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Formation of GABOB From 2-Hydroxyputrescine and Its Anticonvulsant Effect

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NAKAO, J., T. HASEGAWA, H. HASHIMOTO, T. NOTO AND T. NAKAJIMA. *Formation of GABOB from 2-hydroxyputrescine and its anticonvulsant effect.* PHARMACOL BIOCHEM BEHAV **40**(2) 359–366, 1991.—To investigate the formation of γ-amino-β-hydroxybutyric acid from 2-hydroxyputrescine in mammalian organs, the radioactive diamine was synthesized and was injected into rats intraperitoneally or intraventricularly. After intraperitoneal injection, the radioactive amino acid was detected in various organs, but formation of the stereoisomer of the amino acid (γ-amino-α-hydroxybutyric acid) was not demonstrated. Intraventricular injection of the radioactive diamine also resulted in the formation of γ-amino-β-hydroxybutyric acid in the rat brain. In vivo experiments using monoamine oxidase or diamine oxidase inhibitors suggested the participation of both enzymes in the formation of the amino acid from the diamine in rat organs other than the brain, where diamine oxidase appeared to play the major role. To investigate the anticonvulsant effect of 2-hydroxyputrescine, the threshold of pentylenetetrazol-induced generalized convulsions was measured in rats after the intraventricular injection of 2-hydroxyputrescine. Both R(−)- and S(+)-2-hydroxyputrescine had an anticonvulsant effect, with a greater elevation of the threshold being observed after injection of the R(−) form. Time course experiments suggested that this anticonvulsant effect depended on the formation of γ-amino-β-hydroxybutyric acid from 2-hydroxyputrescine in the rat brain. The anticonvulsant action of γ-amino-β-hydroxybutyric acid was also examined, and the stimulation of Cl^- influx plus the inhibition of GABA uptake into brain membrane vesicles were indicated to be involved.

2-Hydroxyputrescine GABOB Monoamine oxidase Diamine oxidase Seizure threshold

SINCE Hayashi and Nagai (10) examined the effects of ω-amino acids on the mammalian motor cortex and reported that the inhibitory action of γ-amino-β-hydroxybutyric acid (GABOB) was more potent than that of γ-aminobutyric acid (GABA) (10), interest has focused on the identification of GABOB in the central nervous system (CNS). Hayashi suggested the occurrence of this compound in the rat brain and the canine cerebrospinal fluid in 1959 (11), and Ohara et al. reported a high concentration of GABOB in the bovine temporal lobe (20), but the identification of this compound in the early studies was incomplete. Subsequent investigations using more precise methods failed to confirm the presence of GABOB in the CNS (4, 6, 16).

In 1986, we identified 2-hydroxyputrescine (2-HO-PUT) in the mammalian brain (9), and then demonstrated the formation of [^{3}H]-GABOB in the rat brain after the intraventricular injection of [^{3}H]-2-HO-PUT (18). However, the biochemical properties of the GABOB formed or the enzymes involved in its formation remained unknown.

In the present series of studies, we examined the formation of [^{3}H]-GABOB in various organs of the rat after the intraperitoneal or intraventricular injection of [^{3}H]-HO-PUT into animals treated with several kinds of enzyme inhibitors in an attempt to clarify the enzymes involved in the formation of GABOB. In addition, the effect of 2-HO-PUT on pentylenetetrazol-induced generalized seizures and its mechanism of action were investigated. On the basis of our findings, the potential clinical applications of 2-HO-PUT are discussed.

METHOD

Animals

Male Wistar rats weighing 180–200 g were used in the experiments. They were housed at 24°C with controlled lighting (lights on: 0800–2000 h) and were given food pellets and tap water ad lib.

Reagents

[^{3}H]-GABA (2.18 TBq/mmol), $Na^{36}Cl$ (111 KBq/mg) and L-[U-^{14}C]-glutamic acid were purchased from Amersham (England), and [^{3}H]-sodium borohydride (0.52 TBq/mmol) was obtained from CEA (France). Horseradish peroxidase was obtained from Toyobo Co. (Japan) and R(−)- and S(+)-GABOB were kindly supplied by Ono Pharmaceutical Co. (Japan). All other reagents used were obtained commercially.

Synthesis of 2-HO-PUT and γ-Amino-α-Hydroxybutyric Acid

2-Oxo-putrescine was reduced by sodium borohydride in 90% ethanol to form the racemic body of 2-HO-PUT using the method previously reported (18).

R(−)- and S(+)-2-HO-PUT were synthesized from L- and

D-malic acid, respectively, by the method of Kullnig et al. (14). The $[\alpha]_D^{25}$ of the synthesized R(−)-2-HO-PUT was −3.14 ±0.07.

γ-Amino-α-hydroxybutyric acid was synthesized from GABA by the method of Bouthillier et al. (3). Elementary analysis of the synthesized compound gave: C, 26.59, H, 6.68 and N, 7.77%. $C_4H_9NO_3 \cdot HCl \cdot H_2O$ required C, 27.68, H, 6.97 and N, 8.07%.

Tritium Labeling of 2-HO-PUT

[^{3}H]-(RS)-2-HO-PUT was synthesized by the reduction of 5 μg of 2-oxo-putrescine in the presence of 185 MB_q of [^{3}H]-sodium borohydride in 90% methanol (18). The solution obtained was acidified, and charged into a 0.5-ml column of Amberlite IR-120 (100–200 mesh, H^+ form). The column was washed with 5 ml of water and 1 M pyridine. [^{3}H]-(RS)-2-HO-PUT was eluted with 10 ml of 3 M NH_4OH, and evaporated to dryness. The dried residue was dissolved in 1 ml of water, charged into a 1×2 cm column of Amberlite-IR 120 (100–200 mesh, NH_4^+ form), and chromatographed with 2 M NH_4OH. Fractions of the eluate (800 μl) were collected successively, and the radioactivity of each fraction was determined with a liquid scintillation counter. The fractions containing [^{3}H]-(RS)-HO-PUT were pooled, and evaporated to dryness. The specific radioactivity of the synthesized [^{3}H]-(RS)-2-HO-PUT was 0.13 TBq/mmol.

Intraperitoneal Injection of [^{3}H]-2-HO-PUT and Preparation of Acidic and Neutral Amino Acid Fractions From Rat Organs

[^{3}H]-2-HO-PUT (370 KBq) was dissolved in 1 ml of saline, and injected intraperitoneally into rats. The rats were killed by decapitation at 3 h after injection, and the organs of interest were quickly taken out. Each organ was homogenized in 10 vol. (v/w) of 10% trichloroacetic acid (TCA), and centrifuged at 2,500×g for 15 min. The supernatant was extracted 3 times with 2 vol. of diethyl ether to remove the TCA. The portion of the aqueous layer equivalent to 200 mg of each organ was then passed through a 1-ml column of Amberlite IR-120 (100–200 mesh, H^+ form). The column was washed with 10 ml of water. Acidic and neutral amino acids were eluted with 5 ml of 1 M pyridine, and were evaporated to dryness for use in the next experiment.

Determination of Radioactivity in the Fraction Corresponding to GABOB in Rat Organs

The dried residue obtained from 0.5–1.5 g of each organ was dissolved in 40 μl of 0.1% GABOB solution. A 10-μl aliquot was subjected to high-voltage paper electrophoresis in pyridine-acetic acid-water (5:50:945, by vol., pH 3.4) at a potential gradient of 20 V/cm for 40 min. The area of the paper corresponding to GABOB was cut out, and its radioactivity was counted using a liquid scintillation counter.

Intraventricular Administration of [^{3}H]-2-HO-PUT and Determination of Radioactivity in the Fraction Corresponding to GABOB or 2-HO-PUT in the Rat Brain

A volume of 2 μl of artificial cerebrospinal fluid containing 30 μg (185 KBq) of 2-HO-PUT was injected intraventricularly into rats. After the injection, rats were killed, and their brains were taken out. The radioactivity in the GABOB fraction prepared from the acidic extract of each brain was then determined by the method described above.

[^{3}H]-2-HO-PUT was eluted from the column of Amberlite IR-120 with 10 ml of NH_4OH, and evaporated to dryness. The dried residue was dissolved in 40 μl of 0.1% 2-HO-PUT solution, and subjected to high-voltage paper electrophoresis. The radioactivity in the 2-HO-PUT fraction was then determined.

Partial Purification of Diamine Oxidase From Rat Small Intestine

Diamine oxidase (DAO, EC 1.4.3.6) was partially purified from rat small intestine by the method of Tsuji et al. (27). A tissue sample (1 g) was washed with saline, homogenized in 10 ml of 0.05 M phosphate buffer (pH 7.4), and centrifuged at 12,000×g for 30 min. The supernatant was brought to 40% saturation by adding saturated ammonium sulfate solution, and was again centrifuged at 12,000×g for 30 min. Partially purified DAO was obtained by dialysis of this supernatant solution against phosphate buffer for 8 h.

Determination of Diamine Oxidase Activity

DAO activity was determined by the method of Snyder and Hendley (25). Partially purified DAO (100 μl) was added to 2.0 ml of an assay solution (0.1 mg/ml of horseradish peroxidase and 0.125 mg/ml of homovanillic acid in 0.125 M phosphate buffer, pH 7.4), and incubated at 37°C for 10 min. Then, 500 μl of the substrate solution containing various concentrations of putrescine (PUT) or 2-HO-PUT was added, and further incubation was performed for 1 h. The enzyme reaction was terminated by the addition of 100 μl of 1 N NaOH, and the fluorescence intensity of the reaction mixture was measured at 430 nm with activation at 323 nm.

Measurement of Convulsion Threshold

The convulsion threshold of rats was measured by a modification of the method of Nutt et al. (19). Saline containing 5 mg/ml of PTZ was infused into the femoral vein via a cannula at a constant rate of 1.7 ml/min. The convulsion threshold was expressed as the total amount of PTZ required to induce generalized convulsions.

Measurement of Cl^- Influx Into Brain Membrane Vesicles

Brain membrane vesicles were prepared from a rat, and the Cl^- influx into the vesicles was measured by the method of Harris and Allan (8). The membrane vesicles obtained from 1 g of rat brain were suspended in 3.5 ml of an incubation buffer (140 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM glucose; 20 mM Tris-HEPES, pH 7.4). Then, 200 μl of the membrane suspension was incubated for 10 min at 30°C. After incubation, Cl^- influx was initiated by adding 200 μl of the incubation buffer containing 1.48 KBq of $Na^{36}Cl$ and various concentrations of R(−)- or S(+)-GABOB, R(−)- or S(+)-2-HO-PUT, or GABA. Ten seconds after addition of the buffer, the influx was halted by adding 5 ml of ice-cold incubation buffer and the mixture was rapidly filtered through a German Type A/E fiber filter. The filter was washed twice with the cold buffer, and then the radioactivity on the filter was counted.

Measurement of GABA Uptake Into Brain Membrane Vesicles

The uptake of GABA into rat brain membrane vesicles was measured by the method of Kuhar (13). Rat cerebral cortex was homogenized in 0.32 M sucrose with a glass-glass homogenizer, and then diluted 100 times (v/w) with the incubation buffer used in the Cl^- influx experiment. A volume of 800 μl of the buffer containing 3.7 KBq of [^{3}H]-GABA and various concentrations

of R(−)- or S(+)-GABOB, or R(−)- or S(+)-2-HO-PUT was incubated at 37°C. [^{3}H]-GABA uptake was initiated by adding 100 μl of the diluted homogenate, and the solution was incubated for 10 min at 37°C. Uptake was then terminated and measured by the same method described in the Cl^- influx experiment.

Determination of the Activity of L-Glutamic Acid Decarboxylase and GABA-α-Oxoglutarate Transaminase

A rat was injected intraventricularly with R(−)- or S(+)-GABOB and was killed by decapitation 3 h later. The brain was taken out and homogenized in 10 vol. of 20 mM phosphate buffer (pH 7.4), containing 0.2% Triton X-100.

The activity of L-glutamic acid decarboxylase (GAD, EC 4.1.1.15) in the brain homogenate was measured by the method of Beaven et al. (2). A 10-μl aliquot of the homogenate was added to 40 μl of the reagent mixture in a 1.5-ml reaction vial. The reagent mixture consisted of 2.66 KBq of L-[U-^{14}C]-glutamic acid, 5 mM α-ketoglutaric acid, 0.2 mM pyridoxal phosphate, 1.25 mM glutathione disulfide and 0.5 M sodium phosphate buffer (pH 6.8). The reaction vial was then placed inside a 20-ml screw-cap liquid scintillation counting vial. To trap the $^{14}CO_2$ formed, 50 μl of hyamine hydroxide was dropped onto a piece of filter paper (1 cm^2) placed at the bottom of the counting vial. The counting vial was then tightly capped and incubated at 37°C for 30 min, after which it was placed on ice and uncapped. Then 50 μl of 3 N perchloric acid was added to the reaction vial to terminate the enzyme reaction, after which the counting vial was quickly recapped and incubated again for 30 min. At the end of the second incubation, the reaction vial was removed, and 10 ml of a scintillation solution was added to the counting vial to assay of the radioactivity.

The activity of GABA-α-oxoglutarate transaminase (GABA-T, EC 2.6.1.19) in the brain homogenate was measured by the method of Schousboe et al. (23). A 10-μl aliquot of the homogenate was added to an assay mixture consisting of 3.7 KBq of [^{3}H]-GABA, 5 mM α-ketoglutaric acid, 0.5 mM succinic acid, 1 mM dithiothreitol, 0.4 mM pyridoxal phosphate, 2.5 mM NAD^+, 10 mM GABA, and 0.2 M Tris-HEPES (pH 8.6). After 30 min of incubation at 37°C, the enzyme reaction was stopped by adding 20 μl of 10% TCA. The mixture was adjusted to pH 4–5 with 0.1 N KOH, and charged into a 0.5-ml column of Amberlite IR-120 (100–200 mesh, H^+ form). The column was washed with 5 ml of water, the effluent and the washings were pooled, and the radioactivity of the pooled solution was determined.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a two-tailed Student's *t*-test.

RESULTS

Formation of GABOB From 2-HO-PUT

Identification of GABOB. Rats were killed by decapitation 3 h after an intraperitoneal injection of 370 KBq of [^{3}H]-2-HO-PUT, and their livers were quickly taken out. Acidic and neutral amino acid fractions were prepared from 6 g of liver tissue.

Ion-exchange chromatography. The fraction containing acidic and neutral amino acids was applied to a 1 × 15 cm column of Amberlite IR-120 (100–200 mesh) previously equilibrated with a mixture of pyridine-acetic acid-water (5:50:945, by vol., pH 3.4), and was developed with the same mixture. Fractions of the effluent (15 ml) were successively collected and 0.5 ml of each fraction was used for the determination of radioactivity. Authentic GABOB or γ-amino-α-hydroxybutyric acid was also chromatographed under the same experimental conditions. A portion of each fraction was evaporated to dryness, and the amino acids in the dried residue were determined by Dubin's method (5). As shown in Fig. 1, radioactivity emerged in the fractions corresponding to GABOB, but not in the fractions corresponding to γ-amino-α-hydroxybutyric acid.

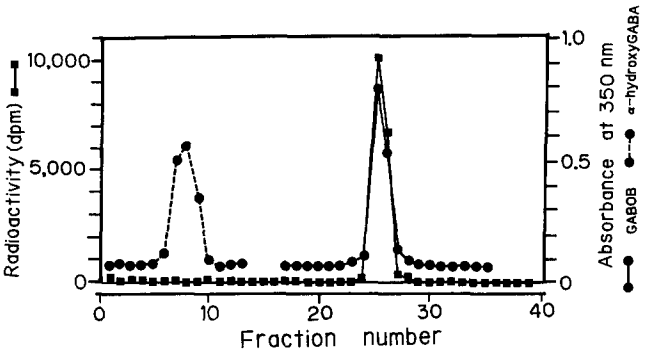

FIG. 1. Ion-exchange chromatography of the acidic and neutral amino acid fraction of rat liver, authentic GABOB, and γ-amino-α-hydroxybutyric acid. Rats were injected intraperitoneally with 370 KBq of [^{3}H]-2-HO-PUT and the fraction containing acidic and neutral amino acids was prepared from 6.0 g of rat liver. The fraction was applied to a 1.0 × 15 cm column of Amberlite IR-120 (100–200 mesh), which had been equilibrated with pyridine-acetic acid-water (5:50:945, pH 3.4). Ion-exchange chromatography was carried out with the buffer and a 0.5-ml aliquot of each 15-ml fraction of the effluent was used for the measurement of radioactivity. Authentic GABOB and γ-amino-α-hydroxybutyric acid were chromatographed under the same conditions and determined by Dubin's method (5).

High-voltage paper electrophoresis. The fractions corresponding to GABOB (No. 25 and 26 in Fig. 1) were collected and evaporated to dryness. The dried residue was then dissolved in 100 μl of 0.1% GABOB solution. A 10-μl aliquot of the solution was subjected to high-voltage paper electrophoresis in pyridine-acetic acid-water (5:50:945, pH 3.4) at a voltage gradient of 20 V/cm for 40 min. The paper was dried, and compounds were visualized with 0.2% ninhydrin in acetone. Radioactivity was extracted into 50% ethanol from 1-cm paper strips and measured. As shown in Fig. 2, the radioactivity was detected in the strip corresponding to GABOB.

Recrystallization. Fractions No. 25 and 26 obtained by ion-exchange chromatography were pooled and evaporated to dryness. The dried residue and 70 mg of authentic GABOB were dissolved in a small volume of water, and then ethanol was added dropwise to obtain crystals. A small portion of these crystals were dissolved in water to count the radioactivity. Recrystallization was repeated five times. The specific radioactivity remained almost constant during recrystallization (Table 1).

The above results indicated that the radioactive substance purified from the rat liver was GABOB, and that the stereoisomer of GABOB (γ-amino-α-hydroxybutyric acid) was not formed from [^{3}H]-2-HO-PUT under our experimental conditions.

Formation of [^{3}H]-GABOB in various rat organs.

Intraperitoneal injection of [^{3}H]-HO-PUT. Rats were intraperitoneally injected with 370 KBq of [^{3}H]-HO-PUT, and the formation of [^{3}H]-GABOB was examined in various organs 3 h later. The highest concentration of the radioactive product was formed in the liver (113,254 dpm/g). Moderate amounts were detected in the kidneys, small intestine and stomach, and a small

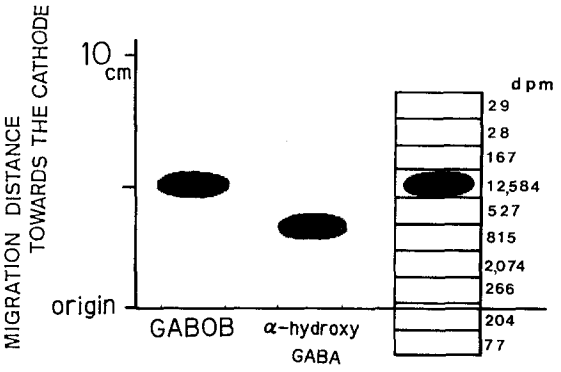

FIG. 2. Paper electrophoresis of the fractions containing [^{3}H]-GABOB from rat liver, authentic GABOB, and γ-amino-α-hydroxybutyric acid. Electrophoresis was carried out at a voltage gradient of 20 V per cm for 30 min in pyridine-acetic acid-water (5:50:945, pH 3.4). Compounds were visualized with 0.2% ninhydrin in acetone. The radioactivity of the paper strip was extracted into 50% ethanol and measured by a liquid scintillation counter.

amount of [^{3}H)-GABOB was also found in the brain.

Intraventricular injection of [^{3}H]-2-HO-PUT (Table 2). 2-HO-PUT (30 μg, 185 KBq) was injected intraventricularly, and changes in the formation of [^{3}H]-GABOB and the persistence of 2-HO-PUT in the brain were examined from 10 min to 2 weeks after the injection. The remaining 2-HO-PUT gradually declined over the course of time, while the formation of [^{3}H]-GABOB reached a maximum 12–24 h after the injection (Fig. 3).

Enzymes Involved in the Formation of GABOB From 2-HO-PUT

Diamine oxidase (EC 1.4.3.6). The formation of GABA from PUT involves the following 2 enzyme reactions.

PUT----------γ-aminobutyraldehyde ---------- GABA
oxidative deamination oxidation

This pathway suggests that DAO is involved in the formation of

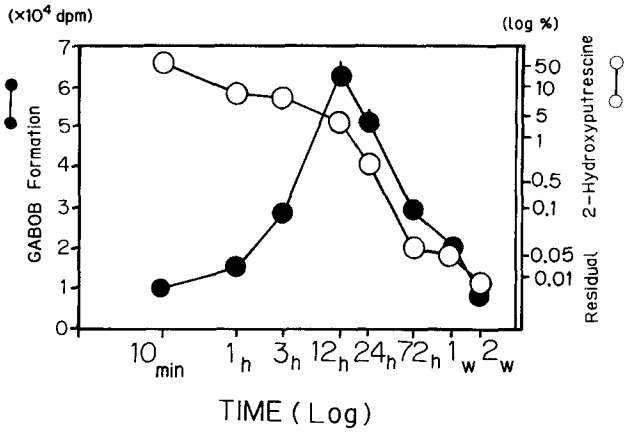

FIG. 3. Changes in the formation of [^{3}H]-GABOB and the persistence of 2-HO-PUT in the brains of rats injected intraventricularly with 30 μg of [^{3}H]-2-HO-PUT (specific radioactivity: 650 KBq/mmol). Brain [^{3}H]-GABOB and [^{3}H]-2-HO-PUT levels were determined at the indicated intervals after the injection of radioactive 2-HO-PUT. Values for [^{3}H]-GABOB formation represent the mean ± S.E.M. of 3–5 experiments. The persistence of 2-[^{3}H]-HO-PUT is represented as the percentage of total radioactivity of 2-[^{3}H]-HO-PUT in rat brain at the indicated intervals.

GABOB from 2-HO-PUT. Therefore, the substrate specificity of 2-HO-PUT for DAO was examined using partially purified DAO (Table 3). Although 2-HO-PUT was metabolized to GABOB, about a 1,000-fold higher concentration of the substrate was required to obtain the same amount of GABOB as GABA from PUT. This enzyme reaction was completely inhibited by 10^{-6} M aminoguanidine, was about 70% inhibited by the same concentration of semicarbazide, and was 10–20% inhibited by pargyline (Table 4).

Pretreatment with monoamine or diamine oxidase inhibitors. According to the report of Seiler and Al-Therib, PUT is acetylated to N-monoacetylputrescine, which is then metabolized to N-acetyl-γ-aminobutyraldehyde by monoamine oxidase (MAO,

TABLE 1

RECRYSTALLIZATION OF RADIOACTIVE GABOB PURIFIED FROM THE LIVER OF A RAT INJECTED INTRAPERITONEALLY WITH RADIOACTIVE 2-HO-PUT

Crystallization	Recovery (mg)	Amount Used (mg)	Specific Radioactivity (dpm/mg)
1	51.9	5.2	340
2	42.4	4.3	295
3	31.6	3.2	297
4	25.4	2.6	292
5	21.6	2.2	313

The fraction containing acidic and neutral amino acids was prepared from the liver, and chromatographed on an Amberlite IR-120 column. Fractions corresponding to GABOB were pooled and evaporated to dryness. The dried residue and 70 mg of authentic GABOB were dissolved in a small volume of water, and ethanol was added dropwise to obtain crystals. A small portion of the isolated GABOB was then dissolved in 0.5 ml of water, and 10 ml of the scintillation solution was added. Radioactivity was determined using the a liquid scintillation counter.

TABLE 2

FORMATION OF [^{3}H]-GABOB IN VARIOUS ORGANS OF RATS INJECTED INTRAPERITONEALLY WITH 370 KBq OF [^{3}H]-2-HO-PUT

Organ	[^{3}H]-GABOB (dpm/g wet weight)
Brain	1,295 ± 273
Heart	6,369 ± 847
Lung	9,096 ± 2,280
Liver	113,254 ± 11,975
Small Intestine	31,814 ± 8,212
Kidney	47,260 ± 8,990
Spleen	12,515 ± 2,330
Muscle	5,535 ± 1,040
Testis	1,329 ± 111
Stomach	17,181 ± 471
Blood	2,028 ± 540

Three hours after the injection, the rats were killed by decapitation and the organs were taken out to measure the radioacivity of [^{3}H]-GABOB. Values represent the mean ± S.D. of 5 experiments.

TABLE 3

FORMATION OF GABOB FROM 2-HO-PUT AND GABA FROM PUT AT VARIOUS CONCENTRATIONS OF THE SUBSTRATES BY DAO PREPARED FROM RAT SMALL INTESTINE

Concentration of Substrate (mM)	2-Hydroxyputrescine Formation of GABOB (nmol/h/mg protein)	Putrescine Formation of GABA (nmol/h/mg protein)
0.005	—	0.032
0.01	—	0.248
0.02	—	0.488
0.05	0.001	0.824
0.075	0.026	0.933
0.1	0.036	1.015
0.2	0.051	1.235
1.0	0.097	1.180
5.0	0.157	0.865

Preparation of DAO and the determinations of GABOB or GABA formation were described in the Method section.

EC 1.4.3.4). The aldehyde is further oxidized to N-acetyl-GABA, which is finally deacetylated to form GABA (24). This metabolic pathway is thought to be important in the brain, where the activity of DAO is low. To investigate the participation of this pathway in the formation of GABOB from 2-HO-PUT, rats were pretreated with intraperitoneal pargyline, and the formation of [^{3}H]-GABOB from [^{3}H]-2-HO-PUT in various organs was compared with that in other rats pretreated with aminoguanidine or semicarbazide. Pretreatment with 1 mg/kg of aminoguanidine inhibited GABOB formation by more than 80%, and pretreatment with 1 mg/kg of pargyline inhibited formation by more than 60% in the liver, $F(2,7)=75.47$, $p<0.01$ and small intestine, $F(2,7)=24.89$, $p<0.01$ (Table 5). These results suggested the participation of both MAO and DAO in the formation of GABOB in these organs. In the brain, pretreatment with 100 mg/kg of pargyline (which inhibited more than 99% of the brain MAO activity) as well as with 100 mg/kg of aminoguanidine (which is poorly transported into the brain) did not significantly inhibit the GABOB formation, while formation was inhibited 32.4% by pretreatment with 100 mg/kg of semicarbazide, $F(2,21)=13.79$, $p<0.05$ (Table 5). From these findings, DAO appeared to play an important role in the formation of GABOB in the rat brain.

TABLE 4

INHIBITION OF THE FORMATION OF GABOB FROM 2-HO-PUT AND GABA FROM PUT BY 1 μM AMINOGUANIDINE, SEMICARBAZIDE, OR PARGYLINE

Substrate	Inhibitor 1 μM Aminoguanidine (%)	1 μM Semicarbazide (%)	1 μM Pargyline (%)
2-Hydroxyputrescine (5.0 mM)	100	73.0	18.9
Putrescine (0.2 mM)	100	68.9	10.2

Values represent the percent inhibition of formation of the products in comparison to enzyme reactions performed without the inhibitors.

Anticonvulsant Effect of 2-HO-PUT and GABOB

Intraventricular injection of 2-HO-PUT or GABOB. The effect of an intraventricular injection of 2-HO-PUT or GABOB on the generalized convulsions induced by intravenous PTZ was examined in rats. R(−)- or S(+)-2-HO-PUT (30 μg) were injected into the lateral ventricle, and the convulsion threshold was measured from 10 min to 2 weeks afterwards. The convulsion threshold reached a peak 12–24 h after the injection of R(−)-2-OH-PUT, and remained high for the following week. Elevation of the threshold after the injection of S(+)-2-HO-PUT was less marked (Fig. 4).

The time course of elevation of the convulsion threshold coincided well with the formation of GABOB after the intraventricular injection of 30 μl of 2-HO-PUT, and no correlation was observed between the threshold and 2-HO-PUT concentration in rat brain (Fig. 3). From these findings, the anticonvulsant effect of 2-HO-PUT appeared to result from the formation of GABOB.

The convulsion threshold after an intraventricular injection of GABOB was examined. As shown in Fig. 5, a marked elevation of the threshold that lasted for over a week was observed after the injection of R(−)-GABOB, while the anticonvulsant effect of S(+)-GABOB was quite weak. These results were consistent with previous findings (1,12). The anticonvulsant effect of R(−)-GABOB was dose dependent from 10 μg to 100 μg (data not shown).

Effect of GABOB on the GABA system. In order to investigate the mechanism of the anticonvulsant effect of GABOB, its actions on the central GABA system were examined.

Effect of GABOB on Cl^- influx into brain membrane vesicles. Since the GABA·benzodiazepine receptor complex is functionally coupled to the Cl^- channel, the $^{36}Cl^-$ influx into rat brain membrane vesicles was examined. Both R(−)- and S(+)-GABOB stimulated the influx of $^{36}Cl^-$, and the potency of these compounds was about one-tenth of that of GABA (Fig. 6). However, 2-HO-PUT did not stimulate Cl^- influx.

Effect of GABOB on GABA uptake into brain membrane vesicles. As shown in Fig. 7, GABOB inhibited the uptake of GABA into the vesicles in a concentration-dependent manner; 50% inhibition was observed with 130 μM R(−)-GABOB and 20 μM S (+)-GABOB. 2-HO-PUT had no effect on GABA uptake.

Effect of GABOB on L-glutamic acid decarboxylase and GABA-α-oxoglutarate transaminase. A rat was killed 3 h after an intraperitoneal injection of 30 μg of R(−)- or S(+)-GABOB, and the brain was taken out to determine the GAD and GABA-T activities. R(−)- and S(+)-GABOB did not affect the activity of either enzyme.

DISCUSSION

The identification of GABOB in the CNS has been a focus of attention in the field of neurochemistry because of its potent inhibitory effect on the CNS. β-Hydroxyglutamic acid was demonstrated to be the substrate of GAD (28), and hydroxyproline was reported to be metabolized to GABOB (21). Furthermore, it was also demonstrated that GABA was directly hydroxylated to GABOB (22). These in vitro findings increasingly stimulated the interest of neurochemists, neuropharmacologists, and neurophysiologists. However, Mitoma denied the occurrence of GABOB in the brain in 1960 (16), and similar negative reports by Elliott and Copper followed (6,4), resulting in a fading of interest in GABOB.

TABLE 5

EFFECTS OF AMINOGUANIDINE AND PARGYLINE ON THE FORMATION OF [^{3}H]-GABOB FROM [^{3}H]-2-HO-PUT IN THE RAT LIVER, SMALL INTESTINE, AND BRAIN

Treatment	Control (dpm/g w.w.)	Aminoguanidine (1 mg/kg IP) (dpm/g w.w., % inhibition)	Pargyline (1 mg/kg IP) (dpm/g w.w., % inhibition)
Liver	113,254 ± 11,975	19,983 ± 4,487† (82.4)	39,847 ± 9,375† (64.8)
Small Intestine	31,814 ± 8,212	4,029 ± 1,088† (87.3)	11,023 ± 1,086† (65.4)
Treatment	Control (dpm/g w.w.)	Semicarbazide (100 mg/kg IP) (dpm/g w.w., % inhibition)	Pargyline (100 mg/kg IP) (dpm/g w.w., % inhibition)
Brain	27,114 ± 2,512	18,265 ± 3,004* (32.4)	23,824 ± 3,952 (12.0)

Rats were pretreated with an intraperitoneal injection of 1 mg/kg of aminoguanidine or pargyline, and then 370 KBq of [^{3}H]-2-HO-PUT was injected intraperitoneally 2 hours later. Three hours after the injection of [^{3}H]-2-HO-PUT, the rats were killed by decapitation and the liver and small intestine were taken out to measure [^{3}H]-GABOB. In the case of the brain, 100 mg/kg of the inhibitors were used. Brains were removed rapidly 1 hour after the injection of 185 KBq of [^{3}H]-2-HO-PUT. Values represent the mean ± S.D. of 6–10 experiments.

*$p<0.05$, †$p<0.01$.

In 1978, Noto et al. reported the formation of 2-HO-PUT from PUT in the rat (17), and then identified 2-HO-PUT in the bovine brain in a series of investigations on polyamine metabolism in mammals. These findings again suggested the occurrence of GABOB in the CNS and that GABOB might be formed from 2-HO-PUT in the same way that GABA is formed from PUT via γ-aminobutyraldehyde. This mechanism was verified by our group in 1978 (27). In 1988, we finally demonstrated the formation of [^{3}H]-GABOB in the rat brain after the intraventricular injection of [^{3}H]-2-HO-PUT (18). However, the biochemical characteristics of the amino acid product remained to be clarified.

In the present series of studies, we confirmed the formation of [^{3}H]-GABOB in various organs of rats injected intraperitoneally with [^{3}H]-2-HO-PUT. The formation of the stereoisomer of GABOB, γ-amino-α-hydroxybutyric acid, was not demonstrated in any of the organs examined. In vivo experiments suggested the participation of both MAO and DAO in the formation of GABOB from 2-HO-PUT in rat organs other than the brain, where DAO appeared to play the main role.

We also demonstrated that both R(−)- and S(+)-2-HO-PUT

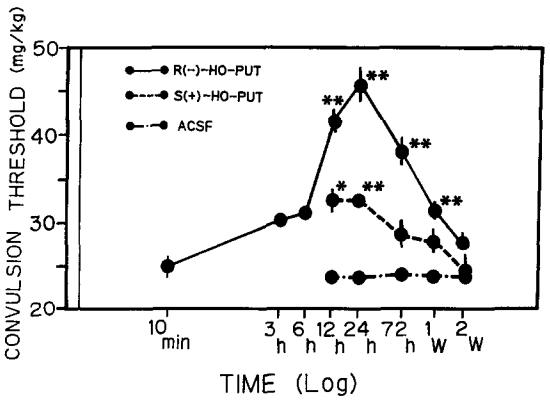

FIG. 4. Changes in the threshold of pentylenetetrazol-induced convulsions in rats after the intraventricular administration of R(−)- or S(−)-2-HO-PUT or artificial cerebrospinal fluid. R(−)- or S(−)-2-HO-PUT (30 μg) or vehicle alone (2 μl) were injected intraventricularly. At the indicated intervals after injection, pentylenetetrazol solution (5 mg/ml) was administered intravenously using a constant flow pump (flow rate: 1.7 ml/min). The threshold indicates the dose of the convulsant needed to induce generalized seizures. Values represent the mean ± S.E.M. of 5–10 experiments. *$p<0.05$, **$p<0.01$.

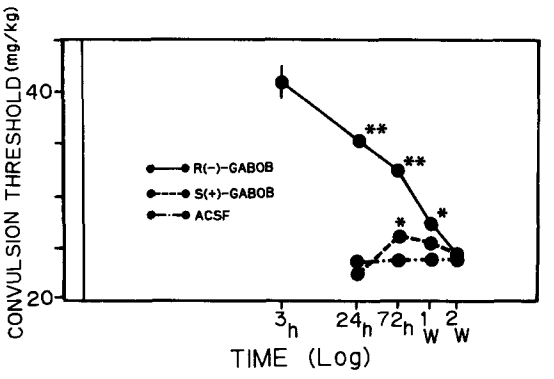

FIG. 5. Changes in the threshold of pentylenetetrazol-induced convulsions in rats after the intraventricular administration of R(−)- or S(−)-GABOB or artificial cerebrospinal fluid. R(−)- or S(−)-GABOB (30 μg) or vehicle alone (2 μl) were injected intraventricularly. At the indicated intervals after injection, pentylenetetrazol solution (5 mg/ml) was administered intravenously using a constant flow pump (flow rate: 1.7 ml/min). The threshold indicates the dose of the convulsant needed to induce generalized seizures. Values represent the mean ± S.E.M. of 5 experiments. *$p<0.05$, **$p<0.01$.

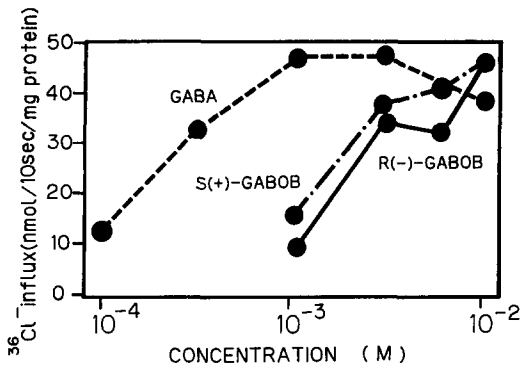

FIG. 6. Effect of R(−)- or S(−)-GABOB or GABA on Cl^- influx into rat brain membrane vesicles. Rat brain membrane vesicles were incubated in the presence of 18.5 KBq of $^{36}Cl^-$ and various concentrations of R(−) or S(−)-GABOB or GABA. $^{36}Cl^-$ influx was then measured as described in the Method section. Values represent the mean of triplicate experiments.

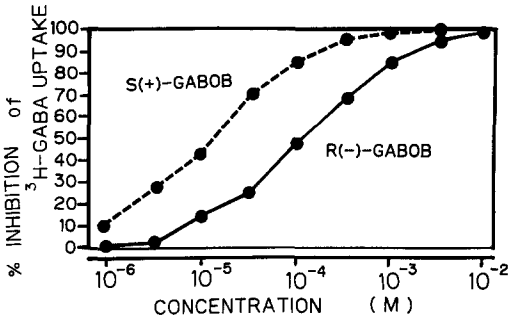

FIG. 7. Effect of R(−)- or S(−)-GABOB on GABA uptake into rat brain membrane vesicles. Rat brain membrane vesicles were incubated in the presence of 370 Bq of [^{3}H]-GABA and various concentrations of R(−)- or S(−)-GABOB. Values represent the mean % inhibition of [^{3}H]-GABA uptake obtained from triplicate experiments.

had an anticonvulsant action with the R(−) form being the more potent of the two. This anticonvulsant effect was apparently derived from GABOB formed in the rat brain. The mechanism of the anticonvulsant effect of GABOB was next examined, and the stimulation of Cl^- influx and inhibition of GABA uptake into rat brain membrane vesicles were shown to probably have at least a partial role in its anticonvulsant activity.

After Hayashi reported the inhibitory effects of GABOB on the generation of impulses in the CNS (10,11), GABOB has been clinically applied to treat patients with epilepsy or hyperkinetic syndrome. However, GABOB does not enter the brain when administered orally and its clinical efficacy has been questioned.

Pharmacological studies of GABOB have elucidated its stimulatory effect on the release of growth hormone, prolactin, and cortisol (7, 15, 26). These findings and those of the present study suggest that the clinical application of GABOB should now be reevaluated, including the development of an effective method of administration.

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