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Stereochemical Action of Mouse Brain Glutamate Decarboxylase

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4-[4-^{2}H]Aminobutyrate was prepared by incubation in ^2H_2O of glutamate with a partially purified glutamate decarboxylase from mouse brain. The 4*R* configuration was assigned to the compound on the basis of ^{1}H nmr analysis of the ω-camphanoylamide of its methyl ester in the presence of Eu(dpm)$_3$. Moreover 4-[4(*S*)4-^{3}H,U-^{14}C]aminobutyrate was shown to be formed from [2(*S*)2-^{3}H,U-^{14}C]glutamate by the same enzyme fraction. It is therefore demonstrated that glutamate decarboxylation catalyzed by this enzyme preparation occurs with retention of configuration.

It was previously shown by us (*1*) that transamination of 4-aminobutyrate (GABA)[1] to succinic semialdehyde (SSA) by mouse brain enzymes implies the abstraction of the 4 *pro-S*-hydrogen. This conclusion was drawn on the basis of loss of the tritium label of [4(*S*)4-^{3}H,U-^{14}C]GABA in its conversion to 4-hydroxybutyric acid (GHBA), catalyzed by enzymes of mouse brain homogenate. Incubations with this homogenate of doubly labeled glutamate were also carried out (*1*), and only a partial loss of tritium was observed in the conversion of this substrate to GHBA. This finding suggested that decarboxylation of GLU by mouse brain enzymes may occur at least partially with inversion of configuration. This appeared, however, in contrast with the behavior of all other PLP-dependent decarboxylases so far tested (*2–5*). This prompted us to check the stereochemistry of GLU decarboxylation by a direct approach. For this purpose the chirality of [4-^{2}H]GABA prepared by incubation of glutamate in ^2H_2O with a partially purified preparation of mouse brain GLU decarboxylase was considered. It had been previously established (*6*) that the resonance of the *pro-R* and the *pro-S*-hydrogens at C_4 of the ω-camphanoylamide of GABA methyl ester was at 4.70 ppm (4 *pro-R*) and 5.02 ppm (4 *pro-S*) when the ^{1}H nmr spectrum of the derivative is examined in the presence of 0.5 mol eq of Eu (dpm)$_3$. Furthermore the ^{1}H nmr spectrum of the ω-camphanoylamide of the methyl ester of [4(*R*)4-^{2}H]GABA obtained from incubation in ^2H_2O of (*S*) GLU with glutamate decarboxylase from *Escherichia coli* showed only the multiplet at δ 5.02 ppm (*6*). Analysis of the derivative of [4-^{2}H]GABA prepared with the brain enzyme preparation, as

[1] Abbreviations used: GLU, glutamate; GABA, 4-aminobutyrate; SSA, succinic semialdehyde; GHBA, 4-hydroxybutyric acid; GSH, reduced glutathione; PLP, pyridoxal-5′-phosphate; GAD, glutamic acid decarboxylase; GABA-T, 4-aminobutyrate: 2-oxoglutarate aminotransferase; Eu (dpm)$_3$, Europium(III)-tris(2,2,6,6-tetromethyl-3,5-heptomedionate).

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described in the Experimental section, also exhibited a multiplet centered at δ 5.02 ppm; whereas no signal was detectable at the resonance of the 4 *pro-R*-hydrogen. This indicates for the monodeuterated compound a stereochemical purity higher than 95% and clearly demonstrates that the tested brain enzyme fraction decarboxylates GLU with retention of configuration.

Owing to the discrepancy between this conclusion and the previously reported suggestion (*1*), [4-^{3}H,U-^{14}C]GABA was also prepared from [2-^{3}H,U-^{14}C]GLU using the partially purified decarboxylase from mouse brain. The results reported in Table 1 show that 80.9% of the tritium label of this compound was lost when it was converted to GHBA by mouse brain enzymes as reported in the Experimental section. The same behavior was observed with these enzymes using as the substrate [4(*S*)4-^{3}H,U-^{14}C]GABA prepared as previously described (*1*) with purified GAD from *E. coli*. The 4*S* configuration was therefore assigned to the doubly labeled GABA prepared with brain enzymes, thus confirming the retention of configuration in its formation reaction from glutamate.

The reasons for the previously observed retention of tritium (*1*) of [2(*S*)2-^{3}H,U-^{14}C]GLU in its overall conversion to GHBA by a crude homogenate of mouse brain are unclear. Despite the accurate purification of GHBA (*7*), contamination of this compound by other labeled molecules cannot be completely excluded. 2-Pyrrolidinone was recently shown to be present in brain (*8*), and its endogenous origin was demonstrated. Such a compound may have been formed from doubly labeled GLU with the crude homogenate of mouse brain and may have followed GHBA during the purification steps.

After the present work had been concluded, a report by Bouclier *et al.* (*9*) appeared, dealing with the stereochemistry of reactions catalyzed by mammalian brain GAD and GABA-T. These authors, using highly purified enzyme preparations, confirmed our previous results (*1*) on the abstraction of the 4 *pro-S*-hydrogen of GABA in its transamination to SSA. Moreover, in agreement with our

TABLE 1

DETERMINATION OF 4-[4-^{3}H,U-^{14}C]AMINOBUTYRATE CHIRALITY BY ITS CONVERSION TO 4-HYDROXYBUTYRATE WITH BRAIN ENZYMES

Origin of [4-^{3}H,U-^{14}C]GABA used as substrate	^{3}H/^{14}C ratio in	
	Substrate	GHBA
GLU decarboxylation by *E. coli* GAD[a]	3.3	0.35
GLU decarboxylation by brain enzymes[b]	1.6	0.30

[a] As already reported (*5*, *6*) [4(*S*)4-^{3}H,U-^{14}C]GABA is formed by *E. coli* GAD.

[b] The P_{13} fraction from mouse brain homogenate (Fig. 1) was used to prepare this substrate as reported in detail in the Experimental section.

present data, they demonstrated the retention of configuration during GLU decarboxylation by brain GAD determining the tritium label release into water during incubation with the stereospecific transaminase of [4-^{3}H]GABA prepared with brain GAD either with [2-^{3}H]GLU in H_2O or with GLU in ^3H_2O.

A particular aspect concerning the inhibition of brain GAD by (*S*)4-aminohex-5-ynoic acid is also discussed by Bouclier *et al.* (*9*), who suggest that the stereospecific inhibition may be related to the ability of brain GAD to abstract the α-hydrogen of (*S*) GLU like an aminotransferase. According to these authors this reaction was not in evidence under their experimental conditions due to the too rapid decarboxylation of GLU by the purified brain GAD. As a matter of fact we reported in our previous paper (*1*) and found in the present research that [4-^{3}H,U-^{14}C]GABA formed by mouse brain enzyme preparations shows a ^{3}H/^{14}C ratio lower than that of [2(*S*)2-^{3}H,U-^{14}C]GLU used as the substrate, indicating the abstraction of the α-hydrogen of (*S*) GLU. Correspondingly, deuterated GABA prepared as reported in the Experimental section was shown to contain a percentage of dideuterated species also attributable to the loss of the α-hydrogen of GLU.

EXPERIMENTAL

Materials. [2(*S*)U-^{14}C]GLU (270 mCi/mmol) and [2(*RS*)2-^{3}H]GLU (4.5 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, United Kingdom). (*S*) GLU, 2-oxoglutaric acid, GHBA, and glutamate decarboxylase from *E. coli* (EC 4.1.1.15) were obtained from Fluka AG (Buchs, Switzerland). Cofactors (NADPH, GSH, PLP) were from Boehringer (Mannheim, West Germany). Cation-exchange resin AG 50W-X2 (100–200 mesh) was purchased from Bio-Rad (Richmond, Calif.). ^2H_2O (99.75% ^{2}H) was from Merck (Darmstadt, West Germany).

Mouse brain homogenate fractionation. Brains from male albino mice (25–30 g body wt) were homogenized in 0.25 *M* sucrose containing 1 m*M* GSH and 0.2 m*M* PLP. Fractionation of the homogenate was carried out according to Wu *et al.* (*10*) as shown in Fig. 1. All steps were carried out at 4°C and enzymes were freshly prepared for each experiment.

Preparation of 4-[4-^{2}H]aminobutyrate. The P_{13} fraction (Fig. 1) obtained from 30 g of mouse brain was suspended in 20 ml of phosphate buffer (50 m*M*, pH 7.2) in ^2H_2O containing 1 m*M* GSH and 0.2 m*M* PLP. The suspension was then dialyzed overnight at 0–4°C against 1000 ml of 50 m*M* phosphate buffer in ^2H_2O containing 1 m*M* GSH. The dialyzed enzyme fraction (18 ml, 36 mg protein) was added with 80 mg of GLU (final concentration 20 m*M*) dissolved in 9 ml of 50 m*M* phosphate buffer (GSH, 1 m*M*) in ^2H_2O and incubated at 37°C for 4 hr under nitrogen. The reaction was ended by the addition of acetic acid and deuterated GABA was isolated and purified as previously described (*1*) by ion-exchange chromatography followed by crystallization from 50% ethanol. The compound (8 mg) was shown to be pure by glc–ms analysis of its trimethylsilyl derivative (*1*) and by silica gel tlc using 70% ethanol as the eluting solvent. Deuterium enrichment was measured by mass fragmentographic analysis of its trimethylsilyl

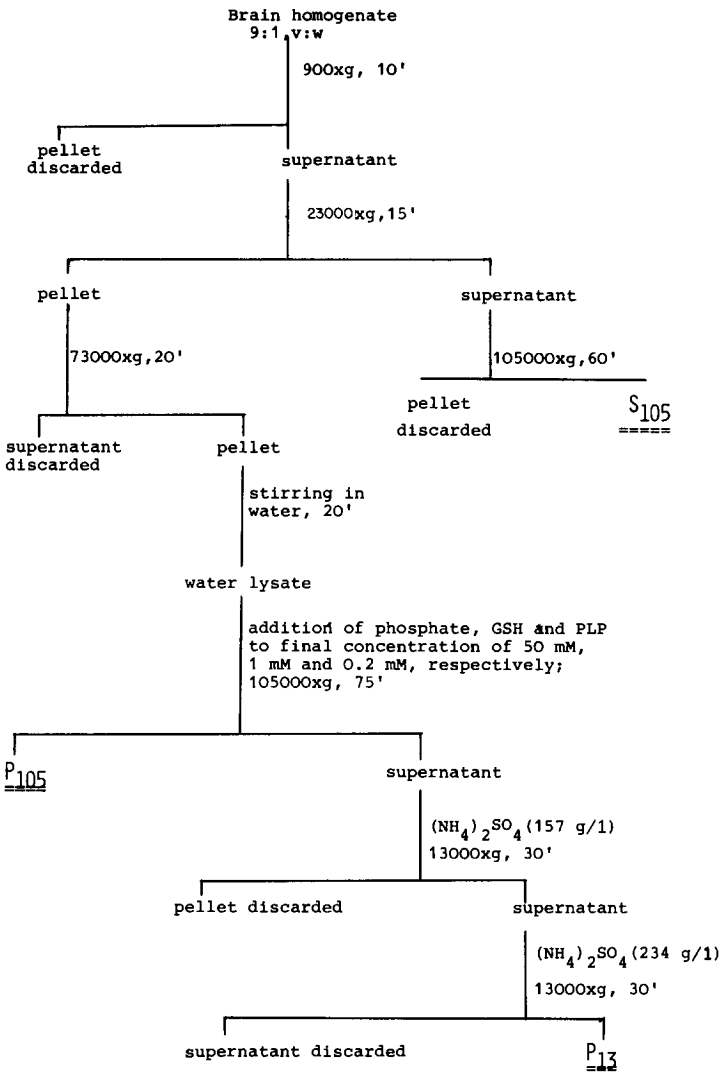

FIG. 1. Scheme of mouse brain homogenate fractionation.

derivative (*1*) focusing the ions at *m/e* 304 (M^+15 of GABA tris–trimethylsilyl derivative), 305, and 306. From the intensity of the three fragments only 1% of nondeuterated GABA was found in the sample. Monodeuterated and dideuterated species were found to be 73 and 26%, respectively.

Chirality of 4-[4-²H]aminobutyrate. (1*S*,4*R*)ω-Camphanoyl chloride (sublimed just prior to use) (16 mg, 0.074 mmol) was dissolved in toluene (0.2 ml). To this solution [4-²H]GABA obtained as described above (5 mg, 0.05 mmol) dissolved in 2 *N* sodium hydroxide (0.025 ml) and 3 *N* sodium hydroxide (0.025 ml) were added and the pH was kept above 7 by dropwise addition of 2 *N* NaOH. After 3 hr at room temperature under stirring, the mixture was washed with ether, acidified,

and extracted with ether (3 × 2 ml). Solvent evaporation gave an oily residue which was treated with diazomethane in ether to afford the methyl ester (*6*); this was purified on a silica gel column by elution with benzene–ethyl acetate (1 : 1, v : v) (yield 13 mg). The ^{1}H nmr registered in the presence of Eu(dpm)$_3$ under the conditions described previously (*6*) to establish the C_4 configuration of [4-^{2}H]GABA prepared as described above. The presence in this compound of dideuterated molecules did not affect the analysis since no signal is derived from these molecules at the chemical shift of the 4 *pro-R*- and 4 *pro-S*-hydrogens of GABA.

Preparation of 4-[4-^{3}H,U-^{14}C]aminobutyrate. The P_{13} fraction (Fig. 1) obtained from 15 g of mouse brain was suspended in 10 ml phosphate buffer and dialyzed under the conditions described above for the preparation of the deuterated GABA. The dialyzed suspension (6 ml, 15 mg protein) was incubated with 6.8 mg (5 m*M* final concentration) of [2(*RS*)2-^{3}H,U-^{14}C]GLU (^{3}H, 63.4 μCi; ^{14}C, 14.8 μCi; ^{3}H/^{14}C of 2(*S*) GLU, 2.14) dissolved in 3 ml of 50 m*M* phosphate buffer containing GSH, 1 m*M*. Incubation conditions and the following GABA purification were as described above for the preparation of [4-^{2}H]GABA. The ^{3}H/^{14}C ratio of purified [4-^{3}H,U-^{14}C]GABA (39 μmol) was shown to be 1.6.

A preparation of [4(*S*)4-^{3}H,U-^{14}C]GABA (18 μmol; ^{3}H/^{14}C, 3.3) was also carried out by incubation of [2(*RS*)2-^{3}H,U-^{14}C]GLU (^{3}H, 40 μCi; ^{14}C, 7.8 μCi; ^{3}H/^{14}C ratio of (*S*) GLU, 2.6) with purified GAD from *E. coli* under previously described conditions (*1*).

Chirality of 4-[4-^{3}H,U-^{14}C]aminobutyrate. [4-^{3}H,U-^{14}C]GABA obtained from doubly labeled GLU both with mouse brain and the bacterial enzyme were converted to GHBA using the mixture of the P_{105} and S_{105} fractions obtained from the same mouse brain homogenate (Fig. 1) as the source of GABA-T (*10*) and SSA reductase (*11*), respectively. The incubation mixture (final volume, 2 ml, pH 7.4) contained 13 mg enzyme protein, [4-^{3}H,U-^{14}C]GABA, 2 m*M*, NADPH, 1m*M*, 2-oxo-glutaric acid, 1 m*M*, GSH, 1 m*M*, PLP, 0.2 m*M*, phosphate, 50 m*M*. After shaking at 37°C for 1 hr, 2 ml of 10% TCA in 1 *N* HCl and 83 mg of sodium 4-hydroxybutyrate were added. The latter compound was converted to γ-butyrolactone which was extracted, distilled under vacuum, and converted into the phenylhydrazide of 4-hydroxybutyric acid as previously described (*7*). The derivative was crystallized four times. After each crystallization step the radioactivity of weighed amounts (3–5 mg) of the crystalline compound in 0.1 ml of methanol was determined in a Packard Model 3385 liquid scintillation counter using 5 ml of Lipoluma (Lumac, Schaesberg, Holland). Enough counts were accumulated to reach a percentage standard deviation of ±2. Counting efficiency was 70, 40, and 7% for ^{14}C, ^{3}H, and ^{14}C spillover, respectively, as determined by measuring the radioactivity of ^{14}C- and ^{3}H-labeled toluene standards prepared in the same scintillation cocktail used for the samples. Quenching was constant in all samples and standards as determined by the external standard ratio. The ^{3}H/^{14}C ratio was found to be constant throughout the four crystallization steps. The enzyme activity was found to be 6.7 nmole of GHBA formed per milligram protein as measured from the amount of ^{14}C radioactivity associated with purified GHBA.

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