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Fluorinated γ-Aminobutyric Acid

Enzymatic Synthesis and Biological Activity of a Potentially Useful Analogue

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SUMMARY

Glutamate decarboxylase (EC 4.1.1.15) of *Escherichia coli* reacts with L-γ-fluoroglutamate to form α-fluoro-γ-aminobutyric acid. This reaction has been characterized, and the two fluorinated amino acids have been investigated as selective inhibitors of γ-aminobutyric acid metabolism in the nervous system. L-γ-Fluoroglutamate inhibits the glutamate decarboxylase from calf brain (K_i = 1.4 × 10^{-2} M) at concentrations comparable to the K_m of this enzyme for glutamate (10^{-2} M). In addition, α-fluoro-γ-aminobutyric acid inhibits the uptake of γ-aminobutyric acid in a nerve-muscle preparation from lobster.

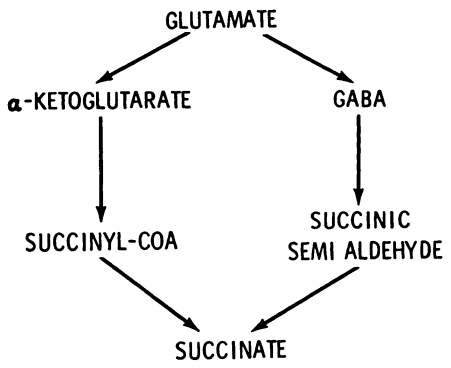
INTRODUCTION

Substitution of a fluorine atom for hydrogen can lead to defined changes in chemical reactivity of biologically important molecules without introducing significant steric changes. For this reason, analyses of the molecular basis of a number of biochemical phenomena have been aided by the availability of the appropriate fluoro analogues (1). Accordingly, when our studies on γ-fluoroglutamate led to the biosynthesis of α-fluoro-γ-aminobutyrate, it seemed of interest to investigate further the possible usefulness of this analogue of γ-aminobutyrate. This approach seemed particularly appropriate, since the function of γ-aminobutyrate in the central nervous system remains to be elucidated.

Speculations on the role of GABA[1] in the central nervous system seem to fall into two categories. In one, GABA would serve as an inhibitory neurotransmitter (2) comparable to its well-documented function in the peripheral nervous system of the lobster (3). In the other, GABA would be an important energy-yielding metabolite (4), arising during the transformation of glutamate to succinate by a pathway of metabolism which bypasses α-ketoglutarate and succinyl coenzyme A (Scheme 1).

Fluorinated analogues are often capable of distinguishing various metabolic functions of the parent compound. Fluoroacetate, for instance, mimics acetate in only some of its metabolic reactions (1). This

[1] The abbreviations used are: GABA, γ-aminobutyric acid; F-GABA, α-fluoro-γ-aminobutyric acid; F-Glu, γ-fluoroglutamic acid. In addition to these abbreviations, D and L with respect to F-Glu refer to the configuration at the α-carbon; on the basis of the specificity of two enzymes (see below) these designations are related to the configuration of glutamate. A second asymmetrical center at the γ-carbon of F-Glu is retained at the α-carbon of F-GABA derived from F-Glu. The asymmetry at the carbon atoms bearing the fluorine substituent in these amino acids has not made itself apparent in studies reported in this paper; hence, no notation with regard to this point has been developed.



SCHEME 1

suggests that F-GABA might provide a way of resolving possible roles of GABA in studies with intact physiological preparations of the central nervous system. For example, F-GABA could be an analogue of GABA which might have the physiological properties of the parent compound without entering into its energy-yielding metabolic pathways.

This paper presents details of the synthesis of F-GABA from F-Glu by glutamate decarboxylase of *Escherichia coli* (EC 4.1.1.15). In addition, a biochemical basis is established for the use of these fluorinated amino acids in the nervous system, since it can be shown that glutamate decarboxylase in calf brain is inhibited by F-Glu and that GABA uptake in a nerve muscle preparation from lobster is inhibited by F-GABA. It thus appears promising that F-GABA, either administered directly or perhaps arising from F-Glu, could play a useful role in studies of the role of GABA in central nervous system physiology.

EXPERIMENTAL PROCEDURE

Materials. Calf brain glutamate decarboxylase was prepared from an acetone powder (5) by extraction at 0° for 1 hr with 10 volumes of 0.1 M potassium phosphate buffer, pH 6.3, containing 5×10^{-3} M 2-mercaptoethanol and 5×10^{-4} M pyridoxal phosphate. The clear supernatant solution obtained after centrifugation for 30 min at 23,000 × *g* was used immediately or stored at −196°. All ion exchange resins were purchased from Bio-Rad Laboratories.

Synthesis of F-Glu. F-Glu was synthesized by the condensation of diethyl fluoromalonate with ethyl acetamidoacrylate, followed by acid treatment of the intermediate, using Scheme 4 of Buchanan, Dean, and Pattison (6). The syrup obtained after the HCl had been removed under vacuum was dissolved in 25 ml of water and applied to a Dowex 1 (AG1-X8) ion exchange column (4.4 × 24 cm, 200–400 mesh) in the acetate form. The column was eluted with 2 N acetic acid, and 20-ml fractions were collected. Fractions were assayed by the photometric ninhydrin method of Troll and Cannan (7), and those containing the product (fractions 41–125) were pooled and evaporated to dryness on a rotary evaporator. Fluoroglutamic acid (4.9 g, 48% yield) was obtained as white crystals from water.

$C_5H_8FNO_4$

Calculated:	C 36.37, H 4.88, N 8.48
Found:	C 36.22, H 5.06, N 8.73

Enzyme assays. Glutamate decarboxylase was assayed by the release of $^{14}CO_2$ from 1-^{14}C-glutamate which contained 0.71 mg of DL-1-^{14}C-glutamic acid (New England Nuclear Corporation) and 33.5 mg of carrier sodium L-glutamate to give a mixture with a specific activity of 3.0×10^4 dpm/μmole. Calculations of the amount of CO_2 released in the enzyme assays were based on one-half this specific activity, since D-1-^{14}C-glutamate is not decarboxylated by this enzyme. The injection of 50% trichloracetic acid (0.1 ml) through a septum stopper was used to terminate the reaction, and $^{14}CO_2$ was collected in filter paper wetted with 0.1 ml of a mixture of equal volumes of methanol and triethanolamine. The plastic center well (Kontes Glass Company, Vineland, N. J., No. K 882320) and the filter paper contained in it were then immersed in Bray's solution (8) and assayed for radioactivity in a Packard liquid scintillation spectrometer. Reaction mixtures in which boiled enzyme was substituted for active enzyme served as controls for nonenzymatic decarboxylation (which was less than 5% of the enzymatic rate).

Decarboxylase activity was also assayed by the appearance of GABA or F-GABA

during decarboxylation. In this procedure an aliquot (0.5 ml) of the reaction mixture was added to a column of Dowex 1 (AG1-X4, 0.4 × 3 cm) in the acetate form, and the column was eluted with 3 ml of water to obtain GABA (or F-GABA), which was assayed by the photometric ninhydrin reaction (7). With this method the color yields on a molar basis were 0.96 for F-Glu and 0.85 for F-GABA, compared to glutamate.

Synthesis of F-GABA and D-*F-Glu.* For the preparation of F-GABA, the reaction mixture contained 330 mg (2 mmoles) of DL-F-Glu neutralized with NaOH, 2 mmoles of pyridine HCl buffer at pH 4.5, 20 μmoles of pyridoxal phosphate, and 50 mg of *E. coli* glutamic acid decarboxylase (Sigma Chemical Company, 880 μl of CO_2 per milligram per 30 min) in a volume of 40 ml. The reaction mixture was incubated at 30°, and aliquots were assayed at various times for the formation of F-GABA (Fig. 1).

After 24 hr the reaction mixture was clarified by centrifugation and added to a Dowex 1 ion exchange column (AG1-X4, 2 × 15 cm, 100–200 mesh) in the acetate form. The column (at neutral pH) was washed with 150 ml of water, and the effluent, containing the F-GABA, was evaporated to dryness. This material was dissolved in water and adsorbed on a Dowex 50 ion exchange column (AG50W-X4, 2 × 50 cm, 200–400 mesh) in the H^+ form. The column was washed with 200 ml of water and then eluted with 1 N HCl. The 5-ml fractions were assayed by the photometric ninhydrin reaction. Fractions (Nos. 88–98) containing the product were pooled, and the HCl and water were removed under vacuum. The hydrochloride of F-GABA was obtained as white crystals from ethanol. The yield of 147 mg represented a recovery of 47%, based on the amount of DL-F-Glu in the reaction mixture.

$C_4H_9ClFNO_2$

Calculated: C 30.49, H 5.76, N 8.89
Found: C 30.32, H 6.05, N 8.79

Recovery of D-*F-Glu.* Only 50% of the F-Glu reacted with glutamic acid decarboxylase, and since this enzyme is specific for L-glutamic acid it was presumed that D-F-Glu would be the species in the racemic F-Glu which was not decarboxylated.

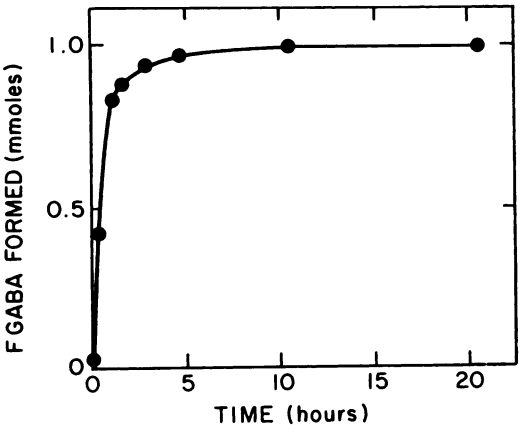


FIG. 1. *Formation of F-GABA from* DL-*F-Glu by glutamate decarboxylase of E. coli*

The reaction mixture and the assay of F-GABA in aliquots of the reaction mixture are described in EXPERIMENTAL PROCEDURE.

D-F-Glu was recovered from the Dowex 1 column mentioned above by elution with 2 N acetic acid. In this procedure, 5-ml fractions were collected, and the photometric ninhydrin assay revealed that D-F-Glu was in fractions 75–120. These fractions were pooled, evaporated to dryness, dissolved in water, and decolorized with activated charcoal. This material was then applied to a Dowex 50 column (AG50W-X4, 2 × 11 cm, H^+ form, 200–400 mesh) and eluted with 2 N acetic acid. The yield of 140 mg represented 42.5% of the DL-F-Glu in the initial reaction mixture. D-F-Glu was then recrystallized from a minimal amount of H_2O. $[\alpha]_D^{25} = -21.6°$ (c, 0.81, in 1 N HCl).

$C_5H_8FNO_4$

Calculated: C 36.37, H 4.88, N 8.48
Found: C 36.35, H 4.94, N 8.67

Formation of lactams of GABA and F-GABA. The lactam of F-GABA was prepared by heating 2–3 mg of F-GABA at 200° in a sealed, evacuated tube, one end of which was maintained at 0°. In this procedure, the volatile lactam which formed in the heated region was collected in the cooled region, where it had condensed. Identical conditions were used to convert GABA to its lactam.

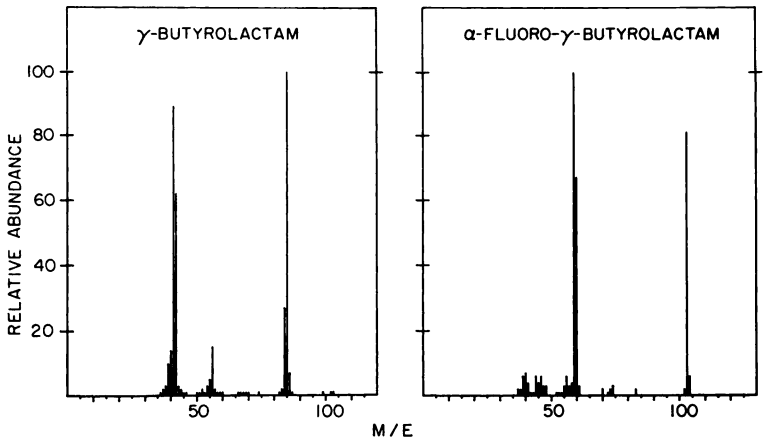


FIG. 2. *Mass spectra of F-GABA and GABA as their lactams*
Low-resolution mass spectroscopy was performed at an ionization voltage of 70 ev in the LKB-9000 gas chromatogram-mass spectrometer after chromatography on 3% OV 17 at 160°. The lactam of GABA is shown at left, while that of F-GABA is at right. We thank William Comstock for this analysis.

Synthesis of N-acetyl-F-Glu. *N*-Acetyl DL-F-Glu, synthesized by a Schotten-Baumann reaction (9), was purified by elution with water from a Dowex 50 column (H^+ form). As judged from gas-liquid chromatography, this material was approximately 95% pure.

Other methods. Amino acid analyses were performed by a slight modification of the method of Spackman (10), using the 4-hr elution system with a column (0.9 × 50 cm) of Beckman UR-30 resin. For basic amino acids, a 0.9 × 20 cm column of Beckman PA-30 resin at 37° was eluted with 0.38 M sodium citrate, pH 4.26, at a flow rate of 68 ml/min.

In the first system, F-Glu was eluted prior to aspartate, and F-GABA, prior to glutamate. The color yields were 0.85 for F-Glu and 0.45 for F-GABA, referred to leucine on a molar basis.[2]

F-GABA was resolved from GABA on a column of Dowex 50 (AG50W-X4, 200–400 mesh, 2 × 55 cm, H^+ form). In this procedure 87 mg of F-GABA were added to the column, which was washed with water and then eluted with 0.5 M KCl in 10^{-3} M HCl; F-GABA was recovered in a sharp peak between 690 and 750 ml of eluting fluid.

Infrared spectra were obtained with a Perkin-Elmer model 21 instrument. Protein was determined by the method of Lowry *et al.* (11).

Lobster nerve-muscle preparation. The effect of F-GABA on the uptake of GABA in a nerve-muscle preparation from the superficial ventral abdominal muscles of the lobster *Homarus americanus* was examined in the system used by Iversen and Kravitz (12). Their procedure was followed exactly, using ^{3}H-GABA (New England Nuclear Corporation) at a concentration of 2 × 10^{-8} M; the effect of inhibitor was examined in four to six muscles, the contralateral muscle serving in each case as a control (12).[3]

RESULTS

Identification of F-GABA. The mass spectrum of the lactam of F-GABA and that of the lactam of GABA are compared in Fig. 2. The two fragmentation patterns are closely analogous in the two compounds, with major and parent peaks differing only by 18 mass units, as would be expected when fluorine (atomic weight 19) is substituted for hydrogen. The infrared spectrum was compatible with that described by Buchanan and Pattison for this compound (13), except that bands were observed at

[2] We are indebted to Barbara Hauck for these analyses.

[3] We thank Dr. E. A. Kravitz for suggesting the use of this system, and for his demonstration of the dissection of the nerve-muscle preparation.

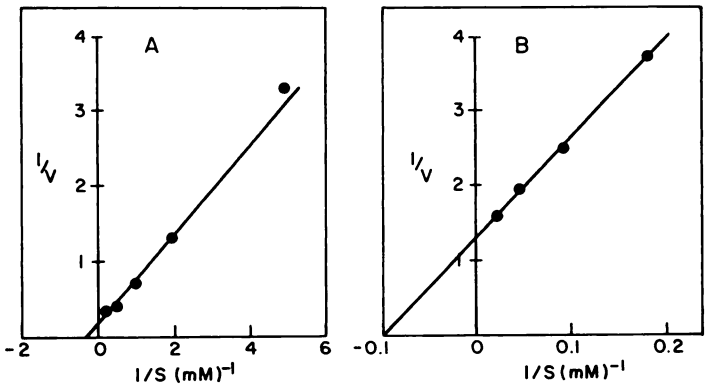


FIG. 3. *Kinetics of glutamate decarboxylase of E. coli*

For the decarboxylation of glutamate (A) the reaction mixture contained 25 μmoles of pyridine HCl buffer at pH 4.5, 0.25 μmole of pyridoxal phosphate, 0.05 mg of enzyme protein, and the amount of 1-^{14}C-glutamate indicated. Incubation in a volume of 0.5 ml was conducted at 30° for 15 min. For the decarboxylation of F-Glu (B), the reaction mixture contained 100 μmoles of pyridine HCl buffer at pH 4.5, 1.0 μmole of pyridoxal phosphate, 2.5 mg of enzyme protein, and the amount of DL-F-Glu indicated. Incubation in a volume of 2.0 ml was conducted for 20 min at 30°. The extent of the reaction was determined by $^{14}CO_2$ collection in part A and by F-GABA release in part B. Kinetic analyses were performed according to Lineweaver and Burk (17).

1070 cm^{-1}(m) and 1030 cm^{-1}(w) instead of at 1054 cm^{-1}(w).

The spectral data, together with the elemental analysis and the nature of the biochemical reaction, establish the identity of the F-GABA made enzymatically.

Stereochemistry of reaction of F-Glu with glutamate decarboxylase of E. coli. The species of DL-F-Glu which is not reactive with glutamate decarboxylase of *E. coli* has been tentatively assigned the D-configuration at the α-carbon. This is based on the assumption that enzymes with a specificity for the L-configuration of glutamate will have a similar specificity for the L-configuration at the α-carbon of F-Glu. The observation that 50% of the DL-F-Glu is decarboxylated (Fig. 1) suggests that only two of the four isomers are reactive. Presumably the reactive isomers are those with the L-configuration at the α-carbon, since this enzyme reacts only with L-glutamate and some of its derivatives (14).

A similar argument in support of this tentative assignment of configuration was obtained from experiments with carboxypeptidase G, an enzyme which is absolutely specific for the hydrolysis of *N*-acyl linkages to L-glutamate (15). Fifty per cent of the racemic *N*-acetyl-F-Glu was hydrolyzed by this enzyme, whose specificity for the deacylation of L-glutamate suggests that the F-Glu released would also be of the L-configuration at the α-carbon. This assignment was in accord with that indicated by glutamate decarboxylase, since F-Glu released by the hydrolysis was completely decarboxylated by the *E. coli* enzyme.

It has been shown that *threo*-hydroxyl-L-glutamate reacts with glutamate decarboxylase of *E. coli* whereas the L-*erythro* isomer does not (14). The tentative assignment of configuration for F-Glu would imply that the decarboxylase can react with both diastereomers of L-F-Glu. This contrast in reactivity, based on the substituent at the γ-carbon of the two glutamate congeners, is consistent with the small steric change introduced when a fluorine atom (in contrast to a hydroxyl group) is substituted for a hydrogen atom (16).

The complete decarboxylation of L-F-Glu racemic at the γ-carbon would produce F-GABA which is racemic at the fluorine-bearing carbon. Failure to observe optical activity ($[\alpha]_D^{25}$ = 0, F-GABA-HCl, *c*, 1.4, in H_2O) is consistent with the formation of racemic F-GABA in the enzymatic reaction.

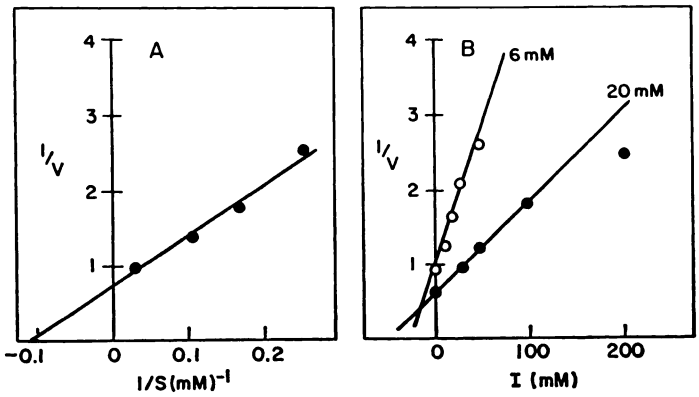


FIG. 4. *Kinetics of glutamate decarboxylase of calf brain*

The reaction mixtures contained 25 μmoles of potassium phosphate buffer at pH 6.3, 0.25 μmole of pyridoxal phosphate, 2.5 μmoles of 2-mercaptoethanol, 1.4 mg of enzyme, and, for part A, the amount of 1-^{14}C-glutamate shown, in a volume of 0.5 ml; $^{14}CO_2$ release was determined after a 60-min incubation at 30°. Similar conditions were used for obtaining the data of part B, except that glutamate was maintained constant at either 6 mM or 20 mM and the amount of DL-F-Glu was varied as shown. The data of part A are plotted according to Lineweaver and Burk (17), while the data of part B are plotted according to Dixon (18).

Kinetics of reaction of F-Glu with glutamate decarboxylase of E. coli The K_m of the enzyme for DL-F-Glu (10×10^{-3} M) is about twice that for glutamate (4×10^{-3} M) (Fig. 3). These experiments were done with racemic F-Glu, but the D-isomer seems to have little effect on the enzyme, as shown by an experiment in which D-F-Glu (5×10^{-2} M) inhibited the decarboxylation of 1-^{14}C-glutamate (5×10^{-4} M) by only 12%. These data indicate that the K_m for L-F-Glu is 5×10^{-3} M, which is about the same as that for L-glutamate.

In separate experiments the rate of formation of F-GABA from DL-F-Glu was compared with the rate of formation of GABA from glutamate, and the V_{max} for the racemic analogue was found to be about 15% of that for glutamate.

Kinetics of glutamate decarboxylase from calf brain. As a basis for the further studies of the biological activity of F-Glu and F-GABA in the mammalian central nervous system, it seemed important to establish the reactivity of a brain glutamate decarboxylase with F-Glu. For the calf brain enzyme, the K_m for glutamate is 10^{-2} M (Fig. 4A), a value similar to that obtained in other mammals (19) and in lobster (20). Because of the greater sensitivity of the assay based on the release of $^{14}CO_2$ in determining the activity of glutamate decarboxylase, the affinity of the enzyme for F-Glu was determined from its K_i (Fig. 4B). In the Dixon plot it can be seen that the inhibition is competitive and that the K_i of the racemic F-Glu (1.4×10^{-2} M) is close to the K_m for glutamate (10^{-2} M). It has not yet been determined whether F-Glu is a substrate for the calf brain decarboxylase.

Inhibition of GABA uptake by F-GABA in the lobster nerve-muscle preparation. The data of Table 1 indicate that at concentrations of 10^{-4} and 5×10^{-4} M, F-GABA is effective in inhibiting GABA uptake. The inhibition with this compound is comparable to that achieved at similar concentrations of β-guanidopropionic acid, which was the most effective of several GABA analogues tested in this system by Iversen and Kravitz (12).

In these experiments tritiated GABA is present at a concentration of 2×10^{-8} M while the inhibitor is added at a level approximately 10^4-fold higher. Therefore contamination of the analogue with GABA at a level as low as 0.02% would produce an artifactual inhibition of tritiated GABA uptake which would be in reality due simply to isotope dilution. This low level of im-

TABLE 1

Effect of F-GABA and β-guanidopropionic acid on uptake of ^{3}H-GABA in the lobster nerve-muscle preparation

Incubation conditions with 2×10^{-8} M ^{3}H-GABA in "saline medium" and the analysis of GABA uptake were the same as described by Iversen and Kravitz (12).

Analogue	Concentration	Inhibition of ^{3}H-GABA uptake
	M	% ± *SEM*
F-GABA	1×10^{-6}	5 ± 4
	1×10^{-4}	42 ± 4.7
	5×10^{-4}	72 ± 1.3
β-Guanidopropionic acid[a]	5×10^{-4}	67.9, 80.0
	1×10^{-3}	78.6

[a] The values for inhibition of GABA uptake by β-guanidopropionic acid are reproduced from the data of Iversen and Kravitz (12).

purity in the analogue would escape detection in an amino acid analysis, and hence F-GABA was purified by chromatography on Dowex 50 (AG50W-X4) under conditions which would resolve it from GABA. F-GABA purified in this way inhibited GABA uptake to the same extent as the -GABA used without this purification.

DISCUSSION

The finding that F-Glu is a competitive inhibitor of brain glutamate decarboxylase suggests the feasibility of inhibiting GABA synthesis with this analogue. This might make it possible to study the altered physiology resulting from lower levels of GABA in the brain. One can also speculate on similar uses for F-GABA. If, for example, this compound inhibits GABA transaminase, it could be useful in depressing GABA removal and thereby have value in studies of the effects of higher GABA levels on the brain. If GABA is, in fact, a neurotransmitter, another possibility to be explored is whether F-GABA can compete with released GABA for receptor sites at the synapses. Clearly, the biochemical studies offered in this paper are only a beginning in the examination of possible uses for these analogues. Another problem with the use of these compounds would relate to their transport across the blood-brain barrier. Although there is evidence that glutamate can enter the brain from the bloodstream (21, 22), there is no information on this point for the fluorinated amino acids.

The inhibition of GABA uptake by F-GABA in lobster neuromuscular preparations suggests its use in mammalian systems, where release of GABA in response to neuronal activity is in doubt. If there is a mammalian GABA transport system which can be inhibited in this way, F-GABA may play a role in its elucidation, in much the same way eserine has been used to enable the detection of acetylcholine release. These speculations on the basis of a few experiments emphasize the need for further studies with these analogues, whose ultimate value can only be determined by investigating their use in the nervous systems of appropriate experimental animals.

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