Resolution of γ-Methyl and γ-Fluoroglutamic Acids. Lack of Stereospecificity of Leucine Aminopeptidase with L-Leucyl-L-*erythro*-γ-substituted Glutamates

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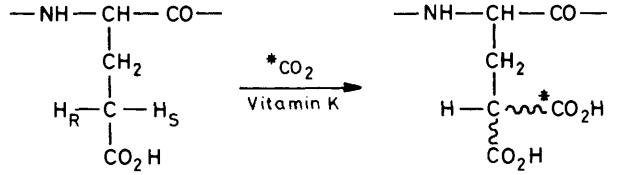
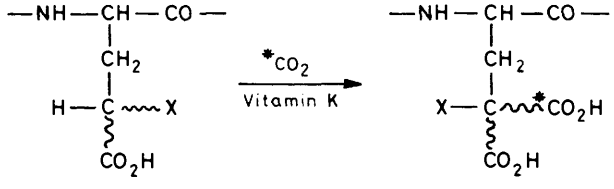
The hydrolysis of L-leucyl-γ-substituted D,L-glutamates by leucine aminopeptidase, from porcine kidney, is stereospecific with *threo*-γ-methyl and *threo*-γ-fluoroglutamate containing dipeptides whereas there is a lack of stereospecificity with the *erythro*-isomers. The optical purities of L-*threo*- and L-*erythro*-glutamate isomers thus obtained have been monitored by gas chromatography, high pressure liquid chromatography, or nuclear magnetic resonance. The optical rotations of optically pure L-isomers have been measured and the discrepancies with former publications are discussed.

In order to determine structure–activity relationships for peptides using unnatural analogues of amino acids, it is necessary to have optically pure enantiomers or diastereoisomers of the analogues. In some cases, data are either missing or even contradictory and then it is not possible to assess the optical purity of a product after synthesis and resolution. It was for this reason that we re-examined the optical purities of γ-methyl- and γ-fluoro-glutamic acids during a study of the stereochemistry of the vitamin K dependent-carboxylation [1-3] of glutamyl residues incorporated in short peptides which we had used in place of the normal substrates, *i.e.*, the endogenous precursor of coagulation factors.[4]



Endogenous precursor or peptide

In order to elucidate the carboxylation stereochemistry it was necessary to determine which of the two diastereotopic hydrogens is involved in the reaction and also the absolute configuration of the γ carbon of γ-carboxyglutamic acid when labelled CO_2 is used. The last part of the determination is difficult because of the easy epimerization at the malonic γ carbon. To overcome this we decided to work with γ substituted glutamic acid-containing peptides.[5,6]



X = Me, F

γ-Methyl and γ-fluoroglutamic acids have already been described and, in both series, *threo*- and *erythro*-isomers have been isolated and identified. Optically active *threo*- and *erythro*-γ-methylglutamic acids have been obtained by Fowden *et al.*[7] and by Kagan and Meister [8] but these groups reported very different optical rotations. Although the optically active *threo*- and *erythro*-γ-fluoroglutamic acids have been prepared by Unkeless and Goldman [9] by hydrolysing the dipeptides L-leucyl-γ-fluoroglutamic acid with leucine aminopeptidase, and their optical purities determined using glutamate decarboxylase, the optical rotations of these acids were not published.

We decided to re-examine the resolution of γ-fluoro- and γ-methylglutamic acids by hydrolysing L-leucylglutamate dipeptides with leucine aminopeptidase and monitoring these resolutions by n.m.r. spectroscopy in the presence of optically active shift reagents,[10] gas chromatography (g.c.) on optically active columns,[11] and high pressure liquid chromatography (h.p.l.c.) with optically active eluants. Furthermore we have tested these products with glutamate decarboxylase. We report here on the problems met during the resolution of those amino acids.

Materials and Methods.—*N*-t-Butoxycarbonyl-L-leucine was from Bachem (Bubendorf, Switzerland), dicyclohexylcarbodi-imide and *N*-hydroxysuccinimide from Fluka (Buchs, Switzerland), leucine aminopeptidase (EC3.4.11.1) (cytosol from Porcine kidney, type III CP) glutamate decarboxylase (EC4.1.1.15) (type V from E.coli), pyridoxal phosphate from Sigma Chemical Co (St Louis, USA), formic acid and acetic acid from Prolabo (Paris, France), AG1X4 and AG1X2 from Biorad (Richmond, USA); γ-methyl glutamic acid and γ-fluoroglutamic acid were synthesized as previously reported [5,6] respectively according to Done and Fowden,[12] Hudlicky,[13] and Buchanan *et al.*[14] All other chemicals were of the highest purity available.

Syntheses of Dipeptides.—L-*Leucyl*-D,L-(threo + erythro)-γ-*methylglutamate*. *N*-t-Butoxycarbonyl-L-leucine *N*-hydroxysuccinimide ester (4.92 g, 14.9 mmol) in ethanol (30 ml) were added to D,L-(*threo* + *erythro*) γ-glutamates (1.2 g, 7.45 mmol) dissolved in water (19 ml) and triethylamine (3.24 ml). The mixture was stirred for 2 h at room temperature and then

the solvent was evaporated under reduced pressure. The residue was dissolved in water and the pH adjusted to 3 with HCl. Extraction with ethyl acetate yielded crude product (2.8 g) which was stirred in a trifluoroacetic acid–methylene chloride (1 : 1; 20 ml) for 2.5 h. Trifluoroacetic acid was removed under reduced pressure and the dipeptide was purified on an AGIX2 column (eluant, 1M-acetic acid; yield 1.57 g, 77%).

L-*Leucyl*-D,L(threo + erythro)-γ-*fluoroglutamate*. The same procedure as above starting with D,L(*threo* + *erythro*)-γ-fluoroglutamate (2.3 g, 14.65 mmol) yielded the corresponding dipeptide (2 g, 50%).

Chromatographic Separations.—D,L-threo-γ-*Methylglutamate and* D,L-erythro-γ-*methylglutamate*. The separation was achieved on an AG1X2 column (104 × 3.6 cm, acetate form). After extensive washing with water the acids were eluted with acetic acid (0.05 mol/l) and, the elution was monitored by titration with ninhydrin.[15] The D,L-*threo*-isomer was eluted first[7] (between 7.0 and 8.2 l) followed by the D,L-*erythro*-isomer (between 8.55 and 10.3 l).

D,L-threo-γ-*Fluoroglutamate and* D,L-erythro-γ-*fluoroglutamate*. The separation was achieved on an AG1X4 column (105 × 3.6 cm, formate form). After extensive washing with water the acids were eluted with formic acid (0.25 mol/l) and the elution monitored by ninhydrin titration.[15] The D,L-*erythro*-isomer eluted first[9] between 11.2 and 12.51 followed by the D,L-*threo*-isomer between 13 and 14.2 l.

L-*Leucyl*-D-erythro-γ-*methylglutamate and* L-*leucyl*-L-erythro-γ-*methylglutamate*. A mixture of both L-leucyl peptides was prepared as above using diastereoisomerically pure D,L-*erythro*-γ-methylglutamate. The separation was achieved on an AG1X2 column (104 × 3.6 cm, acetate form). After washing with water, elution was performed with acetic acid (2 l of 0.03 mol/l followed by 0.05 mol/l). L-Leucyl-D-*erythro*-γ-methylglutamate was eluted first (*vide infra*) between 2.9 and 3.1 l followed by L-leucyl-L-*erythro*-γ-methylglutamate between 3.5 and 4.0 l. L-Leucyl-D-*erythro*-γ-methylglutamate, $[\alpha]_D^{20}$ 63° (*c* 1, acetic acid); δ_H(D_2O, external reference: hexamethyldisilazane) 1.26 (d, 6 H, CH_3Leu), 1.50 (d, 3 H, CH_3 MeGlu), 1.98 (m, 4 H, CH_2), 2.71 (m, 1 H, $CHCH_3$), 4.28 (t, 1 H, $CHNH_2$), and 4.53 (q, 1 H, $CHNH_2$). L-Leucyl-L-*erythro*-γ-methylglutamate, $[\alpha]_D^{20}$ 10° (*c* 1, acetic acid); δ_H(D_2O, external reference: hexamethyldisilazane) 1.26 (d, 6 H, CH_3Leu), 1.49 (d, 3 H, CH_3 MeGlu) 2.02 (m, 4 H, CH_2), 2.78 (m, 1 H, $CHCH_3$), 4.30 (t, 1 H, $CHNH_2$), and 4.56 (q, 1 H, $CHNH_2$).

L-*Leucyl*-D-erythro-γ-*fluoroglutamate and* L-*leucyl*-L-erythro-γ-*fluoroglutamate*. A mixture of both L-leucyl peptides was prepared as above using diastereoisomerically pure D,L-*erythro*-γ-fluoroglutamates. The separation was achieved on an AG1X4 column (54 × 2.2 cm, formate form). Elution was performed with water (300 ml) followed by formic acid (0.25 mol/l). L-Leucyl-L-*erythro*-γ-fluoroglutamate was eluted first (*vide infra*) between 0.75 and 0.9 l followed by L-leucyl-D-*erythro*-γ-fluoroglutamate between 0.9 and 1.0 l. L-Leucyl-L-*erythro*-γ-fluoroglutamate, $[\alpha]_D^{20}$ 10.4° (*c* 0.5, 1M-HCl), δ_H(D_2O, external reference: hexamethyldisilazane) 1.24 (d, 6 H, CH_3 leucine), 1.87 (m, 1 H, CHγleucine), 2.04 (d, 2 H, CH_2 leucine), 2.68 (m, 2 H, CH_2γFGlu), 4.32 (t, 1 H, CHαleucine), 4.96 (m, 1 H, CHαγFGlu), and 5.29 (m, 1 H, CH F); δ_F (D_2O, external reference: trifluoroacetate) 112.2 (q, 1,2,2,2,1; J_{H_1F} 50, J_{H_2F} 22). L-Leucyl-D-*erythro*-γ-fluoroglutamate, $[\alpha]_D^{20}$ 29.8° (*c* 0.5, 1 M-HCl); δ_H(D_2O, external reference: hexamethyldisilazane) 1.24 (d, 6 H, CH_3 leucine), 1.87 (m, 1 H, CHγleucine), 2.01 (d, 2 H, CH_2 leucine), 2.66 (m, 2 H, CH_2γFGlu), 4.32 (t, 1 H, CHαleucine), 4.88 (m, 1 H, CHαγFGlu), and 5.26 (m, 1 H, CHF); δ_F (D_2O, external reference trifluoroacetate) 112.2 (q, 1,2,2,2,1; J_{H_1F} 50, J_{H_2F} 22).

Resolutions with Leucine Aminopeptidase.—*Resolution of a mixture of* L-*leucyl*-D,L-(threo + erythro)-γ-*methylglutamates*. The dipeptide (1.5 g) was hydrolysed at 37 °C in Tris buffer (230 ml, 0.1 mol/l, pH 8.9) containing magnesium sulphate (98 μmol/l) by leucine aminopeptidase (96 units). The reaction was monitored by t.l.c. (silica gel, eluant: butanol–pyridine–water–acetic acid, 6 : 6 : 4.8 : 1.2). After 2.5 h, the reaction slowed and the mixture was poured onto an AG1X2 column (100 × 2 cm, acetate form). Leucine was eliminated by washing with water and the elution performed with acetic acid (0.03 mol/l) yielded successively two dipeptides containing fractions, between 1.33 and 1.93 l (0.250 g corresponding presumably to L-leucyl-D-*erythro*-γ-methylglutamate) and between 2.16 and 2.83 l (0.260 g corresponding presumably to dipeptides with L and D-*threo*-γ-methylglutamate along with unchanged L-*erythro*-containing dipeptide) followed by two γ-methylglutamic acid containing fractions, between 4.30 and 4.86 l (0.188 g, L-*threo*-γ-methylglutamate) and between 5.13 and 5.76 l (0.294 g, L-*erythro*-γ-methylglutamate).[7] L-*threo*-γ-Methylglutamate, $[\alpha]_D^{20}$ 22° (*c* 5, H_2O); $[\alpha]_D^{20}$ 36.6° (*c* 2.5, 6M-HCl); δ_H(D_2O, external reference hexamethyldisilazane) 1.54 (d, *J* 7, 3 H, CH_3), 2.34 (m, 2 H, CH_2), 3.2 (m, 1 H, $CHCH_3$), and 4.15 (t, 1 H, $CHNH_2$). L-*erythro*-γ-Methylglutamate: this batch was only 70% optically pure (*vide infra*) and has an $[\alpha]_D^{20}$ 16° (*c* 2.5, 6 M-HCl); δ_H(D_2O, external reference hexamethyldisilazane) 1.54 (d, *J* 7, 3 H, CH_3), 2.20 (m, 1 H, *H*CH), 2.55 (m, 1 H, HC*H*), 2.96 (m, 1 H, $CHCH_3$), *and* 4.12 (t, 1 H, $CHNH_2$).

Hydrolysis of L-*Leucyl*-L-erythro-γ-*methylglutamate*.—The dipeptide (220 mg) was hydrolysed at 37 °C in Tris buffer (30.8 ml, 0.1M, pH 9) containing magnesium sulphate (0.103 mmol/l) by leucine aminopeptidase (25 units). After 1 h the hydrolysis was almost complete and the mixture was poured onto an AG1X2 column. Elution as above yielded L-*erythro*-γ-methylglutamate (118 mg, 91%). L-*erythro*-γ-Methylglutamate, $[\alpha]_D^{20}$ 20° (*c* 1, 6M-HCl), $[\alpha]_D^{20}$ 22.7° (*c* 2.5, 6M-HCl).

Hydrolysis of L-*Leucyl*-D-erythro-γ-*methylglutamate*.—The dipeptide (120 mg) was hydrolysed at 37 °C in Tris buffer (16.8 ml, 0.1M, pH 9) containing magnesium sulphate (0.103 mmol/l) by leucine aminopeptidase (13 units). After 1 h further enzyme (13 units) was added and the mixture incubated for a further hour. The crude product was poured onto an AG1X2 column as above. Leucine was eluted with water, unchanged dipeptide with 0.05M-acetic acid and D-*erythro*-γ-methylglutamate with 0.5M-acetic acid. Lyophilisation yielded the acid (20.2 mg, 29%). D-*erythro*-γ-Methylglutamate, $[\alpha_D]^{20}$ −19° (*c* 1, 6M-HCl).

Resolution of a mixture of L-*leucyl*-D,L-(threo + erythro)-γ-*fluoroglutamates*. The dipeptide (2.1 g) was hydrolysed at 36 °C in Tris buffer (300 ml, 0.1 mol/l, pH 9.0) containing magnesium sulphate (0.1 mmol/l) by leucine aminopeptidase (160 units). After 3.5 h, the mixture was applied to an AG1X4 column (105 × 2.6 cm, formate form). Leucine was eliminated by washing with water (2 l) followed by 0.025M-formic acid. Elution with 0.025M-formic acid yielded successively L-*erythro*-γ-fluoroglutamate (278 mg between 5.10 and 5.84 l) and L-*threo*-γ-fluoroglutamate (142.5 mg between 6.30 and 7.20 l).[9] Intermediate fractions were pooled and resubmitted to chromatography on the same column. L-*threo*-γ-Fluoroglutamate, $[\alpha]_D^{20}$ 14.6° (*c* 1, 1M-HCl); δ_H (CF_3CO_2H, external reference hexamethyldisilazane) 2.75 (dt, J_{HH} 5.4, J_{HF} 23.4, 2 H, CH_2), 4.5 (m, 1 H, CH), 5.26 (dt, J_{HF} 48, $J_{1H} = J_{2H}$ = 5.4, 1 H, CHF), and 7.42 (s, 3 H, H_3N^+); δ_F(D_2O–CD_3OD, external reference: trifluoroacetate): 114.4 (q 1,2,2,2,1, J_{HF} 47.3, $J_{1HF} = J_{2HF}$ =

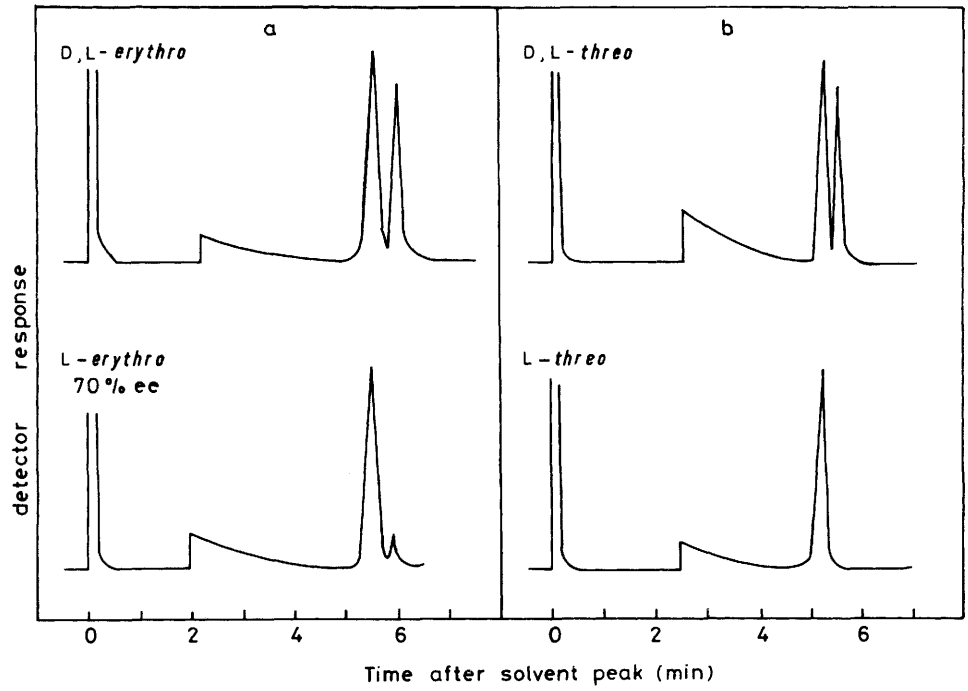


Figure 1. Gas chromatography determination of *erythro*- and *threo*-γ-methylglutamic acid optical purities [27]

24.4).* L-*erythro*-γ-Fluoroglutamate: this batch was only 87% optically pure (*vide infra*) and has an $[\alpha]_D^{20}$ 28° (*c* 1, 1M-HCl); $\delta_H(CF_3CO_2H$, external reference hexamethyldisilazane) 2.63 (m, 2 H, CH_2) 4.54 (m, 1 H, CH) 5.35 (td, J_{HF} 48, J_{1H} 9.2, J_{2H} 3, 1 H, CHF), and 7.44 (s, 3 H, NH_3^+); $\delta_F(D_2O,CD_3OD$, external reference: trifluoroacetate) 114.9 (sextuplet, 1,1,1,-2,1,1,1, J_{HF} 49.6, J_1 33.4, J_2 16.6).*

Hydrolysis of L-*leucyl*-L-erythro-γ-*fluoroglutamate*. The peptide (475.4 mg) was hydrolysed at 37 °C in Tris buffer (71.3 ml, 0.1M, pH 9.0) containing magnesium sulphate (0.100 mmol/l) by leucine aminopeptidase for 5.5 h. Enzyme (178 units) was added initially followed by aliquots after 1 h (178 units) and 3.5 h (178 units). The crude mixture was poured onto an AG1X4 column. Washing with water followed by elution with 1M-formic acid yielded the acid (250.3 mg). L-*erythro*-γ-Fluoroglutamate, $[\alpha]_D^{20}$ 32° (*c* 1, 1M-HCl).

Epimerization of threo- *and* erythro-γ-*Methylglutamate*.—Diastereoisomerically pure D,L *erythro*-γ-methylglutamate (2.8 mg) was treated with 6M-HCl (1 ml) at 110 °C in a sealed tube for 24 h. After careful elimination of HCl the crude product was analyzed on an AG1X2 column (76 × 1 cm, acetate form), with 0.5M-acetic acid as eluant. Elution was monitored with ninhydrin. *threo*-γ-Methylglutamate (23%) eluted between 0.56 and 0.68 l whereas *erythro*-γ-methylglutamate (77%) eluted between 0.68 and 0.89 l.

Optical Purity Determinations.—Gas chromatography on optically active columns.[11] Separation of enantiomers of *threo* and *erythro*-γ-methylglutamic acids was achieved on *N*-trifluoroacetyl isopropyl esters on capillary columns (50 m × 0.5 mm) coated with the docosanoyl-t-butylamide of D-valine at 180 °C. Controls were run with L-valine-coated columns.

High Performance Liquid Chromatography.[17–19]—Analyses of all the γ substituted glutamic acids were performed on a reverse-phase Nucleosil C 18 column (24 × 0.6 cm) with *N,N*-di-n-propyl-L-alanine (8 mmol/l) and cupric acetate (4 mmol/l) in the aqueous mobile phase. Detection was achieved by post-column derivatization with *ortho*-phthalaldehyde and fluorescence determination.

^{1}H *N.m.r. in the Presence of Optically Active Shift Reagent.*[10]—Spectra were recorded on a FT80A Varian spectrometer in deuteriated chloroform with tetramethylsilane as internal reference. Increasing concentrations of tris [3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III) were added to the racemic and optically active isomers of γ-methylglutamates (α-methyl, γ-benzyl diesters) (0.1M), until a clean separation of the methyl ester signal was obtained. Titration was achieved by comparison of the area of the signal (Figure 2).

Glutamate Decarboxylase.—In all the experiments, decarboxylation was achieved in pyridine–HCl buffer (50 mmol/l, pH 4.4) at 30 °C in the presence of pyridoxal phosphate. Aliquots were sampled at the times indicated in Tables 1 and 2 and analyzed. In the γ-methylglutamate series, unchanged γ-methylglutamate was separated from the crude reaction mixture by anion exchange chromatography. An aliquot was poured onto an AG1X2 column (7 × 1.4 cm, acetate form). 2-Methyl-4-aminobutyric acid was eluted with water (50 ml) and the unchanged γ-methylglutamic acid with 1M-acetic acid (50 ml).

Following concentration under reduced pressure the glutamic acid was titrated with ninhydrin [15] with the corresponding γ-methylglutamic acid as standard (we observed that the response to the ninhydrin titration was identical for the *threo*- and *erythro*-isomers).

* Bergmann and Chun-Hsu [16] reported a ^{19}F n.m.r. spectrum of γ-fluoroglutamic acid without mentioning the presence of one or two diastereoisomers in their product. The parameters of their spectrum q(1,2,2,2,1) J_1 49.6; J_2 23.48 resemble closely those we found for the *threo*-isomer. It is thus probable that their synthesis and work-up lead almost exclusively to the *threo*-isomer.

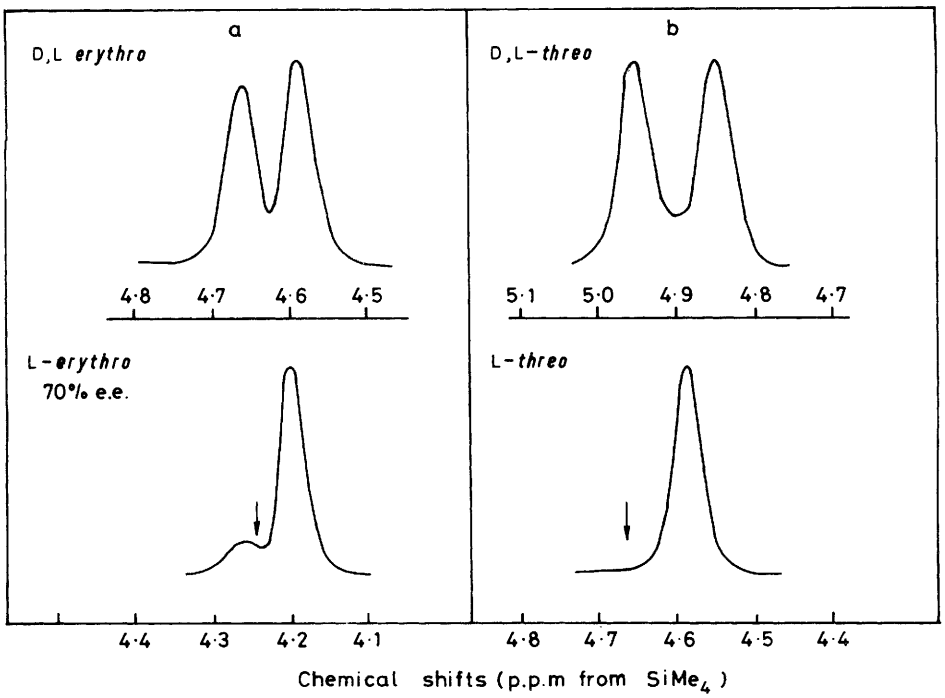


Figure 2. Determination of the optical purities of *threo*- and *erythro*-γ-methylglutamates by ^{1}H nuclear magnetic resonance in the presence of optically active shift reagent.

The spectra of α methyl γ benzyl diesters of γ-methylglutamates were recorded in deuteriated chloroform. These figures show the part of the spectra corresponding to the methoxy group. The amount of shift reagent was not exactly the same in all the experiments and the arrows appearing in the spectra of L-amino acids correspond to the chemical shifts of the D isomers evaluated from curves correlating the chemical shift of the L amino acid signal and the separation of L and D amino acid methoxy groups measured at different shift reagent concentrations (curve not shown)

In the γ-fluoroglutamate series the reaction was monitored by titration of 2-fluoro-4-aminobutyric acid with ninhydrin [15] using a 0.85 ratio compared with glutamic acid.[20] After sampling, the aliquot was applied to an AG1X4 column (7 × 1.4 cm, formate form) and the 2-fluoro-4-aminobutyric acid was eluted with water (50 ml) before determination.

Results

Leucine Aminopeptidase.—We have observed, that in both γ-methyl and γ-fluoroglutamate series, the L-*threo*-isomers (2*S*,4*S*) obtained after hydrolysis of dipeptides L-leucine-X (where X is a mixture of racemic *threo*- and *erythro*-isomers) with leucine aminopeptidase (EC 3.4.11.1) were optically pure. This is illustrated for the L-*threo*-γ-methylglutamate by gas chromatography (Figure 1b) and ^{1}H n.m.r. (Figure 2b) and for the L-*threo*-γ-fluoroglutamate by h.p.l.c. [Figure 3b). In neither case, could the D-isomer be detected.

However, unexpectedly, the L-*erythro*-isomers (2*S*,4*R*) were not optically pure, as shown for the L-*erythro*-γ-methylglutamate by gas chromatography (Figure 1a) or by ^{1}H n.m.r. (Figure 2a) and for the L-*erythro*-γ-fluoroglutamate by h.p.l.c. (Figure 3a). Quantitation using peak areas indicated that optical purities were only 70% for the L-*erythro*-γ-methylglutamate and 87% for the L-*erythro*-γ-fluoroglutamate.

Optically pure L-*erythro*-isomers have been obtained through hydrolysis of pure L-leucyl-L-*erythro*-γ-fluoroglutamate and L-leucyl-L-*erythro*-γ-methylglutamate thoroughly separated from their diastereoisomers by anion exchange chromatography, the L-leucyl-L-*erythro*-γ-substituted glutamate being eluted first in the γ-fluoro series and second in the γ-methylglutamate series as revealed by the optical rotations of the γ-substituted glutamates obtained from each isomer by hydrolysis (*vide infra*).

We observed that treatment of D,L-*erythro*-γ-methylglutamate by HCl yielded a mixture of *threo*- and *erythro*-isomers (23 : 77) so that it was impossible to use HCl hydrolysis of these leucyl peptides to recover pure L-*erythro*-isomers. Hydrolysis of diastereoisomerically pure dipeptides was achieved under very mild conditions using leucine aminopeptidase, leading thus to optically pure L-*erythro*-isomers of γ-fluoroglutamate and γ-methylglutamate. In addition we observed that leucine aminopeptidase could hydrolyse L-leucyl-D-*erythro*-γ-methylglutamate, although at a slower rate than L-leucyl-L-*erythro*-γ-methylglutamate, yielding D-*erythro*-γ-methylglutamate, confirming the low specificity of the enzyme.

Glutamate Decarboxylase.—Glutamate decarboxylase (EC 4.1.1.15), which catalyzes the decarboxylation of glutamic acid into γ-aminobutyric acid, is reported to be very specific for glutamates with the L-configuration and is commonly used for optical purity determinations. In each series, both D,L- and L-*threo* and -*erythro*-isomers were tested. Results are quoted in Table 1 for the γ-methylglutamate and in Table 2 for the γ-fluoroglutamate. It appears that the enzyme is specific for the L-isomers in both series. The reaction was blocked at 50% with all the D,L-isomers, whereas, although we have not waited for a 100% decarboxylation, the reaction went far beyond the 50% value with the L-isomers. In both series, the *threo* was decarboxylated faster than the *erythro*-isomer.

Discussion

The hydrolysis of dipeptides L-leucyl-X (where X is the D,L *threo*- or D,L *erythro*-isomer of γ-methyl or γ-fluoroglutamate)

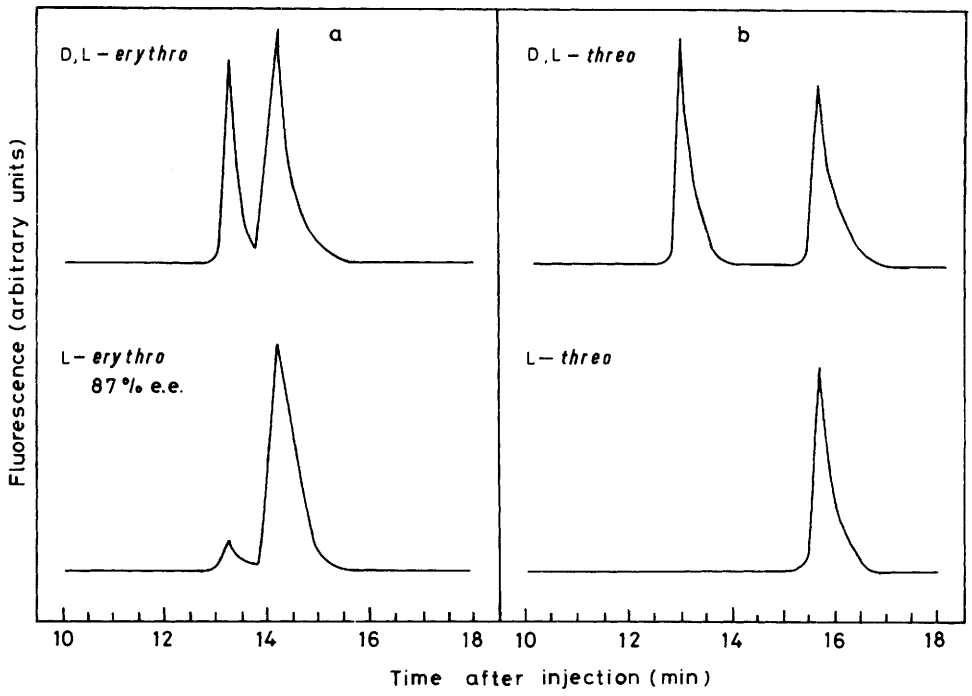


Figure 3. High pressure liquid chromatography determination of *erythro*- and *threo*-γ-fluoroglutamic acid optical purities

Table 1. Decarboxylation of γ-methylglutamic acids with glutamate decarboxylase *

Acid	[Acid] (mmol/l)	Glutamate decarb. (units/ml)	% Decarb-oxyl-ation
D,L-*threo*-γ-MeGlu	13	5.15	53.6
L-*threo*-γ-MeGlu	13	5.15	96
D,L-*erythro*-γ-MeGlu	5	5.15/9.84 †	41
L-*erythro*-γ-MeGlu	5	5.15/9.84 †	89

* *threo*-γ-Methylglutamates were incubated at 32 °C for 22 h in pyridine–HCl buffer (50 mmol/l, pH 4.5) containing pyridoxal phosphate (0.5 mmol/l). After incubation, unchanged γ-methylglutamate was isolated and determined with ninhydrin as described in the Experimental section. Blanks were run without enzyme. † *erythro*-γ-Methylglutamates were incubated under the same conditions (5.15 units/ml) for 22 h. Enzyme was added again (final glutamate decarboxylase: 9.84 units/ml) and the incubation continued for 24 h.

Table 2. Decarboxylation of γ-fluoroglutamic acids with glutamate decarboxylase *

Acid	Time (h)	% Decarb-oxylation
D,L-*erythro*-γ-F Glu	0.5	52.5 †
D-*erythro*-γ-F Glu	0.75	51
	3	69
	18	83
D,L-*threo*-γ-F Glu	0.5	46 †
L-*threo*-γ-F Glu	0.75	80

* γ-F-Glu (12 mmol/l) was incubated at 30 °C in pyridine–HCl buffer (50 mmol/l, pH 4.4) containing pyridoxal phosphate (0.5 mmol/l) with glutamate decarboxylase (50 units/ml). 2-Fluoro-4-aminobutyric acid was isolated and determined with ninhydrin as described in the Experimental section. † Values corresponding to a plateau (constant from 0.5 to 24 h).

by leucine aminopeptidase yields optically active γ-substituted glutamates. The analyses of the optical purities of these amino acids by different methods yielded consistent results and revealed, that both L-*threo*-γ-substituted glutamates were optically pure, whereas unexpectedly, the L-*erythro*-γ-methylglutamate and the L-*erythro*-γ-fluoroglutamates were respectively 70 and 87% optically pure. The data concerning the specificity of the enzyme are still limited to the first papers by Smith *et al.*[21-25] and, in particular, the specificity toward L-leucyl-L-amino acids *versus* L-leucyl-D-amino acids has only been tested on a limited number of examples.[23,25] The lowest specificities were observed for L-leucyl-alanine and L-leucyl-phenylalanine for which the rates of hydrolysis of the L-D peptides were respectively 1.7 and 3.8% of the corresponding rate for L-L peptides.[25]

More recently Unkeless and Goldman used leucine aminopeptidase for the resolution of γ-fluoroglutamates and obtained optical purities greater than 98% for both *threo*- and *erythro*-isomers.[9] The origin of the discrepancy between their study and ours is not clear: they measured optical purities of both L-isomers with glutamate decarboxylase and we have checked that this enzyme is specific for both L-*threo* and L-*erythro*-γ-fluoroglutamate (Table 2). Was their leucine aminopeptidase different from ours (cytosol, Porcine kidney type III CP from Sigma Chemical Co.) and more specific? This hypothesis, although not very likely cannot be excluded.

Our study suggests that leucine aminopeptidase must be used cautiously for resolution but can be very useful in obtaining pure amino acids, since it hydrolyses diastereoisomerically pure dipeptides under very mild conditions.

With optically pure L-*erythro*-γ-methylglutamic acid at our disposal, it is now possible to suggest the origin of erroneous optical rotations reported in previous studies (Table 3). Blake and Fowden hydrogenated γ-methyleneglutamic acid from a natural source and separated the *threo*- and *erythro*-γ methylglutamates by chromatography on a Dowex X1 column.[7] The

Table 3. Comparison of optical rotations for γ-methylglutamic acids

	threo (*c*, solvent)	*erythro* (*c*, solvent)	Ref.
$[\alpha]_D^{20}$	36.6 (2.5, HCl 6M)	22.7 * (2.5, HCl 6M)	This study
	22.2 (2.5, HCl 6M)	19.2 (2.5, HCl 6M)	7
	30.2 (0.23, HCl 5M)	22 (0.66, HCl 5M)	8

* The optical rotation is dependent on concentration (see Experimental section)

optical purities of both L-diastereoisomers depend on the optical purity of the γ-methyleneglutamate. This latter was initially not optically pure since its optical rotation, 12.8°[7] was lower than the 14° rotation reported for a sample obtained by resolution of racemic γ-methyleneglutamate.[26] The optical purities of *threo*- and *erythro*-γ-methylglutamates, may then reflect an extra epimerization or contamination by an impurity during their isolation.

On the other hand, Kagan and Meister isolated the L-*threo*- and L-*erythro*-γ-methylglutamates from a mixture of natural isomers.[8] After treatment with glutamin synthetase which transforms *threo*-γ-methylglutamic acid into the corresponding glutamin but which is inactive on the *erythro*-isomer, the crude product was heated at 100 °C to cyclize the γ-methylglutamin and unchanged *erythro*-γ-methylglutamic acid was isolated by recrystallization. This isomer is almost optically pure but it is impossible to correlate with the optical purity of the starting material since the authors crystallized it at the end of the isolation.

The L-*threo*-γ-methylglutamate was obtained after isolation of the L-*threo*-γ-methylglutamin produced by action of glutamin synthetase.[8] The hydrolysis was achieved at 100 °C during 2 h in 3M-HCl,[8] a procedure which induces, as we have observed, an equilibration of *threo*- and *erythro*-γ-methylglutamates. It is highly probable that Kagan and Meister measured the optical rotation of a mixture of L-*threo*- and L-*erythro*-γ-methylglutamates corresponding to an apparent optical purity of 84% for L-*threo*-γ-methylglutamates.[8]

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