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The Diastereomers of γ-Fluoroglutamate: Complementary Structural Analogues

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SUMMARY

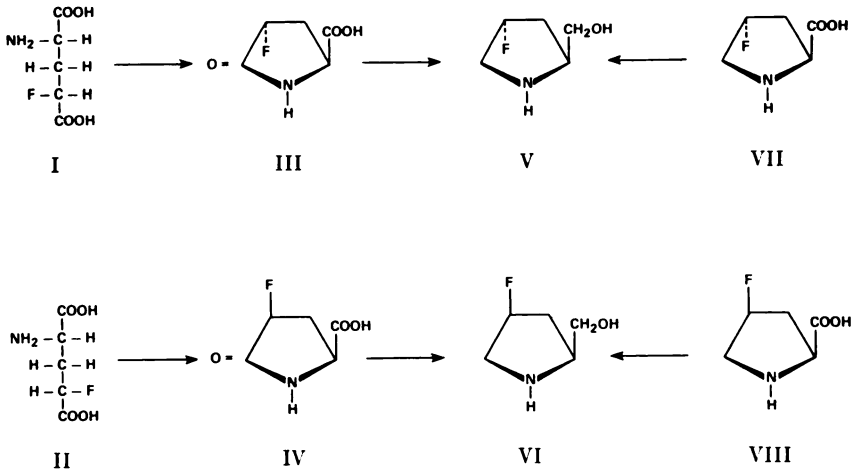
The racemates of the diastereomers of γ-fluoroglutamate have been separated by ion-exchange chromatography, and the relative configuration of the 2 asymmetrical carbon atoms has been established by an unambiguous chemical method. The two diastereomers of L-γ-fluoroglutamate were prepared enzymatically, and both (or their appropriate derivatives) are substrates for the following enzymes: glutamate dehydrogenase (EC 1.4.1.3), glutamine synthetase (EC 6.3.1.2), glutamate decarboxylase (EC 4.1.1.15), carboxypeptidase G(EC 3.4.2.6), and leucine aminopeptidase (EC 3.4.1.1). The reactions of these enzymes and D-glutamate cyclase (EC 4.2.1.54) confirm the tentative assignment of configuration at the α-carbon of γ-fluoroglutamate that was proposed previously. Preliminary kinetic studies with several of these enzymes normally reactive with glutamate indicate that the *threo* tends to be more reactive than the *erythro* diastereomer. Possible biological and biochemical applications of these glutamate analogues are discussed.

INTRODUCTION

The "lethal synthesis" of fluorocitrate from fluoroacetate (1) by a metabolic pathway normally used for acetate shows how structural characteristics of biologically active molecules can be retained when fluorine selectively replaces hydrogen in a bond to carbon. Yet such structural similarities can lead to profound biochemical effects, as illustrated by fluorocitrate, which inhibits aconitase (EC 4.2.1.3), an enzyme normally reactive with citrate (2, 3). This prototype of lethal synthesis has led to the exploration of possible similar applications of other fluorinated analogues. Among several examples one might mention the use of fluoro analogues of pyrimidines (4), amino acids (5), and Krebs cycle intermediates (6) as either substrates or inhibitors of enzymes.

In addition to their value as selective inhibitors of various metabolic pathways, fluoro analogues have been used to define critical structural features of macromolecules. Recently, for example, the inhibitory isomer of fluorocitrate has been incorporated into a model of the stereochemical and conformational features of catalysis by the enzyme aconitase (7). Other examples of this use of fluoro analogues in defining the molecular basis of biological phenomena have recently been reviewed (8).

These considerations suggested that γ-fluoroglutamate might be useful in biological studies either as a selective inhibitor of some aspect of glutamate metabolism or in defining critical structural features of enzymes normally reactive with glutamate. This analogue could have particular interest, since glutamate participates in many biosynthetic and energy-yielding reactions and



SCHEME 1

since a number of enzymes reactive with glutamate have been used as models of enzyme catalysis (9) and regulation (10). An additional value of a fluoro analogue in the study of enzyme catalysis is the possible use of fluorine NMR in defining the detailed relationships between enzyme and the fluorinated substrate or inhibitor.

The anticipated similarity between glutamate and γ-fluoroglutamate is illustrated by the action of glutamate decarboxylase (EC 4.1.1.15) on FGlu[1] (11) and by the hydrolysis of *N*-acetyl-FGlu by carboxypeptidase G (EC 3.4.2.6) (11); both these enzymes react only with glutamate, among the amino acids normally found in proteins (12, 13). The stoichiometry of these reactions (11) had indicated that these enzymes were reactive with both diastereomers of L-FGlu.[2] However, in the earlier studies the diastereomers were not resolved, and the relative reactivity of the two forms of L-FGlu was not studied.

If the two diastereomers of L-FGlu are reactive, they can be regarded as complementary probes of the interaction between enzyme and substrate. The two diastereomers represent two structural variations of glutamate and offer the opportunity to place the fluorine atom at different positions in an enzyme-substrate (or enzyme-inhibitor) complex. This feature of the diastereomers might make it possible to define the detailed interaction of these substrates with the active centers of the enzymes. Therefore we present in this paper the resolution of the diastereomers of L-FGlu and the assignment of the relative configuration of the 2 asymmetrical carbons of the molecule. The potential value of the diastereomers is illustrated by the finding that they are substrates for three additional enzymes: glutamate dehydrogenase (EC 1.4.1.3), glutamine synthetase (EC 6.3.1.2), and D-glutamate cyclase (EC 4.2.1.54). In order to

[1] The abbreviations used are: FGlu, γ-fluoroglutamic acid; FPCA, 3-fluoro-2-pyrrolidone-5-carboxylic acid.

[2] The designations D and L with respect to FGlu refer to the configuration at the α-carbon. This has been related to the configuration at the α-carbon of glutamate by the specificity of the enzymes carboxypeptidase G and *Escherichia coli* glutamate decarboxylase (10). This tentative assignment of configuration appears consistent with the specificity of several additional enzymes that have been tested with FGlu or its derivatives in experiments described in this paper. In addition to the asymmetrical center at the α-carbon, FGlu has a second asymmetrical center at the γ-carbon (Scheme 1). The relative configuration of these asymmetrical centers can be designated *erythro* (I) and *threo* (II) in accordance with conventions used previously to refer to the diastereomers of γ-hydroxyglutamate (13). On pyrolysis, *erythro*- and *threo*-FGlu cyclize to *trans*- (III) and *cis*- (IV) FPCA, respectively.

compare these previously uncharacterized analogues with glutamate, a preliminary study of the kinetics of the resolved diastereomers with these enzymes is presented.

EXPERIMENTAL PROCEDURE AND RESULTS

Materials. FGlu was synthesized by the condensation of diethyl fluoromalonate with ethyl α-acetamidoacrylate (15) and purified as reported previously (11). All ion-exchange resins were purchased from Bio-Rad Laboratories and are identified by the manufacturer's designation. Column separations were done at room temperature unless specified otherwise. Amino acids were quantitated by a photometric ninhydrin reaction, modified from that of Moore and Stein (16) by the addition of 0.8 g of $SnCl_2$ per liter to the ninhydrin reagent in place of hydrindantin. With this method FGlu had a color yield of 0.91 relative to leucine when measured at a wavelength of 570 mμ. To assay FPCA, 20-μl aliquots were hydrolyzed with 5 N HCl (20 μl) at 100° for 1 hr and then assayed as above.

Separation of racemates of FGlu. FGlu was converted to FPCA by pyrolysis in an open beaker at 170°, and the racemates of FPCA were then separated on a column of Dowex 1. The product of the pyrolysis reaction on 4 g of FGlu was dissolved in 100 ml of water, decolorized with Norit, adjusted to pH 7 with 2 N NaOH, and added to the column (6 × 143 cm, AG1-X4, 200–400 mesh, formate form). After washing with 1 liter of water, the column was eluted with dilute formic acid (64 ml of 90% formic acid per liter); 20-ml fractions were collected and assayed for FPCA (Fig. 1). The fractions eluting between 9.6 and 10.3 liters (*trans*-FPCA) and between 10.7 and 11.5 liters (*cis*-FPCA) were pooled and lyophilized. The racemates, whose stereochemistry was determined as described below, were obtained in the following yields after crystallization from water: *trans*-FPCA, 1.23 g; *cis*-FPCA, 1.22 g. The melting points (with some decomposition) were: *trans*-FPCA, 198–203°; *cis*-FPCA, 168–171° (micro hot stage, corrected).

	$C_5H_6FNO_3$
Calculated:	C 40.8, H 4.08, N 9.52
Found:	
trans-FPCA	C 40.84, H 4.34, N 9.76
cis-FPCA	C 40.83, H 4.11, N 9.73

The *erythro* and *threo* racemates of FGlu were also separated by chromatography on Dowex 1. For this procedure FGlu (4 g) was dissolved in 100 ml of H_2O, adjusted to pH 7.0 with 2 N NaOH, and applied to the column as above. The column was washed initially with 1.6 liters of H_2O and then eluted with dilute formic acid (21.4 ml of 90% formic acid per liter). The eluate was analyzed for the presence of ninhydrin-reactive material. After lyophilization the weight of material obtained in the peak between 13 and 14 liters (*erythro*-FGlu) was 1.3 g, while that between 14.2 and 15.1 liters (*threo*-FGlu) was 1.4 g.

A portion of each racemate of FGlu was converted to FPCA and related to the racemates of FPCA which had been separated on Dowex 1. This was possible by gas-liquid chromatography of the methyl esters of FPCA under conditions (6-foot column of 3% OV-17 maintained at 175°) in which each racemate had a different retention time.

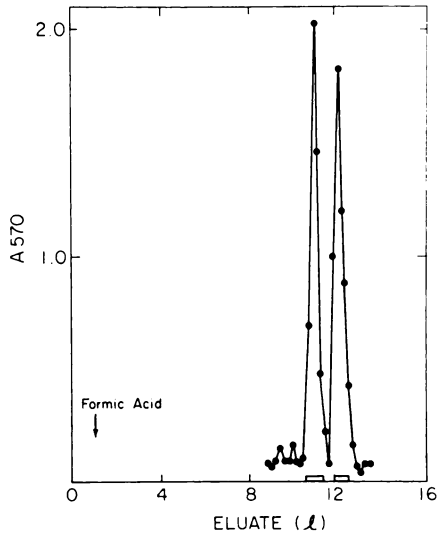


FIG. 1. *Resolution of racemates of FPCA*
Conditions for elution from the Dowex 1 column and for assay of FPCA in the eluate are described in the text. Shaded areas indicate the fractions pooled to obtain racemates of *trans*-FPCA (the first peak) and *cis*-FPCA (the second peak).

This procedure established that the racemate of FPCA which was eluted first from Dowex 1 (*trans*) was related to the first peak in the elution of the racemates of FGlu (*erythro*). In terms of Scheme 1, *erythro*-L-FGlu (I) is eluted ahead of *threo*-L-FGlu (II) when separated as the free amino acid, while *trans*-L-FPCA (III) is eluted ahead of *cis*-L-FPCA (IV) if the separation is done as the lactam.

Preparation of L-threo-FGlu and L-erythro-FGlu. After separation of the diastereomers of FGlu by Dowex 1 chromatography, L-*threo*-FGlu and L-*erythro*-FGlu were obtained from their respective racemates by the action of leucine aminopeptidase (EC 3.4.1.1). In this procedure the racemate was converted to its *N*-L-leucyl derivative and then subjected to the action of the peptidase, which is generally more reactive with *N*-L-leucyl derivatives of L- rather than D-amino acids (17). As indicated below, leucine aminopeptidase appears to be essentially specific for the L-leucyl derivative of the enantiomers of FGlu presumed to have the L-configuration at the α-carbon.

The leucyl dipeptides of DL-FGlu were synthesized by allowing DL-FGlu (either *erythro* or *threo*) to react with a 2-fold excess of the *N*-hydroxysuccinimide ester of *tert*-butyloxycarbonyl-L-leucine (Cyclo Chemical Company) (18); for this reaction, in 50% ethanol, the pH was maintained at 8–9 by the addition of triethylamine. After removal of the *tert*-butyloxycarbonyl group with trifluoracetic acid, and removal of the trifluoracetic acid under vacuum, the reaction product was dissolved in water, neutralized with NaOH, and applied to a Dowex 1 column (1 × 6 cm, AG1-X4, 200–400 mesh, acetate form). The excess leucine was removed by washing with 40 ml of H_2O, and then the dipeptide was eluted with 5 N acetic acid (30 ml). The solvent was removed under vacuum, and the residue was redissolved in water and lyophilized. The yield of L-Leu–DL-FGlu (131–147 mg) was approximately 80% of theoretical.

The reaction mixture for the enzymatic hydrolysis of L-Leu–L-*erythro*-FGlu contained 1 mg of leucine aminopeptidase (Sigma, type III; specific activity, 86 μmoles of L-leucinamide hydrolyzed per minute per milligram), 1.8 mmoles of Tris-HCl buffer (pH 9.0), 18 μmoles of $MgSO_4$, and 450 μmoles of L-Leu–DL-*erythro*-FGlu in a total volume of 18 ml. The incubation, which was conducted at 37° for 90 min, was terminated by applying the reaction mixture to a Dowex 1 column (2 × 40 cm, AG1-X4, 200–400 mesh, formate form). The column, which was washed initially with 90 ml of H_2O, was eluted with dilute formic acid (21.4 ml of 90% formic acid per liter). The L-*erythro*-FGlu (in 77% yield) was eluted in a symmetrical peak between 350 and 390 ml.

L-*threo*-FGlu was obtained under identical reaction conditions except that the substrate consisted of 450 μmoles of L-leu–DL-*threo*-FGlu, and 2 mg of leucine aminopeptidase were used in an incubation which lasted for 2 hr. Under these conditions the yield of L-*threo*-FGlu was 92%.

The purity of the L-enantiomer obtained by this method was examined by subjecting each isomer to the action of *Escherichia coli* glutamate decarboxylase, an enzyme active with both diastereomers tentatively assigned the L-configuration at the α-carbon (11). After exhaustive treatment with this enzyme, no more than 2% of the original FGlu could be detected by amino acid analysis (11), indicating that the purity of the L-isomer was at least 98%.

Relation of configuration of FPCA to 4-fluoroproline. The configuration of each of the racemates of FPCA was related to either *cis*- (VIII) or *trans*- (VII) L-4-fluoroproline (19)[3] by an unequivocal chemical transformation in which both the racemates of FPCA and the authentic diastereomers of L-4-fluoroproline were reduced to their respective *cis*- (VI) or *trans*- (V) 4-fluoroprolyl alcohols (Scheme 1). As the *N*-benzoyl derivative of a 4-fluoroprolyl alcohol, each racemate of FPCA could be related to either *cis*- or *trans*-4-fluoroproline by its characteristic retention time on gas-liquid chromatography.

To reduce a racemate of FPCA, 25 mg were dissolved in 1 ml of methanol and methylated with ethereal diazomethane. After

[3] We thank Dr. B. Witkop for a gift of authentic *cis*- and *trans*-L-4-fluoroproline.

TABLE 1

Comparison of retention times of 4-fluoroprolyl alcohols used to establish configuration of racemates of FPCA

The starting compounds shown below were converted to the *N*-benzoyl derivatives of their respective prolyl alcohols as described in the text. Gas-liquid chromatography was performed on a 6-foot column of 1% OV-17 maintained at 215° in a Glowall 320 instrument.

Starting compound	Retention time
	min
trans-L-4-Fluoroproline[a]	33.4
3-Fluoro-2-pyrrolidone-5-carboxylic acid (peak I)[a]	35.6
cis-L-4-Fluoroproline[a]	41.6
3-Fluoro-2-pyrrolidone-5-carboxylic acid (peak II)[a]	42.0
L-Proline[b]	39.7
2-Pyrrolidone-5-carboxylic acid[b]	38.6

[a] Mass spectra of these compounds indicated the fragmentation pattern of *N*-benzoyl-4-fluoroprolyl alcohol. We thank Mr. William Comstock, who obtained these analyses on an LKB gas-liquid chromatography mass spectrometer.

[b] Mass spectra of these compounds indicated the fragmentation pattern of *N*-benzoylprolyl alcohol.

the solvent was removed under a stream of N_2, the residue was dissolved in 1 ml of dioxane, and to this solution were added 30 mg of $LiAlH_4$ suspended in 1 ml of ether. The mixture was heated on a steam bath for 15 min, after which the reaction was terminated by the cautious addition of H_2O (0.12 ml). The product was extracted twice with ethyl acetate. After evaporation of the ethyl acetate, the product was dissolved in 1 N NaOH and converted to the *N*-benzoyl derivative, using a 2-fold excess of benzoyl chloride. The *N*-benzoyl derivative was extracted into ethyl acetate for gas-liquid chromatography.

For the reduction of authentic *cis*- or *trans*-4-fluoroproline, the methyl ester was first prepared by dissolving 0.5–0.8 mg of the 4-fluoroproline in 100 μl of methanolic HCl. After 2 hr at 25°, the solvent was completely removed by repeated evaporation of small additions of methanol under a stream of N_2. The residue was then dissolved in ether, and $LiAlH_4$ (2 mg) was added. After 15 min at 25°, 20 μl of H_2O were added, the mixture was extracted twice with 1.5-ml portions of ethyl acetate, and the *N*-benzoyl derivative was formed as described above.

Table 1 shows the retention times of the benzoyl derivatives of the 4-fluoroprolyl alcohols. These data indicate that the reduction of the first peak of FPCA in the Dowex 1 eluate according to Scheme 1 gives a 4-fluoroprolyl alcohol with the same relative configuration of substituents as that derived from the reduction of *trans*-4-fluoroproline. Similarly, the reduction of the second peak of FPCA yields the racemate of 4-fluoroprolyl alcohol with the same relative configuration as that derived from *cis*-4-fluoroproline. On the basis of these findings, FPCA in the first peak of the Dowex 1 eluate is considered *trans* with respect to the relative positions of fluorine and carboxyl substituents, while that in the second peak is assigned the *cis*- configuration.

The retention time of prolyl alcohol is also shown in Table 1, since this compound was formed as a side product during the reduction of FPCA, particularly with the *trans* racemate.

Enzyme assays. The kinetics of the reaction of beef liver L-glutamate dehydrogenase was monitored in a Gilford recording spectrophotometer at 340 mμ (20). The reaction mixture at 25° contained 100 μmoles of potassium phosphate buffer (pH 7.6), 0.5 μmole of DPN, and enzyme (Sigma, type 1; 2.4 international units/mg) in a total volume of 1 ml.

Glutamine synthetase was measured by the release of orthophosphate from ATP (21). The reaction mixture at pH 8.0 contained 2.5 μmoles of imidazole, 2.5 μmoles of triethanolamine, 5 μmoles of NH_4Cl, 0.75 μmole of ATP, enzyme, and substrate in a total volume of 0.1 ml. Incubation was carried out at 37° for 15 min. The enzyme was a homogeneous preparation from *E. coli* containing 1 adenyl group/12 subunits (22).[4]

[4] We thank Dr. Richard E. Miller for his gift of this enzyme and for his advice on the assay procedure.

The assay of D-glutamate cyclase partially purified from mouse liver (23) was based on the formation of ninhydrin-positive FGlu obtained when FPCA is hydrolyzed (23).

Reaction of diastereomers of L-FGlu and DL-FPCA with several enzymes. The kinetics of the reaction of FGlu with glutamate dehydrogenase has been studied previously (24) but can now be examined in more detail, using the separate diastereomers of L-FGlu. The preliminary studies shown in Fig. 2 indicate that both diastereomers of L-FGlu react with the enzyme and that under these experimental conditions both analogues have Michaelis constants comparable to that for the natural substrate (Table 2).[5] Of interest is the V_{max} with *threo*-L-FGlu (Table 2), which suggests that this analogue may rival L-norvaline, the best amino acid substrate other than glutamic acid found previously (25).

Both diastereomers of L-FGlu are also substrates for glutamine synthetase of *E. coli*, and a preliminary study of the kinetics of the reaction of the two diastereomers was made under the reaction conditions described (Fig. 3). The kinetic constants for the analogues are summarized in Table 2. As with glutamate dehydrogenase, the V_{max} of *threo*-L-FGlu, although not as great as for L-glutamate, is greater than that for *erythro*-L-FGlu. Under these experimental conditions the Michaelis constants of the analogues are comparable to that of glutamate. The fluoro

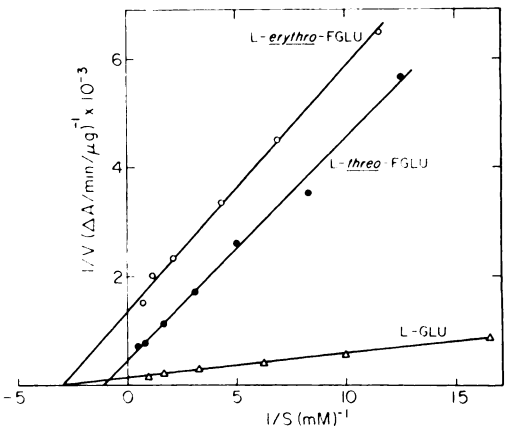


FIG. 2. *Double-reciprocal plot of reaction of L-glutamate dehydrogenase with L-glutamate and the two diastereomers of L-FGlu*

Assay conditions are described in the text. For the reaction with L-glutamate, each reaction mixture contained 3 μg of enzyme protein, while for the diastereomers of L-FGlu the reaction mixtures contained 30 μg of protein.

analogues are apparently transformed to fluoroglutamine by the crystalline enzyme, since a new ninhydrin-reactive compound is obtained when the reaction product is subjected to thin-layer chromatography (Eastman 6064 cellulose plate developed with methanol–H_2O–pyridine, 20:5:1).

Both diastereomers of FPCA are substrates for D-glutamate cyclase. Whereas the Michaelis constant for the *cis* diastereomer is comparable to that for 2-pyrrolidone-5-carboxylic acid, that for the *trans* diastereomer is considerably higher. In spite of this difference in affinity for the enzyme, the V_{max} for hydrolysis of both fluorinated substrates is much greater than that of 2-pyrrolidone-5-carboxylic acid.

The relative rates of the reaction of *E. coli* glutamate decarboxylase with the two diastereomers of L-FGlu were also examined under conditions previously described (11). With DL-FGlu at a concentration of 50 mM, the *threo* diastereomer was decarboxylated 3–4 times faster than the *erythro* diastereomer.

DISCUSSION

Previously it was possible to relate the configuration of the α-carbon of L-FGlu to

[5] Differences in substrate ionization are probably not significant as a basis for the difference in reactivity of glutamic acid and its analogues. For example, the pK'_2 of glutamic acid is 4.25 and that for FGlu is considerably lower, owing to the fluorine substituent. Hence the carboxyl groups of glutamate and the diastereomers of FGlu are completely ionized in the pH range 7.6–8.0. Pronation of the amines of glutamic acid and FGlu might be slightly different in this pH range, since the pK'_3 of glutamic acid (9.65) is slightly higher than that of FGlu (9.45, determined by half-titration). However, even at pH 8.0 the amine of FGlu would be 96% protonated compared to the 98% protonation of glutamic acid. Presumably the diastereomers of FGlu do not differ greatly in their respective pK values, since their separation on both anion- and cation-exchange resins was achieved with difficulty.

TABLE 2

Comparison of kinetic constants for various enzymes of glutamate or pyroglutamate and its fluoro analogues
Assay conditions are described in the text.

Substrate	Glutamate dehydrogenase[a]		Glutamine synthetase[a]		D-Glutamate cyclase	
	K_m	V_{max}[b]	K_m	V_{max}[b]	K_m	V_{max}[b]
	mM		*mM*		*M*	
L-Glutamate	0.5	1.0	4	1.0		
L-*threo*-FGlu	0.9	0.21	3	0.57		
L-*erythro*-FGlu	0.3	0.07	4	0.09		
DL-2-Pyrrolidone-5-carboxylic acid					0.09	1.0
DL-*cis*-FPCA					0.2	56
DL-*trans*-FPCA					1.5	56

[a] Each substrate was tested with different amounts of enzyme (see legends to Figs. 2 and 3); hence the calculation of relative V_{max} among the three substrates is made on the assumption that the reaction rate is linearly related to enzyme concentration within this range. Careful studies with glutamine synthetase indicate that this assumption is not strictly correct, possibly because of differences in subunit aggregation (R. E. Miller and E. R. Stadtman, personal communication). Nevertheless, it seems useful to portray the relative properties of the substrates in this way.

[b] Values of V_{max} are expressed relative to L-glutamate for glutamate dehydrogenase and glutamine synthetase and relative to DL-2-pyrrolidone-5-carboxylic acid for D-glutamate cyclase; for these substrates the value of V_{max} is arbitrarily set at 1.0.

that of L-glutamic acid by the specificity of two enzymes, glutamate decarboxylase and carboxypeptidase G. The tentative assignment of the configuration of FGlu made on that basis now appears to be consistent also with the specificity of several additional enzymes. These include L-glutamate dehydrogenase and D-glutamate cyclase (23, 26), both of which are specific for a particular configuration of glutamate. This tentative assignment is also in accordance with the actions of glutamine synthetase (21) and leucine aminopeptidase (17), which react well with the isomers of FGlu tentatively assigned the L-configuration. Although the configuration of FGlu must still be considered provisional, it should be noted that all enzymes reactive with the analogue support the assignment.

Our preliminary enzyme studies suggest some potentially interesting additions to previous work. For example, the observation that *threo*-L-FGlu is more reactive with glutamate decarboxylase than the *erythro* diastereomer correlates with the finding that L-*threo*-γ-hydroxyglutamate is a substrate for this enzyme while L-*erythro*-γ-

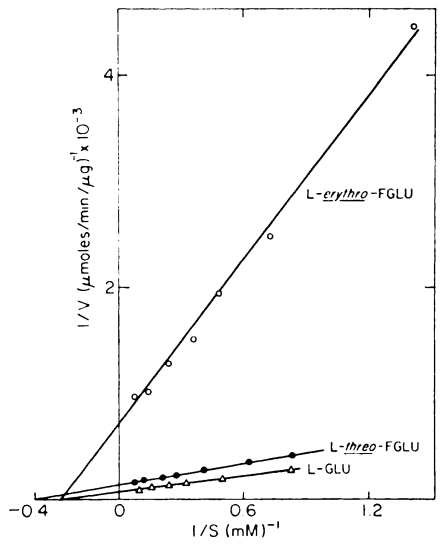


FIG. 3. *Double-reciprocal plot of reaction of glutamine synthetase with* L-*glutamate and the two diastereomers of L-FGlu*

For assay conditions, see the text. With L-glutamate as substrate the reaction mixture contained 0.25 μg of enzyme, while 1.0 μg was used with L-*threo*-FGlu and 7.4 μg for L-*erythro*-FGlu.

hydroxyglutamate is not (13). Presumably the interaction of the enzyme with substrate is diminished more when 1 of the 2 hydrogens at the α-carbon of L-glutamic acid is replaced by the slightly larger fluorine atom. However, when the substituent at this position is the larger hydroxyl group, steric considerations make the resulting glutamate derivative no longer suitable as a substrate.

With D-glutamate cyclase, both *cis*- and *trans*-FPCA are hydrolyzed more rapidly than 2-pyrrolidone-5-carboxylic acid, an effect which probably can be attributed to the electronegativity of fluorine and the consequent withdrawal of electrons from the lactam bond. Since this effect is the same for both diastereomers, it arises from the presence of fluorine at the γ-carbon and is not stereospecific. On the other hand, the stereospecificity of the fluorine substituent affects the affinity of the enzyme for the substrate, since the K_m of *trans*-FPCA is almost an order of magnitude greater than that of *cis*-FPCA. Perhaps this kind of information, together with knowledge of the conformational differences between the two fluorinated diastereomers, can eventually help to define relationships between the various substrates and the catalytic site of the enzyme.

FGlu has now been shown to react with a sizable number of enzymes, suggesting that its effect on the metabolism of whole organisms may not be as selective as that of fluoroacetate. FGlu is nontoxic to mice, and no antibacterial, antiviral, or antitumor activity has been found (16). Nevertheless it may exert some interesting physiological effects since in spinal neurons FGlu exerts a longer excitatory effect than glutamate.[6]

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