Cerebral Metabolic Compartmentation

ESTIMATION OF GLUCOSE FLUX VIA PYRUVATE CARBOXYLASE/PYRUVATE DEHYDROGENASE BY $^{13}$C NMR ISOTOPOMER ANALYSIS OF D-[U-13C]GLUCOSE METABOLITES*

Aviva Lapidot‡ and Asher Gopher

From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

A method is presented for determining the compartmentation of amino acid metabolism in the brain. $^{13}$C NMR spectroscopy, and more specifically, homonuclear $^{13}$C-$^{13}$C spin coupling patterns of $^{13}$C-labeled amino acids were used to measure the relative flux of label from D-[U-$^{13}$C]glucose through the anaplerotic pathway versus the oxidative pathway. Glucose flux through the pyruvate carboxylase pathway was quantitated following primed dose constant infusion of D-[U-$^{13}$C]glucose to young rabbits at a rate of 1 mg/kg body weight per min. We demonstrate, for the first time, that multiplet spectra of three adjacent $^{13}$C isotomers in 1,2,3-$^{13}$C, glutamine and glutamate, which are derived from [1,2,3-$^{13}$C]pyruvate, present different isotopomer populations in glutamine in comparison to that in glutamate. This is due to two different metabolic compartments characterized by the presence or absence of glutamine synthetase activity and two different tricarboxylic acid cycles, one preferentially mediated by pyruvate carboxylase and the other by pyruvate dehydrogenase.

Our results indicate that the anaplerotic pathway accounts for 34% of glutamate synthesis and only 16% of glutamate and $\gamma$-aminobutyric acid syntheses in metabolic and isotopic steady state conditions. These results support the concept, and provide a quantitative measure, that glutamine and/or tricarboxylic acid cycle intermediates are supplied by astrocytes to neurons to replenish the neurotransmitter pool of $\gamma$-aminobutyric acid and glutamate.

It is well known that glucose is the main organic source for brain energy and an efficient precursor of glutamate and $\gamma$-aminobutyric acid (GABA). The flow of glucose carbons to the neurotransmitter glutamate and the turnover time of the glutamate pool depend on both the rate of glycolysis and rate of entry of glucose carbons into the tricarboxylic acid cycle (Garfinkel, 1966). The maintenance of glycolysis depends entirely on glucose reserve and to some extent on glycogen storage. Glutamate metabolism occurs in at least two morphologically distinct cellular compartments (Berl and Clark, 1969). It is now known that two key anabolic enzymes in the brain, glutamate synthetase and pyruvate carboxylase, reside in the glial cells and may reflect the involvement of astrocytes in replenishing and regulating the neurotransmitter pools of glutamate and GABA.

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‡ To whom correspondence and reprint requests should be addressed. Tel: 972-8-343413; Fax: 972-8-344142.

The abbreviations used are: GABA, $\gamma$-aminobutyric acid; NAA, N-acetyl aspartate.

EXPERIMENTAL PROCEDURES

Materials—D-[U-$^{13}$C]Glucose (99%) was prepared in our laboratory from algae grown on $^{13}$CO$_2$ (>99% enriched; Lapidot and Kahana, 1986). Ion exchange resins were from Fluka AG. All other reagents were of analytical grade.

Animal and Glucose Administration Procedures—Young, male albino rabbits, 40–45 days old (1.5–1.8 kg), were fasted 14–15 h (overnight) prior to each experiment. Each rabbit received an isotonic solution (0.3...
ml/kg body weight/min) of [U-13C]glucose (99% enriched) dissolved in sterile saline solution as primed dose constant infusion into the marginal ear vein at a range of 0.2 to 1.2 mg/kg/min for 10 to 70 min. The primed dose was 25% of the total amount of [U-13C]glucose given throughout the study. Control animals were infused with unlabeled n-glucose dissolved in sterile saline solution at 1.0 mg/kg/min for 60 min, following the primed dose of unlabeled n-glucose. A total of 16 animals were studied, 10 with [U-13C]glucose and 6 with n-glucose. Blood samples were taken from the contralateral ear vein at several time intervals during [U-13C]glucose infusion for blood glucose levels and 13C enrichment measurements. The rabbits were fasted prior to glucose infusion to minimize variability in blood glucose levels and incorporation of the 13C-labeled glucose. All animal use procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by our local animal care committee.

**Brain Extracts and Blood Samples Preparation for 13C Measurements—**Animals were anesthetized by a 30 mg/kg body weight intravenous dose of sodium pentobarbital (Nembutal) 30 s prior to the end of [U-13C]glucose infusion. The cranium of each animal was opened by scissors dissection, and the brain was removed and rapidly frozen in liquid nitrogen. The frozen brain (~7 g) was pulsed in a metal mortar and pestle under liquid nitrogen, and the brain powder was immediately transferred to a centrifuge tube with 1.5 ml of chilled perchloric acid (6%) and homogenized with a polytron homogenizer for 20 s. The extracts were centrifuged at 4000 rpm for 10 min at 2000 rpm. The supernatants were neutralized to pH 7.5 with K2HPO4 (28%) and left for 10 min on ice before centrifugation to remove KClO4 precipitate. Cerebral glycogen was removed from the extract by adding 2 volumes of ethanol and centrifuged for 10 min at 2000 rpm. Brain extract was finally concentrated under reduced pressure by Speed Vac concentrator to ~1 ml, more KClO4 was removed, and the supernatant was evaporated to dryness to remove traces of ethanol and was then dissolved in 1 ml of deionized water for 13C NMR measurement. Amino acid concentrations of brain extract were measured by amino acid analyzer (Dionex D-500).

Brain content of glutamate, glutamine, GABA, aspartate, and other b-chain metabolites were not affected by the glucose infusion protocol. The 13C NMR spectra of other isotopomers of glutamate are produced by glutamate dehydrogenase, and the other isotopomers of glutamine can be aminated to [4,5-13C2]glutamine by glutamine synthetase or deaminated to [1,2-13C2]GABA by glutamate decarboxylase. As a result of randomization of the reversible part of the tricarboxylic acid cycle, two more glutamate (or glutamine) isotopomers, 3-13C and 1,2-13C2, will be obtained from the second turn of the tricarboxylic acid cycle. The corresponding isotopomers of glutamine should be 3-13C and 1,2-13C2.

**RESULTS**

**High Resolution 13C NMR Spectroscopy of Natural Abundance and 13C-labeled Rabbit Brain Extracts—**The 13C spectrum (125.76 MHz) of neutralized brain extract obtained from two control rabbits infused with nonlabeled glucose, is shown in Fig. 2A. The most prominent signals arise from inositol carbons in the region of 70–75 ppm. Less intense resonances, corresponding to glutamate, glutamine, GABA, N-acetyl aspartate, aspartate, lactate, and taurine, are clearly resolved as shown in Fig 2A. The 13C NMR spectra taken from nonlabeled brains were remarkably similar. Well resolved resonances of N-acetyl aspartate (NAA) C-3 from GABA C-4 are presented in the expanded view of Fig. 2A. The well resolved resonances were used to estimate relative pool sizes of glutamate, glutamine, GABA, and aspartate (Table 1). Results similar to those derived from 15N NMR: 9.2, 5.1, 1.5, and 1.0 ppm.
and 2.1 μmol/100 g of protein for glutamate, glutamine, GABA, and aspartate, respectively, measured by an amino acid analyzer, are in the range of 7.8–12.5, 2.1–5.6, 0.8–2.3, and 1.5–2.7 μmol/100 g of cerebral protein, presented by others (McIlwain and Bachelard, 1985). The well resolved natural abundance $^{13}$C resonances in Fig. 2A allowed us to estimate the $^{13}$C enrichments of each labeled carbon in Fig. 2B (Table II).

The high resolution spectrum of a neutralized extract of rabbit brain infused with $\text{n-}[U-^{13}\text{C}]$glucose (99% enriched) at a rate of 1.1 mg/kg/min for 60 min is depicted in Fig. 2B. The carbon resonances of glutamate, glutamine, GABA, aspartate, alanine, and lactate display multiplet structures in contrast to singlet resonances of the natural abundance carbon resonances of inositol, NAA, creatine, taurine, and other small signals from natural abundance metabolites. GABA C-4 is clearly resolved from the nonlabeled NAA C-3 resonance as is presented in Fig. 2A. The significant difference in the relative signal intensities of NAA C-3 and GABA C-4 of this spectrum (Fig. 2B), in com-
**FIG. 2.** Proton-decoupled $^{13}$C NMR spectrum (125.78 MHz) of rabbit brain extracts. A, brain extracts obtained from two rabbits after infusion of nonlabeled $\mu$-glucose (1 mg/kg/min for 60 min). The spectrum consists of 4000 accumulations at 2-s repetition time and presents the region of 10 to 80 ppm. The extended view in the region 40.1–40.5 ppm indicates spectral resolution of NAA C-3 (40.44 ppm) from GABA C-4 (40.11 ppm). Most natural abundance $^{13}$C resonances were assigned. B, brain extract of one animal after infusion of $\nu$-U-1$^{13}$C-glucose (99% enriched) at a rate of 1.1 mg/kg/min for 60 min. The expanded view of the region 40.1–40.5 ppm corresponds to the $^{13}$C natural abundance resonance of NAA C-3 (at 40.44 ppm) and to the $^{13}$C-enriched GABA C-4 (at 40.11 ppm). In most cases, the $^{13}$C-labeled resonances appear as multiplets. The $^{13}$C NMR peaks include the following: Ala, alanine; Lac, lactate; NAA, N-acetyl aspartic acid; GABA, y-aminobutyric acid; Gln, glutamine; Glu, glutamate; Asp, aspartate; Tau, taurine; Cr, creatine and inositol carbons.

**TABLE I**

$^{13}$C enrichments of Glu(C-4), Gln(C-4), GABA(C-2), and Asp(C-2), derived from brains of rabbits infused with $\nu$-U-1$^{13}$C-glucose and from rabbits infused with nonlabeled glucose, in comparison with $^{13}$C NMR doublet/singlet peak area ratios reflecting their $^{13}$C enrichments.

<table>
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<tr>
<th>Experiment</th>
<th>Plasma glucose-C-1 d/s</th>
<th>Inositol C-4, C-6</th>
<th>Glutamate C-4</th>
<th>Glutamine C-4</th>
<th>GABA C-4</th>
<th>Aspartate C-2</th>
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<td>Nonlabeled glucose</td>
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<td>R.I.</td>
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<td>0.44</td>
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<td>5.7</td>
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<td>0.34</td>
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Results are the average of six nonlabeled glucose experiments. Relative brain amino acid concentrations were derived from the relative $^{13}$C NMR peak areas of the amino acids to that of inositol C-4, C-6 in the nonlabeled glucose experiments.

**Comparison to Fig. 2A**, is a direct result of the $^{13}$C enrichments of GABA carbons, including GABA C-4.

**Measurement of Isotopic Steady State Condition of Brain Metabolites**—In order to derive useful biochemical information from the relative population of the isotopomers of brain glutamate, glutamine, and GABA, analysis of cerebral compartmentation was obtained from experiments carried out at isotopic steady state conditions. Most of the results are presented for infusion rates at −1 mg/kg/min for 60 min. Spectra taken after 15 min of [U-1$^{13}$C]glucose infusion at the rate of 1 mg/kg/min (not shown) revealed a praisotopical steady state condition of plasma glucose and low $^{13}$C enrichments of glutamate and glutamine, as observed from the $^{13}$C NMR multiplet resonances—doublet/singlet (d/s) values of glutamate and glutamine C-4 and GABA C-2. Their resonance intensities increased with time of infusion and achieved a steady state condition after 30 min of infusion (Fig. 3A). Five rabbits were studied during infusion rates of 1 mg/kg body weight/min for 15 to 70 min, to estimate plasma glucose and cerebral glutamate, glutamine, and GABA isotopic steady state conditions. Plasma glucose concentration was not elevated throughout the study. Isotopic steady state condition of plasma glucose was achieved after 25–30 min of [U-1$^{13}$C]glucose infusion (Fig. 3A). The $^{13}$C enrichment of plasma glucose C-1 was estimated from its $^{13}$C NMR spectra. The resolved $\beta$-glucose C-1 multiplet resonances of glucose plasma spectra permit measurements of the fractional doublet peak area. The doublet/singlet peak areas (d/s) ratio of glucose C-1 reflect its $^{13}$C enrichment as was shown previously (Kalderon et
al., 1989b; Gopher et al., 1990a). The fractional enrichment of infusion rates in the range of 0.2-1.2 mg/kg/min for 60 min. enrichments of glutamate and glutamine C-4 and GABA C-2 reflect their 13C enrichments. 13C enrichments in the range of 1-6 atom% excess were found for glutamate, glutamine C-4, and GABA C-2, as a function of [U-13C]glucose infusion rates in the range of 0.2-1.2 mg/kg/min for ~60 min. Our results reveal that at isotopic steady state conditions, the isotopic steady state condition of glutamate and GABA C-2 was achieved after 30 min of glucose infusion (Fig. 3A), while the isotopic steady state condition for glutamate C-3 took 40 to 50 min, as revealed from the 13C NMR peak ratios of glutamate C-3/C-4 as a function of time of [U-13C]glucose infusion (Fig. 3B). The 13C enrichment of glutamate C-3 is only 55% of that of C-4. This value remained unchanged even at an infusion rate of [U-13C]glucose (1 mg/kg/min for 70 min) (Fig. 3B, Tables I and II). Glutamine C-4 enrichment is about 70% of that of glutamate C-4 (Fig. 3A), the ratio of glutamine C-3/C-4 is 0.75 even at 70 min of [U-13C]glucose infusion, indicating different routes for glutamate and glutamine syntheses in the two compartments.

**Measurements of 13C Enrichments of Brain Amino Acids as a Function of [U-13C]Glucose Infusion Rates—13C enrichments of brain amino acids at different [U-13C]glucose infusion rates were carried out on seven rabbits. A linear increase of 13C enrichments of glutamate and glutamine C-4 and GABA C-2 was obtained as a function of increased rates of [U-13C]glucose infusion (0.9-1.2 mg/kg/min) (Fig. 4), whereas cerebral amino acid concentrations remained constant (determined by an amino acid analyzer).

Despite the low 13C enrichments of glucose metabolites, the resolved multiplet resonances of glutamate, glutamine C-4, and GABA C-2 enabled us to measure the peak area ratios of d/s of these carbons. The d/s ratios of glutamate and glutamine C-4 and GABA C-2 reflect their 13C enrichments. 13C enrichments in the range of 1-6 atom% excess were found for glutamate, glutamine C-4, and GABA C-2, as a function of [U-13C]glucose infusion rates in the range of 0.2-1.2 mg/kg/min for ~60 min. Our results reveal that at isotopic steady state conditions, the 13C enrichments of glutamate C-4 and GABA C-2 are 1.4-fold higher than glutamine C-4 (Fig. 3A, Table I).

**Measurements of Individual Carbon 13C Enrichments of Glutamate, Glutamine, GABA, and Aspartate—The relative intensities of individual glutamate, glutamine, GABA, and aspartate carbons were derived from six experiments of 13C-enriched rabbit brain extracts (Table II). For the calculation of 13C enrichment presented in this study (e.g. Tables I and II), the peak areas were normalized to account for partial saturation and differences in NOE enhancement by comparing peak areas to standard solutions of glutamate, glutamine, GABA, and aspartate. The most intense peaks of glutamate and glutamine are C-4 and C-5. The 13C enrichments of glutamate and glutamine C-1, C-2, and C-3 are similarly distributed, and their 13C enrichments are 55-60% and 70% of glutamate and glutamine C-4 (or C-5), respectively. The most intense peaks of GABA are C-1 and C-2. GABA C-3 and C-4 are similarly 13C-labeled and their enrichments are 60% of that of GABA C-1 or C-2. Aspartate carbon enrichments are similarly distributed in the four carbons.

In the present study, the center peaks of glutamate and glutamine C-4 and GABA C-2 arise at best mainly from the non-enriched molecules, and the doublet resonances arise from glutamate and glutamine C-4 coupled to C-5, and from GABA C-2 coupled to C-1. Thus, the d/s ratio of glutamate and glutamine C-4 and GABA C-2 are the reflection of their enrichments (Table I). In each experiment, the 13C enrichment of glutamate C-4 is about 70% of the corresponding plasma glucose 13C enrichment.

The d/s values of glutamate, glutamine C-4, and GABA C-2 were compared with the 13C enrichments calculated from 13C
NMR peak area ratios of labeled/nonlabeled glucose experiments. The results derived from the peak area ratios were about 0.3% higher than the d/s (1.1%) values of glutamate, glutamine C-4, and GABA C-2 (Table I). The slight 13C enrichments of the singlet resonances may arise from plasma [13C]glucose recycling in the liver prior to its transport to the brain (Kalderon et al., 1986, 1989). The results were further verified by comparing the singlet resonances of C-4 glutamate and glutamine and GABA C-2 versus inositol C-4, C-6 resonances. As seen in Table III, the ratio of the singlet resonances of glutamate C-4, derived from the ratio of labeled glucose/nonlabeled glucose experiments (0.59/0.44), is 1.3, similar values are presented for glutamine C-4 (0.31/0.23), 1.3, and for the singlet peak areas of GABA C-2 (0.8/0.06), 1.3.

From the d/s values and from the relative peak area values, it is clearly shown that the 13C enrichment of glutamate C-4 and C-5 (Table I) is approximately 70% of that of glutamate, while the 13C enrichment of GABA C-2 is the same as glutamate C-4. GABA C-3 enrichment is very close to that of glutamate C-3 (Tables I and II), indicating that GABA is not derived from the same pool where glutamine synthesis takes place.

**Contribution of the Oxidative Pathway as Revealed by the Isotopomer Populations of Glutamate, Glutamine [13C]4, and GABA [13C]4**—The expanded views of the 13C NMR multiplet resonances of glutamate, glutamine, GABA, and aspartate carbons (Fig. 2B) are depicted in Fig. 5. The multiplet resonances of glutamate and glutamine in comparison to the nonlabeled glutamate, and aspartate, based on the 13C NMR
**TABLE IV**

Isotopomer populations (%) of individual carbons in glutamate, glutamine, GABA, and aspartate derived from U-13C-glucose. Analysis of the multiplet spectra observed for glutamate, glutamine, GABA, and aspartate at different carbon resonances permits quantitation of the two metabolic pathways of glucose associated with pyruvate dehydrogenase or pyruvate carboxylase activities.

<table>
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<tr>
<th>Carbon</th>
<th>Isotopomer populations</th>
<th>Multiplicity</th>
<th>Glutamate</th>
<th>Glutamine</th>
<th>GABA</th>
<th>Aspartate</th>
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<tr>
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<td>1,2-13C_2</td>
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<td>52</td>
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As seen in Table IV, the isotopomer population of glutamate or glutamine arises only when [U-13C]glucose is metabolized via the anaerobic pathway, where pyruvate carboxylase and glutamine synthetase reside. Our results indicate that the population of this isotopomer is significantly higher in glutamine (16%) than in glutamate (9%) (Table IV). Similarly, the isotopomer population of glutamine is significantly higher in glutamine (33%) than in glutamate (24%) (Table IV). The quartet resonance was not detected at lower infusion rates of [U-13C]glucose, since the probability of recombination declined with the decrease of [13C]glucose enrichment. The major isotopomer populations of 4,5-13C_4 of glutamate and glutamine and 1,2-13C_2 of GABA indicate that [1,2-13C_2]acetyl-CoA is the only precursor of these carbons (as presented in Fig. 1). The isotopomer distribution of glutamate C-2, 1,2-13C_2/1,2-13C_3 is 4.25, similar to the isotopomer ratio of glutamate C-4, 4,5-13C_4/4,5-13C_3, which is 4.5, or to glutamate C-5 ratio 4,5-13C_5/4,5-13C_4, 4.3, but markedly different from glutamine C-4 isotopomer ratio of 4,5-13C_4/4,5-13C_3, which is only 2.84. Thus, the isotopomer populations of [4,5-13C_4]glutamate and [1,2-13C_2]GABA reflect glucose flux mediated by pyruvate dehydrogenase activity.

**Fig. 6. Schematic presentation of isotopomer populations of glutamate and glutamine C-4 and GABA C-2.** Glutamate and glutamine C-4 coupled to either one doublet resonance or two adjacent 13C carbons; [4,5-13C_4]glutamate with J_2 = 51.0 Hz; and glutamine with J_1,2 = 49.5 Hz; [1,2,3-13C_3]glutamate and glutamine with J_1,3 = 34.5 Hz. GABA C-2 coupled to either one or two adjacent 13C carbons: [1,2,3-13C_3]gaba; [4,5-13C_4]gaba; [4,5-13C_3]gaba; [4,5-13C_2]gaba; [4,5-13C_1]gaba. Otherwise, isotopomer populations presented in Table IV were obtained from the highest rate of [U-13C]glucose infusion used in this study, 1.2 mg/kg/min; thus, as a result of the relatively "high" infusion rate, a small isotopomer population of 3,4,5-13C_3 could be resolved. The contribution from the quartet resonances to the total peak areas of glutamate (or glutamine) C-4 were not more than 6% and 3%, respectively (Table IV). The quartet resonance was not detected at lower infusion rates of [U-13C]glucose, since the probability of recombination declined with the decrease of [13C]glucose enrichment. The major isotopomer populations of 4,5-13C_4 of glutamate and glutamine and 1,2-13C_2 of GABA indicate that [1,2-13C_2]acetyl-CoA is the only precursor of these carbons (as presented in Fig. 1). The isotopomer distribution of glutamate C-2, 1,2-13C_2/1,2-13C_3 is 4.25, similar to the isotopomer ratio of glutamate C-4, 4,5-13C_4/4,5-13C_3, which is 4.5, or to glutamate C-5 ratio 4,5-13C_5/4,5-13C_4, 4.3, but markedly different from glutamine C-4 isotopomer ratio of 4,5-13C_4/4,5-13C_3, which is only 2.84. Thus, the isotopomer populations of [4,5-13C_4]glutamate and [1,2-13C_2]GABA reflect glucose flux mediated by pyruvate dehydrogenase activity.

**Table IV**

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Isotopomer populations</th>
<th>Multiplicity</th>
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<th>Glutamine</th>
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<tr>
<td>5</td>
<td>5-13C</td>
<td>s</td>
<td>17</td>
<td>24</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4,5-13C_2</td>
<td>d</td>
<td>83</td>
<td>76</td>
<td>ND</td>
<td>4</td>
</tr>
</tbody>
</table>
The correlation of d/s of GABA C-3 to Glu and Gln C-3 and GABA C-4 to Glu and Gln C-2

<table>
<thead>
<tr>
<th></th>
<th>Glu C-3</th>
<th>Gln C-3</th>
<th>GABA C-3</th>
<th>Glu C-2</th>
<th>Gln C-2</th>
<th>GABA C-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d/s)</td>
<td>0.32 ± 0.02</td>
<td>0.53 ± 0.02</td>
<td>0.32 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>0.53 ± 0.01</td>
<td>0.22 ± 0.02</td>
</tr>
</tbody>
</table>

a The measured GABA C-4 (d/s) values are compared to the calculated values of Glu C-2 and Gln C-2. CC The multiplet patterns of Glu C-2 or Gln C-2 are used in the communication by Lapidot and Gopher (1991) to represent the relative contribution of the anaplerotic oxidative pathways to their syntheses.

RESULTS

The origin of GABA carbons—The isotopomer population predicted for GABA is also presented in the scheme of Fig. 1. We used the d/s values of glutamate and glutamine C-4 to compare with those of GABA C-2. The d/s of these carbons reflect their 13C enrichments. Our results indicate that the isotopomer population of GABA C-2 (d/s) is similar to that of glutamate C-4 (4.2 and 4.5, respectively), but significantly different from that of glutamine C-4 (2.7) (Fig. 6, Table I). Similarly, GABA C-3 d/s values are as those of glutamate (0.35) and markedly different from glutamine C-3 (0.53). The results presented in Table V reveal identical values for GABA C-4 and calculated d/s glutamate C-2, which are ~2.5-fold lower than that of glutamine. These data, and the 13C enrichment of GABA C-2, when compared to glutamate and glutamine C-4 (Table I and Fig. 6), indicate that all GABA carbons are directly derived from glutamate.

Estimation of the Anaplerotic Pathway in Comparison with the Oxidative Pathway—The ratio of the isotopomer populations of glutamate and glutamine at carbon positions C-2/C-4 (or C-3/C-4): 2,3-13C2/1,2,3-13C3, 4,5-13C2/3,4-13C3, and 3,4-13C3 are the main value, 16% of glutamate and GABA synthesis under metabolic and isotopic steady state conditions (Table VI). The relative contribution of the anaplerotic pathway to GABA synthesis was calculated from GABA isotopomer ratio C-4/C-2. The ratio of the isotopomers 3,4-13C2/1,2,3-13C3 gave the same value, 16%, as that of glutamate (using the corresponding carbons isotopomers), which is expected from the similarity of the corresponding isotopomer populations. These results quantitatively support the concept that astrocytes supplied glutamate to neurons to replenish the neurotransmitter pool of GABA and glutamate.

DISCUSSION

The results presented in this study relate to the nature of metabolic compartmentation in the brain, as well as to metabolite transport between astrocytes and neurons. The strategy used in this study has been to administer low concentrations of [U-13C]glucose to young rabbits and to analyze brain extracts. Our studies show an almost identical labeling pattern for GABA and glutamate carbons, indicating that they are probably labeled in the same metabolic compartment. In contrast, the labeling pattern of glutamine is quite different, indicating that a lesser fraction of glutamine is formed via pyruvate de-
hydrogenase relative to pyruvate carboxylase emphasizing the quantitation of the two pathways.

Compartmentation of GABA Synthesis—The metabolic pathway for the synthesis of GABA from [1-13C]glucose was studied by Brainard et al. (1989) in the rat brain by 13C NMR. But little, if any, 13C from glucose was incorporated into GABA via a pathway in which pyruvate was converted to acetyl-CoA. The authors suggested that GABA is synthesized via an anaplerotic pathway, and the acetyl-CoA required by this pathway is derived from an unlabeled pool of glucose. Unfortunately, this conclusion was derived from poorly 13C-labeled brain amino acids, low sensitivity, and resolution of 13C NMR spectra. The infusion protocol used in our study enables the detection by 13C NMR of all 13C-labeled amino acids and lactate carbons. The NMR resonances of GABA C-4 and N-acetyl aspartate C-3 presented in our study, are resolved, while these resonances coincided in the study of Brainard et al. (1989), and it was considered to be the most intense resonance of GABA carbons. Comparison of the 13C labeling pattern of GABA carbons to the corresponding precursor carbons of glutamate in our study permits the conclusion of whether the label arose as a result of oxidative decarboxylation of [1,2,3-13C]pyruvate by pyruvate dehydrogenase activity or via carboxylation by pyruvate carboxylase (Fig. 1). The similar 13C labeling patterns observed for glutamate and GABA indicate that the large pool of glutamate compartment, localized with the neurons and lacking glutamate synthetase, does serve as the precursor of GABA. Comparison of resonance areas, which are not completely resolved and originated from significantly different pool sizes (e.g. cerebral glutamate level is 7-fold higher than that of GABA), may result in some ambiguous data (e.g. those of Brainard et al. (1989); in contrast, the isotopomer analysis used in our study for cerebral [1-13C]glucose metabolism, and [1,2-13C]lactate metabolism by Cerdan et al. (1990), is a unique approach for unraveling metabolic compartmentation by quantitation isotopomer population ratios of the labeled carbons of interest originating from different metabolites.

Shank et al. (1993) reported that at 15 min post-intraperitoneal single-dose injection suggest that the anaplerotic pathway did not contribute to GABA synthesis. Yet, at longer time periods, an appreciable contribution by this pathway was noted, ~10%, significantly higher than that in glutamate (~3%), but lower than that in glutamine (~25%). Since the data presented in our studies were obtained during metabolic and isotopic steady state conditions, the contribution of the anaplerotic pathway to glutamate and GABA synthesis was the same (16%), which is significantly lower than that in glutamine (34%). Glutamine is undoubtedly formed in astrocytes from glutamate and used as a glutamate precursor in neurons, but this is not the only pathway of glutamate synthesis. The isotopomer distribution of glutamate, found in this study, reflects mainly the isotopomer population of the tricarboxylic acid cycle intermediate α-oxoglutarate (Fig. 1) formed via the oxidative pathway (Tables IV and V). Thus, the labeling pattern of glutamate metabolites, such as GABA, should reflect glutamate isotopomer population at the compartment of its production and not glutamine carbons which equilibrate within the large pool of glutamate carbons after deamination. Our results are consistent with previous results, that pyruvate carboxylase, the primary anaplerotic enzyme, is exclusively located in astrocytes (Hertz and Shousboe, 1988), and most anaplerotic activity must occur in these cells.

The Relative Glucose Fluxes via Pyruvate Carboxylase/ Pyruvate Dehydrogenase as Reflected by Glutamate and Glutamine Compartmentation—[U-13C]Glucose is metabolized into pyruvate labeled in 3-carbon positions, which subsequently enter the pool through two different pathways, mediated by pyruvate dehydrogenase and pyruvate carboxylase activities (Fig. 1). The probability of condensation of two labeled metabolites, oxalacetate with acetyl-CoA, is negligible at a low rate of glucose infusion. We demonstrate, for the first time, that multiplet spectra of three adjacent 13C isotope in [1,2,3-13C]glutamine (and [1,2,3-13C]glutamate, which are derived from [1,2,3-13C]pyruvate, present different isotopomer populations in glutamine in comparison to that in glutamate. This is due to two different metabolic compartments characterized by the presence or absence of glutamine synthetase activity and two different tricarboxylic acid cycles, one preferentially mediated by pyruvate carboxylase and the other by pyruvate dehydrogenase. The labeling patterns and 13C isotopomer populations of glutamate and GABA carbons indicate that these amino acids were preferentially synthesized in the compartment where glucose is metabolized to acetyl-CoA by pyruvate dehydrogenase for entry into the tricarboxylic acid cycle. In contrast, the labeling pattern and 13C isotopomer population of glutamate indicate that this amino acid is preferentially synthesized in the compartment where glucose is metabolized to pyruvate followed by carboxylation to oxalacetate by pyruvate carboxylase. Our results show that even at isotopic steady state conditions, the enrichment of glutamate C-4/C-3 is U0.5. The source of dilution is mainly from the nonlabeled oxalacetate pool and other nonlabeled tricarboxylic acid cycle intermediates, or amino acids entering the tricarboxylic acid cycle.

Based on previous findings that glutamine synthetase is localized exclusively in astrocytes, and pyruvate carboxylase is exclusively active in astrocytes, glutamine isotopomer population determined in this study can be used as a measure of pyruvate carboxylase activity in astrocytes. The relative glucose fluxes via pyruvate carboxylase and pyruvate dehydrogenase activities are reflected by the ratio of the isotopomer populations of glutamate and glutamine C-3/C-4 or C-2/C-4 (Table VI). The ratio of these isotopomer populations is 2.7-fold higher.
in glutamine than in glutamate. The net synthesis of glutamate from glutamine via the pyruvate carboxylase pathway is demonstrated in this study. This synthesis occurs in the glial compartment and is reflected by glutamine isotopomer population. The anaplerotic pathway contributed 34% to glutamine synthesis, and only 12–16% to the large pool of glutamate and its product GABA.

In a recent study on [1-\(^13\)C]glucose metabolism in rats, Shank et al. (1993) reported that the anaplerotic pathway contributed very little (<3%) to glutamine synthesis. Their explanation was that the concentration of glutamate in astrocytes is so small that the enrichment in glutamate C-2 relative to C-3 in these cells was nearly obscured by the much larger amount of glutamate labeled via the oxidative pathway in neurons. It seems that the approach presented by Shank et al. (1993) is still an inexact method, as the resonances used cannot be exclusively attributed to anaplerotic activity. The isotopomer population approach used in our studies revealed a significantly higher value (~5-fold) for the contribution of the anaplerotic pathway to glutamate and 50% more to glutamine than that reported by Shank et al. (1993).

To estimate pyruvate carboxylase activity in neuronal and glial cell cultures and tumor cell lines, Brand et al. (1992) used pyruvate labeled in either C-2 or C-3 positions. The authors found ratios of pyruvate carboxylase/pyruvate dehydrogenase of 46% in primary glial and 28% in primary neurons. The pyruvate carboxylase/pyruvate dehydrogenase ratio was calculated from the sum of labeling in glutamate C-2, C-3 and aspartate C-2, C-3 versus glutamate C-5 only. Although glutamate C-1 and aspartate C-1 and C-4 are also labeled by the pyruvate dehydrogenase activity, they were not taken into account.

Thus, the method used by Brand et al. (1992) to calculate the pyruvate carboxylase/pyruvate dehydrogenase ratio may have contributed to an overestimation of the results.

The quantitative approach used in our study for metabolic compartmentation is related to metabolic trafficking between astrocytes and neurons, the flux of glutamate and GABA from the neurons to the astrocytes and the flux of glutamate (and a tricarboxylic acid cycle intermediates) from astrocytes to neurons. Although the pool of glutamate localized in the neurons is larger (80% of brain glutamate) than its precursor, the brain glutamine, which is located within the astrocytes, consists of about 40% of glutamate molecules derived from their precursor glutamine. In spite of the small pool of glutamate/glutamine in astrocytes, the rapid turnover of this pool can supply its carbon skeleton to the large neuronal pool.

It is believed that in the brain glutamine synthetase detoxifies excess ammonia, resulting in an increase in brain glutamine concentration. Therefore, it was of interest to investigate whether pyruvate carboxylase activity is induced to replenish the neurotransmitter pool. We have found (Gopher and Lapidot, 1991; Lapidot and Gopher, 1992) an increase in pyruvate carboxylase activity in rabbit brain under hyperammonemic conditions, presumably related to increased glutamine synthesis. The contribution of pyruvate carboxylase activity to glutamate synthesis increased from 34% (control) to 52% in hyperammonemic rabbits, while its contribution to glutamine synthesis increased from 12% (control) to 24% in hyperammonemic rabbits.

No Evidence for the Existence of Cerebral Pyruvate Recycling—It was suggested by Cerdan et al. (1996) in a study of [1,2,\(^13\)C]lactate metabolism in rat brain that the pyruvate recycling system is associated with the tricarboxylic acid of the neuronal compartment. This conclusion was based on the detection of an isotopomer of glutamate labeled either in C-4 or

\(^2\) A. Lapidot and A. Gopher, manuscript in preparation.

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In summary, our results indicate that the \(^13\)C isotopomer of cerebral [U-\(^13\)C]glucose metabolites can be used to determine a quantitatively metabolic event. The isotopomer populations of amino acid neurotransmitters reflect the activity of pyruvate dehydrogenase and pyruvate carboxylase associated with two tricarboxylic acid cycles localized in the neurons and astrocytes, respectively.

Quantitation of cerebral pyruvate carboxylase activity in rabbits based on \(^13\)C isotopomer populations in glutamate, glutamine, GABA, and aspartate originating from one uniformed \(^13\)C-labeled precursor, glucose, the main source for brain metabolism, is presented here for the first time. The interconversion of glutamate to glutamine and GABA in the different cerebral compartments was determined by \(^13\)C NMR isotopomer analyses of the various metabolites. GABA was found to be synthesized from glutamate in the oxidative compartment while the glutamine carbon isotopomer population reflects the net production of glutamate from the tricarboxylic acid cycle intermediate by the activity of pyruvate carboxylase in the synthetic compartment. The strategy for probing pyruvate carboxylase flux under normal physiological conditions, described in this study, is being used for investigating the effect of metabolic rearrangement of the brain on pyruvate carboxylase activity.

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