Detection of the *in vivo* Conversion of 2-Pyrrolidinone to γ-Aminobutyric Acid in Mouse Brain†

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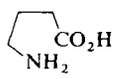
Labeled γ-aminobutyric acid was detected in mouse brain following intravenous injections of deuterium labeled 2-pyrrolidinone. [$^2H_6$]Pyrrolidinone was prepared by the reduction of [$^2H_4$]succinimide with lithium aluminum deuteride. Quantification was accomplished by a gas chromatography mass spectrometry assay method. γ-Aminobutyric acid and internal standard, 5-aminovaleric acid, were converted to volatile derivatives by treatment with *N,N*-dimethylformamide dimethyl acetal. Quantitative estimates were derived from peak area measurements obtained from monitoring the parent ions of the γ-aminobutyric acid and internal standard derivatives by repetitive scanning during the GC run. The conversion of pyrrolidinone to γ-aminobutyric acid may provide a method for labeling central γ-aminobutyric acid pools.

INTRODUCTION

γ-Aminobutyric acid (GABA, **1**) is thought to be an important inhibitory neurotransmitter in the vertebrate central nervous system.[1,2] Systemically administered GABA has been shown to be effectively excluded from the CNS by the blood brain barrier.[3,4] 2-Pyrrolidinone (**2**), the lactam of GABA, is more lipophilic than GABA and penetrates readily into the CNS.[5] Although pyrrolidinone has been reported to exhibit no direct synaptic activity when applied to exposed cortex,[6] in concept hydrolysis of pyrrolidinone in the brain would produce the active inhibitory substance GABA. Tower has shown that incubation of [^{14}C]pyrrolidinone with cat cortical slices results in the enzymatic formation of [^{14}C]GABA.[7] The absorption of pyrrolidinone into the



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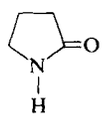

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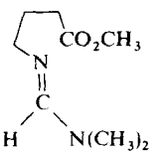

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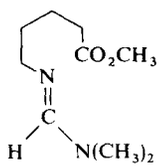

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brain and the finding that brain slices have the capacity to hydrolyze pyrrolidinone to GABA suggest that pyrrolidinone might serve as a pro-drug[8] or precursor of GABA. However, attempts to increase whole brain GABA concentrations with single large doses of pyrrolidinone have been unsuccessful.[9] The observation that pyrrolidinone appears to be a natural constituent in mouse brain[10] has led us to study in more detail the possibility of a relationship between pyrrolidinone and the functioning GABA-system. In this communication, evidence is presented which suggests that intravenously administered pyrrolidinone is converted to GABA in mouse brain.

† GABA = γ-aminobutyric acid; AVA = 5-aminovaleric acid; DMF-DMA = *N,N*-dimethylformamide dimethyl acetal.

EXPERIMENTAL

All reagents were obtained from Aldrich Chemical Co. unless specified otherwise and were used as received.

[3,3,4,4-$^2H_4$]Succinimide

Succinimide (8 g) deuterium oxide (99% deuterium, 8 ml) and anhydrous pyridine (1 ml) were sealed in a glass ampule and heated for 30 days at 85 °C. Every 5 days during that period the solvents were replaced. The solvents were then removed and the residue was stirred for 15 min with hot acetonitrile (50 ml) and filtered. The residue contained mainly [3,3,4,4-$^2H_4$]succinamic acid. The filtrate was evaporated to leave a white solid which was recrystallized from EtOH to give 1.5 g of labeled succinimide, m.p. 120–122 °C. Isotopic incorporation was 83% $^2H_4$ and 15% $^2H_3$. Unlabeled succinimide was less than 0.1% of [$^2H_4$]succinimide.

2-[3,3,4,4,5,5-$^2H_6$]Pyrrolidinone

To a mixture of [$^2H_4$]succinimide (1.8 g) dissolved in 100 ml of dry tetrahydrofuran (distilled from $LiAlH_4$) was cautiously added lithium aluminum deuteride (0.6 g, Merck, Sharp & Dohme, isotopic purity 99%). After heating the mixture to reflux for 75 min, water (1 ml) was carefully added to terminate the reaction. Salts were removed by filtration and the filtrate was

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reduced to dryness by distillation. The residue was fractionally distilled in a short path still to yield labeled pyrrolidinone: 145 mg, b.p. 84 °C per 0.7 mm. After back exchange of the N–H with H_2O, the product showed isotopic purity of 75% $^2H_6$, 21% $^2H_5$ and less than 0.1% unlabeled material.

4-[2,2,3,3,4,4-$^2H_6$]Aminobutyric acid

A solution of [$^2H_6$]pyrrolidinone (20 mg) in 10 ml of 20% deuterium chloride in deuterium oxide was held at reflux for 3 h. The volatile solvents were removed under vacuum and the residue purified by ion exchange chromatography (Dowex 50) as described previously.[10] Isotopic purity: 77% $^2H_6$, 15% $^2H_5$ and less than 0.1% unlabeled material.

Preparation of derivatives of GABA and 5-aminovaleric acid

Dry samples of GABA and 5-aminovaleric acid (AVA) were treated with 50 μl of a solution of *N,N*-dimethylformamide dimethyl acetal (DMF-DMA) in acetonitrile (2:1, v/v) and the mixtures heated gently with a heat gun for about 1 min during which time the residue dissolved in the reagent. Aliquots (2–3 μl) of the derivatives, methyl 4-(*N,N*-dimethyl-*N'*-formamidino)-butanoate (**3**) and methyl 5-(*N,N*-dimethyl-*N'*-formamidino)pentanoate (**4**) in the reagent were analyzed directly by GCMS. Standard curves were generated for varying amounts (0–100 nmol) of GABA or deuterium enriched GABA and standard AVA (50 nmol). An example of such a standard curve is seen in Fig. 1. A plot of the ratio of the peak area of the parent ions of **3** and **4** versus concentration of GABA yielded a linear relationship.

Preparation of samples

Male ICR mice (25–30 g) were injected in the tail vein with [$^2H_6$]pyrrolidinone (200 mg kg^{-1}) in water (0.1 ml) or [$^2H_6$]GABA (200 mg kg^{-1}) in water (0.1 ml). After 30 min, the animals were decapitated and the brains immediately removed. The brains were homogenized in 5% trichloroacetic acid (2 ml) containing standard and the mixture centrifuged at 12 000 **g** for 6 min. The supernatant was separated into GABA and pyrrolidinone fractions by ion exchange chromatography (Dowex 50) as described previously.[10] The evaporated GABA fraction was derivatized directly with DMF-DMA. The pyrrolidinone fraction was held at reflux for 2 h with an equal volume of conc. HCl. This procedure results in the hydrolysis of pyrrolidinone to GABA. Measurement of GABA in this solution provides an estimate of the amount of pyrrolidinone in the sample.

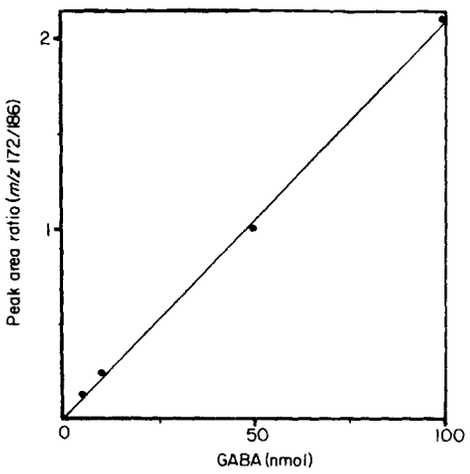

Figure 1. A typical standard curve for varying concentrations of GABA with AVA (50 nmol) as internal standard. Each point represents a mean value for four determinations of a ratio of peak areas of the parent ions of **3** and **4**. Correlation coefficient 0.995.

Gas chromatography mass spectrometry

A DuPont 21-490 mass spectrometer operating at 70 eV and source temp. 190 °C was used for all experiments. Gas chromatography was carried out on a Varian 1400 gas chromatograph interfaced with the mass spectrometer through a heated glass jet separator. GC conditions were: 3% OV-17 on Chromosorb W.H.P., 2 m × 3 mm glass column; helium carrier gas, 30 ml min^{-1}; injector and detector 270 °C; column 120–270 °C programmed at 15 °C min^{-1}; and separator temp. 210 °C. A DuPont-094 data system was used for data acquisition and processing. Data from repetitive scans during each GC run were collected at a rate of one scan each 4 s over a mass range of 219 to 51. Each GC peak was scanned 5 to 7 times. Quantification was accomplished from a ratio of the areas of the reconstructed ion current profiles for the parent ions of the compounds of interest.[11] Approximation of areas were determined by summing the ion intensities of *m/z* 172 for the GABA derivative **3** (*m/z* 178 for [$^2H_6$]GABA) and *m/z* 186 for the AVA derivative **4**.

RESULTS AND DISCUSSION

Mass spectral and stable isotope labeling studies have been carried out in an effort to evaluate the possibility that 2-pyrrolidinone is converted to GABA in animals.

A GCMS assay which measures brain concentrations of GABA was developed using the GABA homologue, 5-aminovaleric acid (AVA), as internal standard. Whole brains from mice were homogenized and GABA separated by ion exchange chromatography. A convenient one step derivatization procedure for GABA and standard was carried out by reaction with *N,N*-dimethylformamide dimethyl acetal (DMF-DMA). This reagent has been used successfully by Thenot and Horning to produce volatile derivatives of amino acids for GCMS analysis.[12] The amino function is converted to a formamidine and the carboxyl group is esterified. In Fig. 2 the mass spectra of the derivative of GABA, methyl 4-(*N,N*-dimethyl-*N'*-formamidino)butanoate (**3**) and the derivative of AVA, methyl 5-(*N,N*-dimethyl-*N'*-formamidino)pentanoate (**4**) are shown. These derivatives form readily, are well resolved by GC, and exhibit mass spectra with prominent parent ions suitable for selected ion measurements. Quantification of GABA

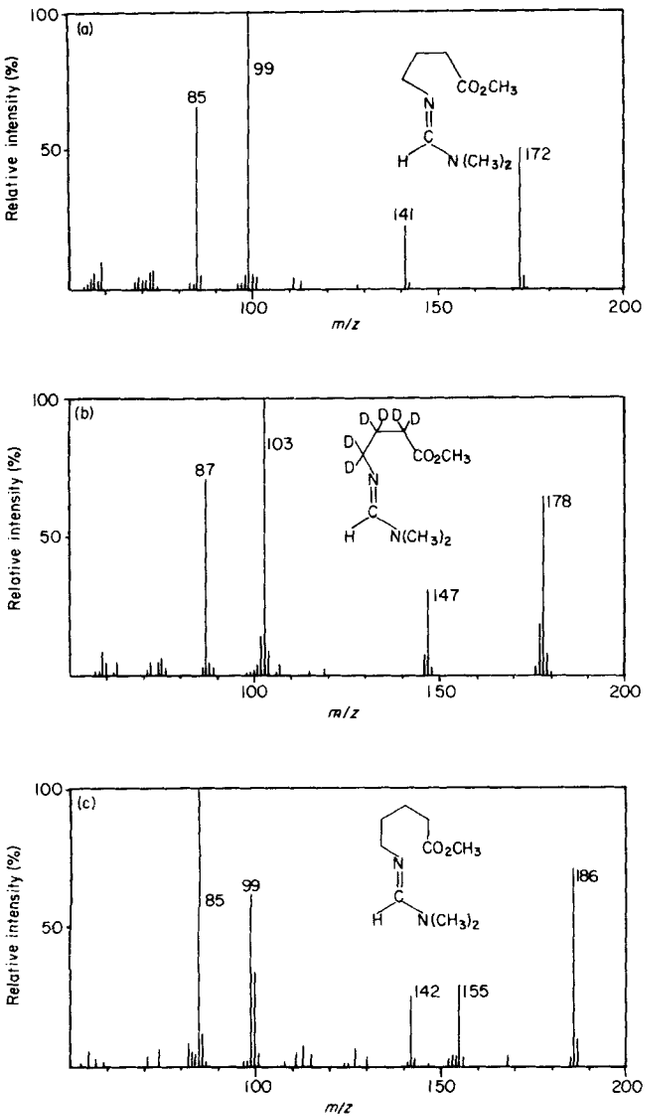

Figure 2. Mass spectra of (a) methyl 4-(*N,N*-dimethyl-*N'*-formamidino)butanoate (**3**), (b) [2,2,3,3,4,4-$^2H_6$]methyl-4-(*N,N*-dimethyl-*N'*-formamidino)butanoate and (c) methyl-5-(*N,N*-dimethyl-*N'*-formamidino)pentanoate (**4**).

was accomplished by the comparison of the peak areas of GABA/AVA (*m/z* 172/186) from brain and blood samples with values from a standard curve made with pure compounds and found to be 2.26 ± 0.10 μmol per g wet tissue (mean of six mice ± SEM). This value is in agreement with published GABA concentrations (2.27 ± 0.16 μmol per g) determined in mice sacrificed by decapitation.[13]

The deuterium labeled analog, [4,4-$^2H_2$]GABA[10], and the GABA homologue, AVA, were both found to serve as satisfactory internal standards for the concentrations of GABA measured in our studies. These results are in agreement with similar studies carried out by Bertilsson and Costa with [2,2-$^2H_2$]GABA and AVA as standards for a GCMS assay for GABA.[14] Since the present studies involved the administration of deuterated compounds and the measurement of deuterated and deuterium free compounds in the same sample, the homolog AVA was chosen as internal standard for quantitative analyses.

Recovery of GABA and AVA from brain samples was found to be essentially complete. Recovery of brain GABA was determined by dividing mouse brains into two equal parts. To one part was added internal standard, to the other internal standard plus a known amount of GABA. By this method, recovery was calculated to be $96.1 \pm 4.7\%$ (mean of 3 animals ± SEM). In a separate experiment, mouse brains were divided into two equal parts and to one half, standard AVA was added at the homogenization step while to the other half, AVA was added at the end of the work-up procedure immediately prior to derivatization. This experiment yielded a recovery of GABA of $98.4 \pm 1.3\%$ (mean of 3 animals ± SEM).

Labeled pyrrolidinone was synthesized and administered to mice as a means of tracing GABA formed from pyrrolidinone in the presence of normal brain GABA levels. [$^2H_6$]Pyrrolidinone was prepared by a modified method of Duffield *et al.*[15] [$^2H_4$]Succinimide, produced by exchange with deuterium oxide in pyridine, was selectively reduced to [$^2H_6$]pyrrolidinone with lithium aluminum deuteride. Following purification by distillation, solutions of labeled pyrrolidinone were injected intravenously into mice and brain concentrations of labeled GABA were measured.

Labeled GABA was identified in mouse brain extracts 30 min after intravenous injections of [$^2H_6$]pyrrolidinone. Figure 3 shows a typical reconstructed ion current profile for four of the major ions arising from derivatized [$^2H_6$]GABA isolated from brain. The approximate ratios of ions *m/z* 178, 177, 147 and 103

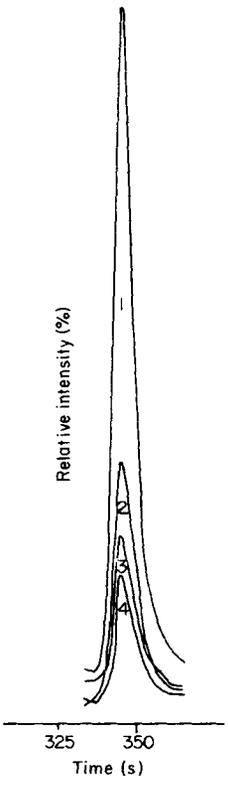

Figure 3. Mass chromatogram of the GABA fraction of a [$^2H_6$]pyrrolidinone treated mouse. Four of the major ions of the [$^2H_6$]GABA derivative, *m/z* 103, 178, 147 and 177, are represented by peaks 1 through 4, respectively. The curves were plotted from computer reconstructed ion current profiles derived from successive scanning.

Table 1. Incorporation of deuterium into brain γ-aminobutyric acid (GABA) following intravenous administration of [$^2H_6$]pyrrolidinone in mice[a]

	μmol/g whole brain
Control GABA	2.26 ± 0.10 (6)[b]
[$^2H_6$]GABA	0.017 ± 0.006 (3)
[$^2H_6$]Pyrrolidinone	1.67 ± 0.18 (3)

[a] [$^2H_6$]Pyrrolidinone (200 mg kg^{-1}) was administered into the tail vein of mice.
[b] Values represent mean ±SEM with the number of animals in parentheses.

are in close agreement with the authentic [$^2H_6$]GABA derivative (Fig. 2). An estimate of the amount of [$^2H_6$]GABA in mouse brain after injection of labeled pyrrolidinone was determined by GCMS analysis and found to be approximately 17 nmol per g wet tissue (see Table 1). About 0.7% of whole brain GABA was accounted for as labeled material. This value is near the lower limit of the sensitivity of the assay procedure carried out by the repetitive magnetic scanning method.[16] At 30 min after injection, unchanged [$^2H_6$]pyrrolidinone was found in brain tissue in a concentration 1.67 μmol per g. This observation supports the assumption that pyrrolidinone crosses into the CNS. The apparent low degree of conversion of pyrrolidinone to GABA may be explained by a slow rate of hydrolysis of pyrrolidinone by brain first observed *in vitro* by Tower[7] coupled with the reported rapid turnover rate of GABA in mammalian brain.[17]

Intravenous injection of [$^2H_6$]GABA in the same concentration and procedure as the pyrrolidinone experiments did not result in a measurable incorporation of label into central GABA stores. These results are consistent with a blood brain barrier which excludes GABA from the CNS,[3,4] and suggest that labeled GABA from [$^2H_6$]pyrrolidinone arises from hydrolysis occurring within the brain.

Detection of labeled GABA in the brain following administration of labeled pyrrolidinone supports a possible relationship between GABA and its lactam in mouse brain. Although it is too early to suggest that such a relationship is of physiological significance, hydrolysis of pyrrolidinone represents a pathway leading to GABA that, unlike the main source of GABA, apparently does not depend on the decarboxylation of glutamic acid.

Methods for studying the dynamics of the functioning GABA-system have been sought for some time.[2] Useful methods usually rely on the labeling of GABA pools with isotopes as part of GABA turnover studies. Collins has labeled brain GABA by the administration of [^{14}C]GABA directly into the central nervous system.[18] Bertilsson *et al.*[17] have estimated GABA turnover rates by labeling GABA in brain nuclei by intravenous infusion of [^{13}C]glucose. Since labeled pyrrolidinone appears to be a source of labeled GABA in concentrations which do not appreciably alter steady state levels of GABA, the conversion of pyrrolidinone to GABA may provide a new method for labeling central stores of GABA.

Acknowledgement

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