolabel from the medium the glycine half-life was 13.3 h, indicating that glycine radioactivity disappeared from the medium faster than glycine mass. However, in the absence of exogenous glycine, but otherwise, under the same conditions routinely used for measurement of the glycine consumption rate, no significant amounts of glycine were released by the cells (data not shown).

As the total intracellular volume of the astroglia-rich primary cultures amounts to 4 µl only (Dringen and Hamprecht, in preparation), in the absence of glycine consumption the complete uptake of the glycine present at an extracellular concentration of 333 µM would result in an intracellular concentration of more than 200 mM. However, as determined by HPLC analysis, the actual concentration of glycine within the cells rose from 4 mM to a maximum of 11.5 mM during 1.5 h of incubation and then declined again to 4 mM during 88.5 h of further incubation. Thus a pathway for glycine degradation must exist within the cells. The incorporation of glycine and its putative metabolites into cellular macromolecules is likely to contribute to total glycine consumption. To assess the extent of this incorporation, [U-^{14}C]labelled glycine was added to the incubation medium, and the distribution of radioactivity between culture medium and cell fractions was determined at various times after the application (Fig. 2). After 36 h, about 13% of label was incorporated into the PCA-insoluble fraction of cell content, whereas 4% was present in the cells as PCA-soluble material. If the incubation period was prolonged these values remained constant.

At the end of the experiment (after 160 h), about 48% of radiolabel had disappeared from the culture dish. A considerable amount of radioactivity (35%) was still found in the culture medium, although glycine had been consumed almost entirely (Figs. 1B, 2), indicating the release of radiolabelled metabolites from the cells. Radiolabelled lactate in the sample was identified by its retention time on an Inertsil ODS column and by its enzymatic conversion to alanine by treatment with Pediococcus lactic oxidase and glutamate:pyruvate ami-

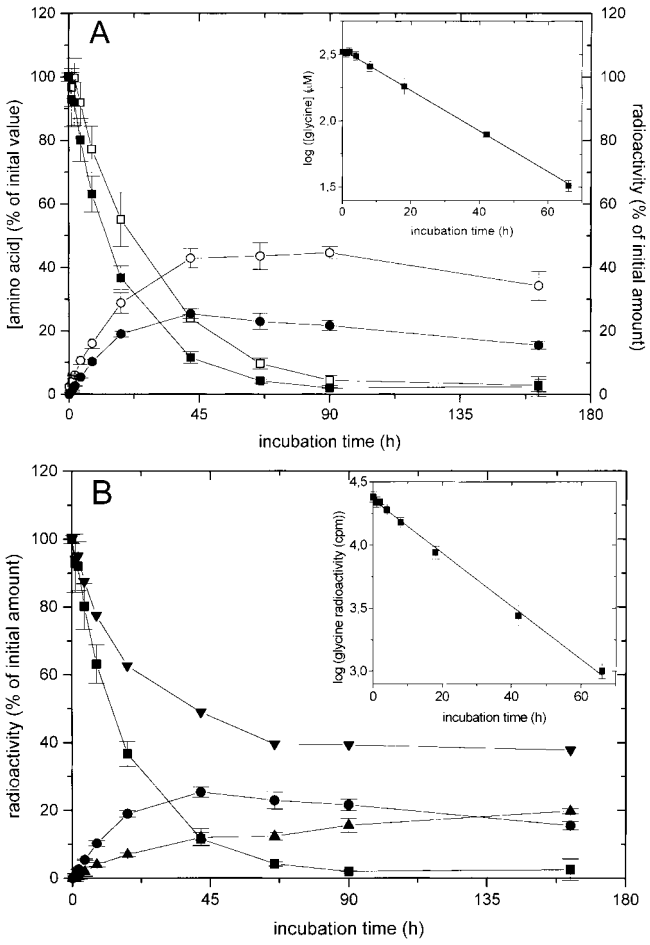

Fig. 1. Time course of the disappearance of radioactively labelled glycine from the culture medium of astroglia-rich primary cultures from rat brain and the accumulation of metabolites as determined by HPLC analysis and liquid scintillation counting. The incubation medium was DMEM lacking the other 14 amino acids, pyruvate, and inositol. Each culture dish contained 333 µM (100% value in A) glycine and 37 kBq [U-^{14}C]glycine (100% value in A and B). The values corresponding to the solid symbols are the mean values ± SD of the counts per minute (cpm) measured in the appropriate HPLC fractions of three 10 µl aliquot parts of the total initial medium volume (3 ml) of three separate culture dishes (50 mm in diameter). The values corresponding to the open symbols are the mean values ± SD of the concentrations as calculated from the peak areas of the HPLC chromatograms. Before the onset of the main incubation, the cells were preincubated in DMEM devoid of amino acids, pyruvate, and inositol for 1.5 h. At the beginning of the experiment the cultures were 18 days old. **A:** Time course of the disappearance of glycine mass (□) and radioactivity (■) and the simultaneous appearance of serine mass (○) and radioactivity (●). **Inset A:** Linearization of the time course of glycine disappearance by semilogarithmic representation of the data shows first-order kinetics for the first 66 h. The correlation coefficient is 0.99. **B:** Time course of the disappearance of glycine radioactivity (■) and the simultaneous appearance of radiolabelled serine (●) and lactate (▲).The downward triangles (▼) depict the total radioactivity present in the medium at the times indicated. **Inset B:** Semilogarithmic representation of the data for the disappearance of glycine radioactivity from the culture medium. Correlation coefficient is 0.99.

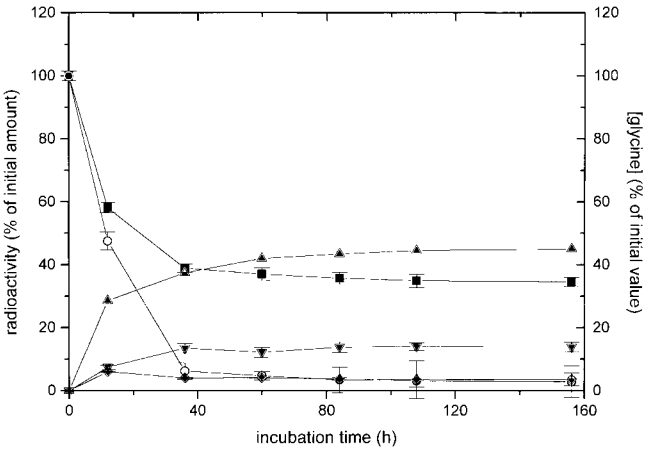

Fig. 2. Time-dependent distribution of total radioactivity between the culture medium (■), the PCA-insoluble (◆) and the PCA-soluble (▼) cell content of astroglia-rich primary cultures after incubation with 0.36 mM glycine and approximately 14 kBq [U-^{14}C]glycine in DMEM devoid of pyruvate, inositol, and other amino acids. Glycine concentration (○) and net loss of radioactivity from the system (▲) are also indicated. At the start of the incubation, the cultures were 18 days old. The medium volume was 3 ml. Prior to the main experiment, the cells were preincubated for 1.5 h with 3 ml DMEM lacking pyruvate, inositol and, amino acids.

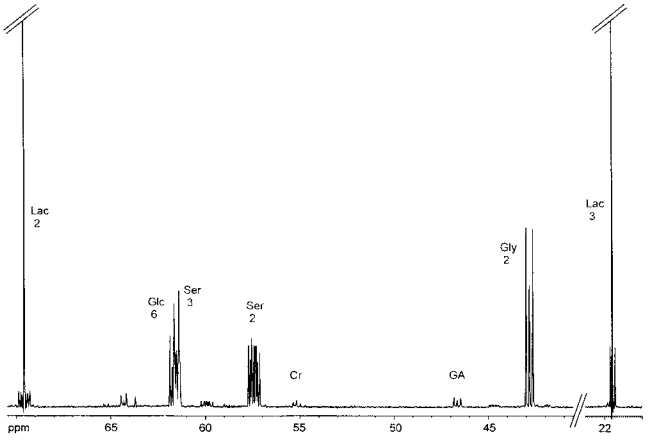

Fig. 3. ^{13}C-NMR spectrum (aliphatic region) of a cell extract from astroglia-rich primary cultures incubated for 24 h in a medium containing [1,2-^{13}C]glycine (1 mM) and unlabelled glucose (5 mM). Various multiply ^{13}C-labelled isotopomers of metabolites are synthesized from [1,2-^{13}C]glycine such as serine labelled at carbon C2 (Ser 2) and carbon C3 (Ser 3), lactate at position C2 (Lac 2) and position C3 (Lac 3), creatine (Cr), guanidinoacetate (GA), and glycine at carbon C2 (Gly 2). Further signals are due to serine carbamate positions C2 and C3 (unassigned) and glucose C6 (Glc 6) in natural abundance. A minor amount of labelled glutathione is located at 44.7 ppm. For clarity's sake, the low field part of the spectrum (carboxylgroup carbons) has been omitted.

notransferase. After such treatment, radioactivity was no longer found in the eluate at the retention time of lactate but passed the column unretardedly. Radiolabelled serine was identified by HPLC of its OPA derivative using a Hypersil ODS column. Addition of [U-^{14}C]serine to the sample increased the amount of radioactivity appearing at the retention time of authentic unlabelled serine.

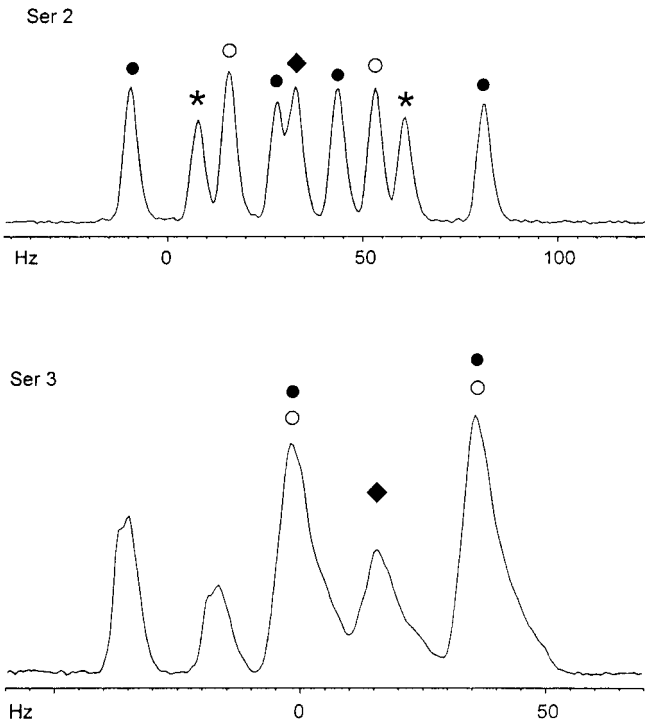

Fig. 4. Expanded plots of serine signals in the ^{13}C-NMR spectrum (Fig. 3) generated from a cell extract derived from astroglia-rich primary cultures that where incubated for 24 h in a medium containing [1,2-^{13}C]glycine (0.4 mM) and glucose (5 mM). The top part shows the position of carbon 2 of serine (Ser 2) and the lower part represents carbon 3 (Ser 3). The different isotopomers are [1,2,3-^{13}C]serine (●), [1,2-^{13}C]serine (*), [2,3-^{13}C]serine (○), [2-^{13}C]serine (◆) or [3-^{13}C]serine (◆).

The identity of the metabolites and in particular their isotopomer composition were also investigated by recording the ^{13}C-NMR spectrum of a culture medium supplemented with 400 µM [U-^{13}C]glycine that had been previously exposed to astroglia-rich primary cultures for 24 h. ^{13}C-labelled metabolites of glycine, serine, lactate, guanidinoacetate, creatine and glutathione were identified in D_2O solutions of lyophilized culture media (Fig. 3). Several isotopomers of [^{13}C]serine were present in the medium such as [1,2,3-^{13}C]serine, [1,2-^{13}C]serine, and [2,3-^{13}C]serine and the monolabelled [1-^{13}C]serine, [2-^{13}C]serine, and [3-^{13}C]serine. The signal intensities of the monolabelled isotopomers are superpositions of the natural abundance signal intensities of serine derived from unlabelled precursors and signal intensities of monolabelled serine synthesized from monolabelled glycine or a labelled C1 unit (Fig. 4). The multiply labelled isotopomers were recognized by the spin spin coupling in both carbon signals of C2 and C3, respectively. Further multiple ^{13}C labelling originating from doubly labelled [U-^{13}C]glycine appeared in [^{13}C]lactate such as [1,2,3-^{13}C]lactate, [1,2-^{13}C]lactate, and [2,3-^{13}C]lactate (Fig. 5). With the exception of the superimposed signal intensities of natural abundance [2-^{13}C]lactate and [3-^{13}C]lactate the multiply labelled isotopomer ratios in lactate and serine were identical (Figs. 6, 7), thus indicating a metabolic pathway lead-

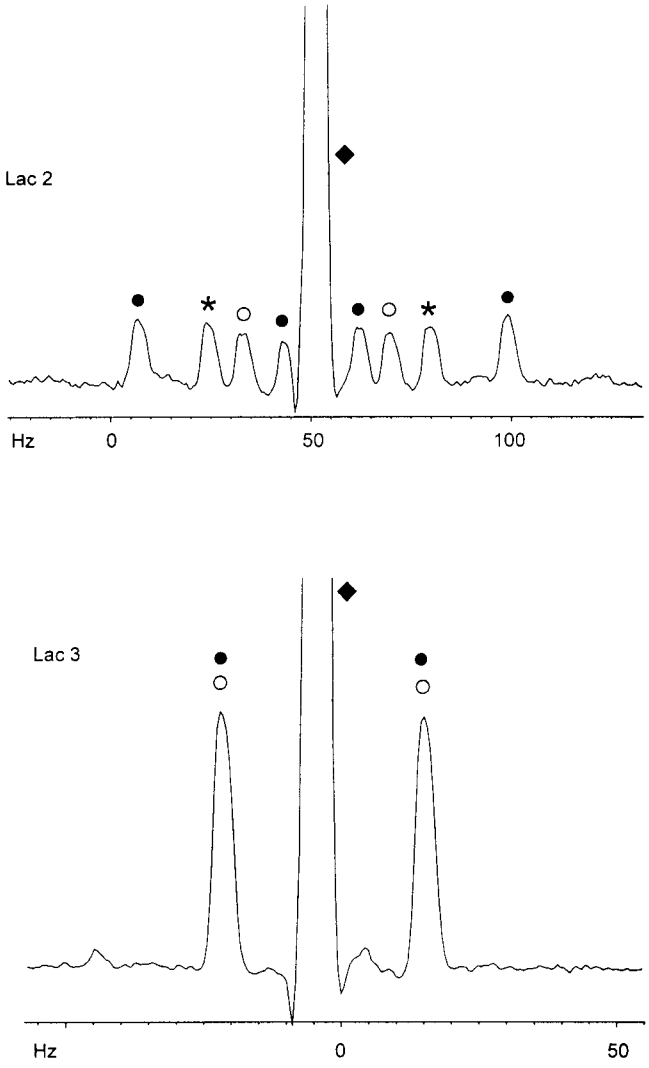

Fig. 5. Expanded plots of lactate signals in the ^{13}C-NMR spectrum (Fig. 3) generated from a cell extract derived from astroglia-rich primary cultures that where incubated for 24 h in a medium containing [1,2-^{13}C]glycine (1 mM) and glucose (5 mM). The top part shows the position of carbon 2 of lactate (Lac 2) and the lower part represents carbon 3 (Lac 3). The different isotopomers are [1,2,3-^{13}C]lactate (●), [1,2-^{13}C]lactate (*), [2,3-^{13}C]lactate (○), [2-^{13}C]lactate (◆) or [3-^{13}C]lactate (◆). The signal intensity of the monolabelled isotopomers [2-^{13}C]lactate (◆) and [3-^{13}C]lactate (◆) is much higher due to a large surplus of lactate in natural abundance derived from unlabelled precursors, in particular glucose.

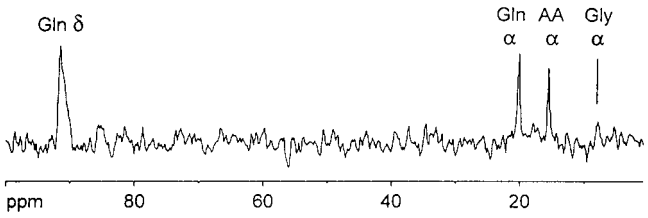

Fig. 6. ^{15}N-NMR spectrum of a cell extract from astroglia-rich primary cultures that were incubated for 24 h in a medium containing [1,2-^{13}C]glycine, [^{15}N]glycine (1 mM), and unlabelled glucose (5 mM). ^{15}N label appears in glutamine in the α position (Gln α) and δ position (Gln δ) and other α positions of amino acids (AA). The labelled glycine has almost disappeared after 24 h of incubation.

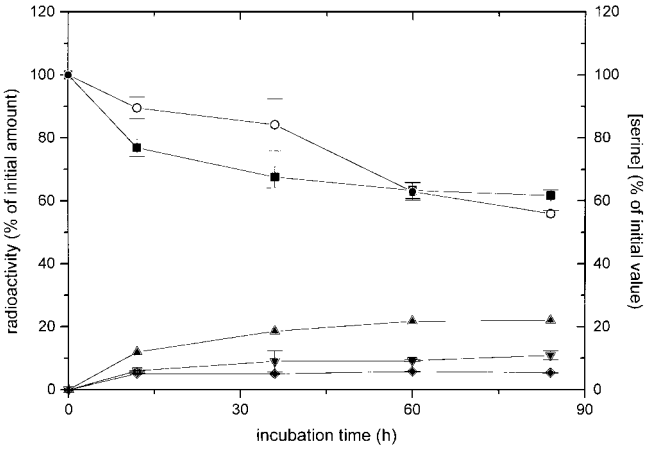

Fig. 7. Distribution as a function of incubation time of total radioactivity between the culture medium (■), the PCA-insoluble (◆), and the PCA-soluble (▼) cell content of astroglia-rich primary cultures after incubation with serine (0.37 mM, corresponding to 100%) and [U-^{14}C]serine (14 kBq, corresponding to 100%) in DMEM lacking pyruvate, inositol and other amino acids. Serine concentration (○) and net loss of radioactivity from the system (▲) are also indicated. At the start of the incubation, the cultures were 16 days old and the medium volume was 3 ml. Prior to the main experiment, the cells were preincubated for 1.5 h with 3 ml DMEM lacking pyruvate, inositol, and amino acids.

ing from serine to lactate without rearrangement of the carbon skeleton.

Double labels were also seen in creatine, guanidoacetic acid and the glycyl moiety of glutathione (Fig. 3). These doubly labelled metabolites correspond to the same monolabelled metabolites found if monolabelled glycine was used as substrate (Dringen et al., 1998).

In another NMR experiment the cells were offered 200 μM [^{15}N]glycine. The glycine nitrogen was subsequently found in glutamine (α and δ positions) and in other α amino acids, which have indistinguishable chemical shifts of their α amino nitrogens (e.g., glutamate and aspartate; Fig. 6). During glycine consumption, the cells released excessive amounts of glutamine, a vehicle for carrying excessive nitrogen into the extracellular space. Under the same conditions, but without exogenously supplied glycine, the glutamine release was much lower (data not shown). If the cells were simultaneously offered 200 μM [U-^{13}C]glycine and 200 μM [^{15}N]glycine the glycine nitrogen was consumed at higher rate than glycine carbon (cf. Figs. 5 and 8), indicating that the deamination step occurs at a rate higher than that of net glycine cleavage.

Upon addition of [U-^{14}C]glycine, labelled serine and lactate appeared simultaneously (Fig. 1B). Serine release from the cells was initially higher than lactate release (Fig. 1A). Serine radioactivity and concentration slightly declined after peaking at 45 h (Fig. 1B), whereas radioactive lactate increased continuously (Fig. 1A). The increase in lactate radioactivity continued even after the cells had essentially removed all glycine from the medium, indicating serine reuptake and metabolism to lactate by the cells. This is corroborated by

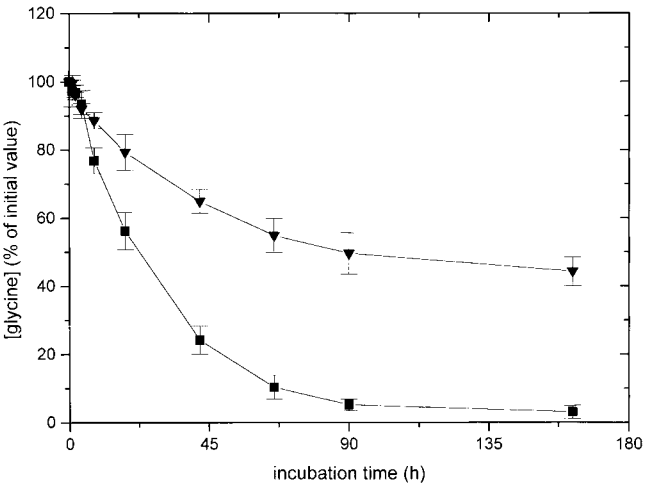

Fig. 8. Time courses of glycine disappearance from the culture medium of astroglia-rich primary cultures in the presence (▼) and absence (■) of aminopterin (100 μM). The initial glycine concentration was 0.37 mM (corresponding to 100%) and the incubation medium was DMEM without pyruvate, inositol, and other amino acids. The cultures were 19 days old at the beginning of the experiment and the medium volume was 3 ml. Before the main incubation, the cultures were preincubated for 1.5 h in DMEM devoid of amino acids, pyruvate, and inositol. In the case of incubation with aminopterin, the preincubation medium already contained 100 μM aminopterin.

the observation that also serine was taken up and metabolized to lactate by astroglia-rich primary cultures (Fig. 7). Serine radioactivity vanished from the medium substantially faster than serine mass, due to the release of unlabelled serine during the uptake of labelled serine and due to the metabolism of labelled serine. The unlabelled serine was at least partially synthesized from glucose, making use of nitrogen liberated from cell protein (S. Verleysdonk, unpublished results). During the synthesis of ^{14}C-labelled serine from labelled glycine, release of unlabelled serine from the cells was also observed (Fig. 1A). Utilization by rat astroglia-rich primary cultures was generally slower for serine than for glycine. This was not due to differences in amino acid transport, as determined by uptake studies with labelled compounds. Transport of either glycine or serine was faster than calculated from the half-lives of these amino acids in the culture medium, indicating that metabolism and not uptake was rate-limiting for removal of glycine or serine from the extracellular space (data not shown).

To establish a metabolic pathway from glycine to serine in neural tissue, homogenates of neural primary cultures and brain were assayed for their content of serine hydroxymethyltransferase and glycine cleavage system. SHMT activity was present in homogenates of rat brain as well as in astroglia-rich and neuron-rich primary cultures, although the specific activities in the homogenates of cultured cells were approximately one order of magnitude higher than the specific activities in rat brain homogenates (Table 1). The specific activity of GCS in brain homogenates was somewhat lower than in homogenates of astroglia-rich primary cultures, whereas no activity could be detected in homogenates of

*TABLE 1. Specific activities of SHMT and GCS in homogenates of whole rat brain and of rat brain cell cultures**

Enzyme	Specific activity in homogenate (mU/mg protein)		
	Rat brain	Astroglia-rich primary culture	Neuron-rich primary culture
SHMT	0.032 ± 0.002	0.24 ± 0.03	0.42 ± 0.03
GCS	1.47 ± 0.180	1.89 ± 0.19	n.d.

*The data for each homogenate are those of one representative experiment of the two (astroglia and neuron homogenates) or three (brain homogenate) experiments carried out. The data are presented as specific activities ± SD calculated from the straight line that depicted the relationship between product formed in the enzymatically catalyzed reaction and the time of incubation; n.d., not detectable.

neuron-rich primary cultures. In the case of SHMT, a rat brain homogenate was also separated in a mitochondrial and a cytosolic fraction and the specific enzyme activity was determined in each fraction. Activity was largely confined to the mitochondrial fraction. Correction for contaminating mitochondrial material revealed that the cytosol did not contain SHMT activity (data not shown).

To gain evidence for the involvement of SHMT in the process of glycine breakdown, the rate of glycine consumption by astroglia-rich primary cultures was monitored in the presence of the dihydrofolate reductase inhibitor aminopterin. A reduction in glycine utilization was observed (Fig. 8). Glycine consumption was negligible when L-serine was present in tenfold excess (4 mM). In contrast, at the same excess concentration, alanine only slightly influenced glycine consumption during an incubation period of 20 h, which corresponds to the biological half-life of glycine in the absence of any other addition (data not shown).

DISCUSSION

It is shown here that astroglia-rich primary cultures from rat brain take up glycine from their culture medium and metabolize it to serine and lactate. Both products are subsequently released into the culture medium. Glycine is eliminated from the medium at a rate comparable to the rates for the elimination of branched-chain amino acids (Bixel and Hamprecht, 1995; Yudkoff et al., 1994) and the removal is almost complete. An initial glycine consumption rate of 0.47 nmol per minute per culture dish (approximately 1 mg of protein) can be calculated. This is in accordance with the glycine consumption rates reported by Bommakanti et al. (1996) for chicken astrocytes. The exponential decrease of glycine concentration in the culture medium suggests that the rate-limiting system for the consumption of glycine has a K_M value higher than 0.4 mM, the initial glycine concentration in the medium.

Two enzymes are generally accepted to be involved in glycine metabolism, the GCS and SHMT (Fig. 9). The mitochondrial coupling of these two enzymes has previously been reported to result in the conversion of glycine to serine (Cowin et al., 1997). GCS is present in brain (Bruin et al., 1973), where it has been localized in astrocytes (Sato et al., 1991). Also SHMT is found in

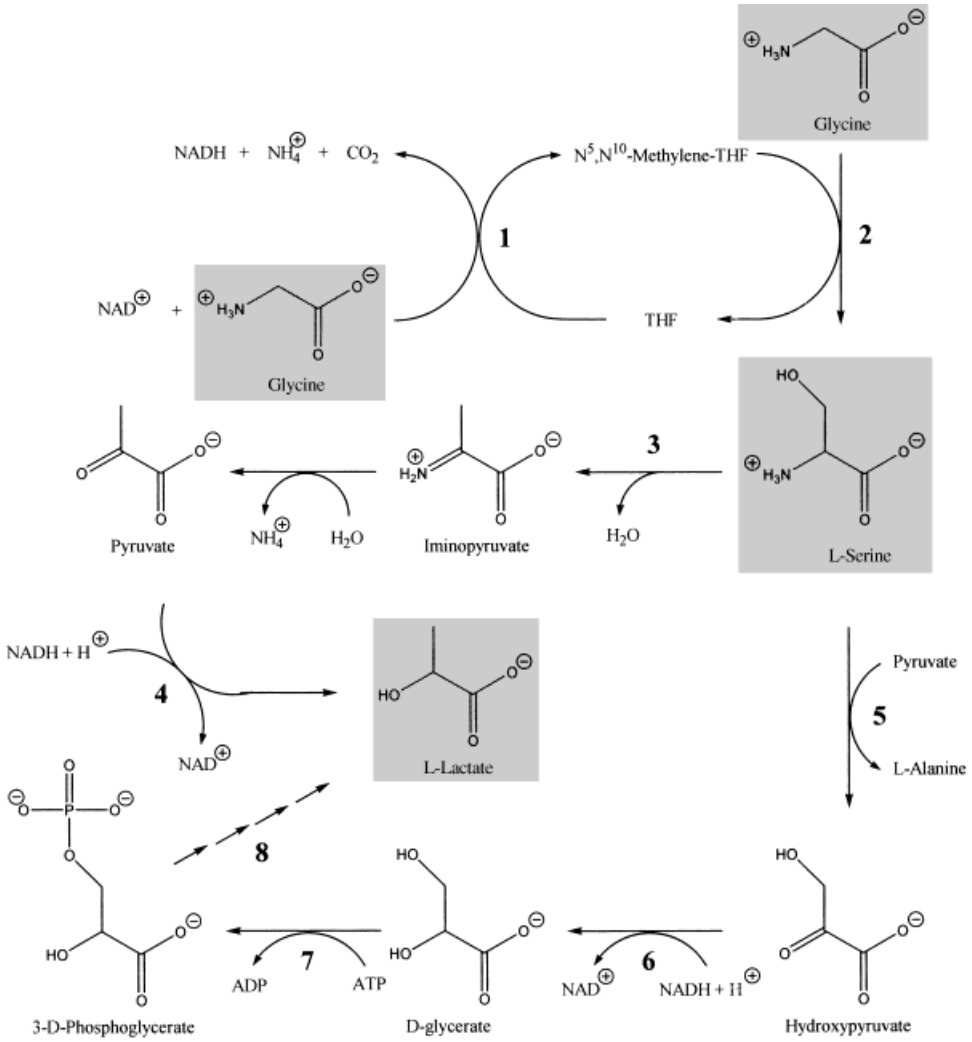

Fig. 9. Pathways of glycine metabolism. The involved enzymes are glycine cleavage system (1), serine hydroxymethyltransferase (2), serine dehydratase (3), lactate dehydrogenase (4), serine:pyruvate aminotransferase (5), hydroxypyruvate reductase (6), glycerate kinase (7), and four steps of the glycolytic pathway (8). The educt glycine and the two products lactate and serine are shaded.

brain (Daly and Aprison, 1974; Rassin and Gaull, 1975). We have detected both enzymatic activities in astroglia-rich primary cultures and therefore propose that glycine is degraded by the cultured cells in the way that one molecule of glycine is cleaved by the GCS, yielding one C1 unit in the form of N^5,N^{10}-methylenetetrahydrofolate. In the second step, the C1 unit is transferred to another molecule of glycine to form serine (Fig. 9), which is substantiated by the ^{13}C labelling experiment The predominantly observed triply labelled [1,2,3-^{13}C]serine (Fig. 3) is formed if an already ^{13}C-labelled methylene group is transferred from N^5,N^{10}-methylenetetrahydrofolate to a C1,2-labelled glycine molecule. As at the onset of the incubation the cells contain residual unlabelled glycine, doubly labelled [1,2-^{13}C]serine is formed to a smaller extent as well. Correspondingly monolabelled [3-^{13}C]serine is produced, if a ^{13}C-labelled methylene group is transferred onto unlabelled glycine. In astroglia-rich primary cultures from rat, the principal candidate for the rate-limiting step of this process is SHMT, which is confirmed by the presence of [2,3-^{13}C]serine. This isotopomer is formed if a labelled and an unlabelled C1 unit combine fast and reversibly in a preceding equilibration step to form [2-^{13}C]glycine. This reacts again with a ^{13}C-labelled methylene group transferred from N^5,N^{10}-methylenetetrahydrofolate to yield [2,3-^{13}C]serine. The latter appeared in approximately equal intensity as [1,2-^{13}C]serine, which means that the GCS reaction has to be faster than the SHMT reaction. The low specific SHMT activity of only 0.24 mU/mg protein contrasts with the considerably higher specific GCS activity of 1.89 mU/mg protein. The reported K_M values of GCS for glycine vary between 3.4 and 40 mM (Hayasaka et al., 1980; Hiraga and Kikuchi, 1980a,b; Fujiwara and Motokawa, 1983). This means that with increasing concentrations of glycine, the activity of the GCS begins to exceed the maximal rate of SHMT at an intracellular glycine concentration of 6

mM. This is less than the 11.5 mM initially found in the presence of 0.4 mM extracellular glycine. The K_M value of 46 μM for glycine, albeit for a bacterial SHMT (Miyazaki et al., 1987), would ensure that the enzyme were saturated in the cells, because the intracellular glycine concentration was found to be about 4 mM even in the absence of extracellular glycine. In the case of rat brain, the differences in specific activities between SHMT and the GCS are such that it is even more unlikely that the rate of the reaction catalyzed by SHMT could ever exceed that of the GCS-catalyzed reaction.

As shown by the labelling experiments with [U-^{14}C]glycine and [U-^{13}C]glycine, part of the serine produced from glycine appeared in the culture medium. This reflects the ability of astroglial cells to release the serine synthesized from the glycine applied. A similar mechanism of net serine production and release concomitant with a net consumption of glycine has previously been suggested to occur in fetal lamb hepatocytes (Narkewicz et al., 1996; Thureen et al., 1995). The biological half-life of radioactively labelled glycine is shorter than that of glycine mass. Therefore, apparently both glycine synthesis and release occur under the conditions used. Glycine synthesis, from unknown sources, has been observed in renal proximal tubule cells (Cowin et al.,1996) and, from serine, in astroglia-rich primary cultures (Dringen and Hamprecht, 1996).

Glycine is not expected to be readily released from astroglial cells, because 1) it is a neuroactive substance the extracellular concentration of which must be tightly controlled and 2) astroglial cells possess an active glycine-specific high-affinity uptake system that is sodium and chloride ion-dependent (Holopainen and Kontro, 1989). Thus, because of the sodium concentration in DMEM of 160 mM, glycine release via the uptake system by cells cultured in this medium would require a very high concentration of intracellular glycine. However, potassium-stimulated glycine release from preloaded astrocytes has been observed in the presence of extracellular sodium (Holopainen and Kontro, 1989), showing that a high extracellular concentration of sodium is not necessarily prohibitive for glycine release. Since the glycine transporter GLYT1 is electrogenic (López-Corcuera et al., 1991), depolarization of the cells might be a prerequisite for glycine release. In this context it is noteworthy that exogenous serine does not lead to a noticeable release of glycine into the medium. This may, however, be explained by the fact that serine (4 mM) in the culture medium does not increase the intracellular glycine concentration (S. Verleysdonk, unpublished results), although serine can be converted into glycine intracellularly (Dringen and Hamprecht, 1996) and both GCS and SHMT catalyze fully reversible reactions. For brain the release of serine from astrocytes might suggest that neighboring cells could take up and use this amino acid for the synthesis of protein or for other purposes.

The observation that glycine nitrogen is likely to be removed from the medium faster than glycine carbon can be explained by either of the following mechanisms: 1) The GCS-catalyzed reaction is much faster than the SHMT-catalyzed reaction and reversible. A ^{15}N-labelled molecule of glycine is cleaved by the GCS to yield N^5,N^{10}-methylenetetrahydrofolate, NADH and $^{15}NH_4^+$. The reverse reaction then occurs with unlabelled NH_4^+. 2) A glycine aminotransferase (Thompson and Richardson, 1967) converts glycine to glyoxylate and back to glycine, thereby equilibrating the labelled amino group with the unlabelled glutamate pool of amino groups. Further investigations in this area would constitute a separate research project to be undertaken in the future.

Although presently the regulation of glycine metabolism is poorly understood, it is obvious that astroglial cells prevent the uncontrolled release of the glycine that they have effectively taken up from the extracellular space by their specific active transport system. Astroglial cells must be considered essential for the removal of this neuroactive compound from the vicinity of susceptible structures, just as they are known to be essential for termination of glutamatergic signal transmission. As in the case of glutamate, astroglial cells not only take up the neuroactive amino acid but they also release a nonneuroactive metabolite, L-serine. It is conceivable that L-serine of astroglial origin is taken up again into neurons to serve as a precursor for the generation of glycine. This would constitute a "glycine-serine cycle" in analogy to the well-established glutamate-glutamine cycle in brain (Berl et al., 1977; Hamberger et al., 1977; Van den Berg et al., 1978; Westergaard et al., 1995; Sibson et al., 1997). The minimum requirements for this cycle are the presence of SHMT in neurons that use the serine for glycine synthesis, and a mode of disposal of the C1 fragment generated. Indeed we have detected SHMT in neuron-rich primary cultures from rat brain at considerable activity.

Serine, however, is not the sole product of glycine degradation by astroglia cells in culture. Lactate is also produced in significant amounts without any further rearrangement of the C3 carbon unit of serine. This immediately excludes a pathway from glycine to lactate via aminoacetone (Ando and Nyhan, 1969; Fubara et al., 1986). Two alternative pathways are compatible with the fact that the isotopomeric patterns of serine and lactate do not differ. One candidate enzyme that might be involved after the transformation of glycine into serine is serine dehydratase, although it should be kept in mind that its mRNA could not be detected in brain (Ogawa et al., 1991). Alternatively, the serine formed from glycine could enter the "transamination pathway" (Sallach, 1956; Ichihara and Greenberg, 1957; Willis and Sallach, 1962) that comprises enzymes 5–8 in Figure 9. Lactate, in turn, has already been reported to serve as fuel for the generation of energy in neurons (Dringen et al., 1993; Tsacopoulos and Magistretti, 1996).

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