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Activity of Glutamine Synthetase toward *threo*-γ-Methyl-L-glutamic Acid and the Isomers of γ-Hydroxyglumatic Acid

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ABSTRACT: Previous studies on the unusual optical specificity of glutamine synthetase led to the hypothesis that L-glutamic acid is oriented on the enzyme in an extended conformation in which the α-hydrogen atom is directed away from the active site of the enzyme. This hypothesis has been examined further by studies on several γ-substituted derivatives of glutamic acid. Only one of the four isomers of γ-methylglutamic acid (*threo*-γ-methyl-L-glutamic acid) is a substrate. Of the four γ-hydroxyglutamic acids, *threo*-γ-hydroxy-L-glutamic acid is the most active substrate, but appreciable activity was also observed with *erythro*-γ-hydroxy-L-glutamic acid and *erythro*-γ-hydroxy-D-glutamic acid. These observations and the finding that *threo*-γ-hydroxy-D-glutamic acid is only slightly active are consistent with the original hypothesis and provide additional clues to the spatial relationships between the substrate and the enzyme. The substrate specificity of glutamine synthetase from peas is substantially the same as that of the enzyme isolated from sheep brain.

Earlier studies in this laboratory have shown that highly purified glutamine synthetase from sheep brain exhibits unusual optical specificity (Table I). Thus, the enzyme acts on both optical isomers of glutamic acid, but exhibits absolute L specificity toward α-methylglutamic acid and absolute D specificity toward *threo*-β-methylglutamic acid. β-Glutamic acid [which possesses a *meso* carbon atom (Schwartz and Carter, 1954)], is converted only to D-β-glutamine. The findings with glutamic acid, β-glutamic acid, and α-methylglutamic acid led to an hypothesis concerning the conformation of the enzyme-bound substrates, according to which L-glutamic acid is oriented on the enzyme in an extended conformation in which the α-hydrogen atom is directed away from the active site of the enzyme. The amino and carboxyl groups of D-glutamic acid (also in an extended conformation) are bound to the same respective sites of the enzyme as the corresponding groups of L-glutamic acid; thus, the α-hydrogen atom of D-glutamic acid is oriented toward the enzyme. The proposed conformations of these substrates are shown in color stereophotographs of models given in a previous communication (Kagan and Meister, 1966). This hypothesis led to prediction of the results subsequently obtained with the isomers of β-methylglutamic acid.

The present studies represent an extension of this work to γ-substituted derivatives of glutamic acid. Examination of the models of the extended conformations of L- and D-glutamic acid (Figure 5, Kagan and Meister, 1966) indicates that the *erythro*-γ-hydrogen atoms of both L- and D-glutamic acids occupy about the same position in space and lie just between the γ-carboxyl and amino groups of these molecules. Study of the models suggests that introduction of an *erythro*-γ-methyl group might provide considerable steric hindrance to formation of an activated γ-carboxyl derivative (*e.g.*, γ-glutamyl phosphate) and also to reaction of the amino group with the enzyme or metal nucleotide complex. The *threo*-γ-hydrogen atom of D-

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TABLE I: Optical Specificity of Glutamine Synthetase.[a]

Substrate	L Isomer	D Isomer	Lit.
Glutamic acid	+	+	Levintow and Meister (1953)
α-Methylglutamic acid	+	0	Kagan *et al.* (1965)
β-Glutamic acid	0	+[b]	Khedouri and Meister (1965)
threo-β-Methylglutamic acid	0	+	Kagan and Meister (1966)
erythro-β-Methylglutamic acid	0	0	Kagan and Meister (1966)
threo-γ-Methylglutamic acid	+	0	Present paper
erythro-γ-Methylglutamic acid	0	0	Present paper

[a] + = active substrate; 0 = inactive. [b] Product.

glutamic acid is very close to the α-hydrogen atom of D-glutamic acid; it might therefore be expected that *threo*-γ-methyl-D-glutamic acid, like α-methyl-D-glutamic acid, would not be a substrate. On the other hand, the *threo*-γ-hydrogen atom of L-glutamic acid is adjacent to the α-hydrogen atom of this molecule and is very close to the position of the *threo*-β-hydrogen atom of D-glutamic acid; replacement of the α-hydrogen atom of L-glutamic acid and of the *threo*-β-hydrogen atom of D-glutamic acid by a methyl group does not lead to loss of enzymatic susceptibility. These considerations suggested that *threo*-γ-methyl-L-glutamic acid would be a substrate for glutamine synthetase. The experimental results are in accord with this suggestion. Thus, we now report that of the four isomers of γ-methylglutamic acid, only one isomer, *threo*-γ-methyl-L-glutamic acid, is a substrate. Studies have also been carried out with the four γ-hydroxyglutamic acids; these experiments seem to provide additional clues as to the spatial relationships between the substrate and enzyme. In addition, the findings offer further support for the hypothesis that there is a specific site on the enzyme for the binding of ammonia.

Experimental Section

Materials. Glutamine synthetase was isolated from sheep brain as previously described (Pamiljans *et al.*, 1962). The enzyme was also isolated from peas; this preparation was carried through the ammonium sulfate fractionation step (sp act. 55.4 units/mg of protein) (Elliott, 1953). Sodium phosphoenolpyruvate, ATP,[1] and crystalline beef liver catalase were obtained from the Sigma Chemical Co. *Crotalus adamanteus* venom was obtained from Ross Allen's Reptile Institute, Inc., Silver Springs, Fla. γ-Methylene-L-glutamine, γ-methyl-L-glutamic acid,[2] *threo*-γ-hydroxy-L-glutamic acid, and pyruvate kinase were obtained from Calbiochem. α-Methyl-L-glutamic acid, α-methyl-D-glutamic acid (Kagan *et al.*, 1965), *threo*-β-methyl-D-glutamic acid (Kagan and Meister, 1966), and β-glutamic acid (Khedouri *et al.*, 1964) were obtained as described. γ-Methylene-DL-glutamic acid was generously supplied by Dr. Abraham Marcus. The authors are indebted to Dr. Elijah Adams for samples of the isomers of γ-hydroxyglutamic acid. These were also obtained from the corresponding racemates by enzymatic resolution (Benoiton *et al.*, 1957). The *threo*-γ-hydroxy-D-glutamic acid was further purified by exhaustive treatment with *Escherichia coli* L-glutamic decarboxylase kindly provided by Dr. J. S. Nishimura. Samples of β,γ-dihydroxyglutamic acid were donated by Dr. E. E. Dekker.

γ-Methylglutamic Acid. Several different preparations of γ-methylglutamic acid were used in these experiments. *Preparations 1* and *2* were synthesized by condensation of methyl methacrylate and diethyl acetamidomalonate (Done and Fowden, 1952); the amino acid was isolated from the acid hydrolysate of the condensation product as described (Meister, 1954). *Preparation 3* was obtained by chloroacetylation of preparation 2; the *N*-chloroacetyl derivative was crystallized from ethyl acetate–petroleum ether (bp 30–60°) and then hydrolyzed by refluxing with 3 N HCl for 3 hr. The product was crystallized from water–acetone as described (Meister, 1954). *Preparation 4* was obtained by recrystallizing preparation 3 twice from water–ethanol. *Preparation 5* was obtained by catalytic reduction of γ-methylene-DL-glutamic acid. The γ-methylene compound (100 mg) was dissolved in 8 ml of water and 500 mg of 10% palladium on charcoal was added. Hydrogenation was carried out essentially as described by Done and Fowden (1952) at atmospheric pressure. After 2 hr, the theoretical quantity of hydrogen had been absorbed and the catalyst was removed by filtration. The solution was evaporated under reduced pressure to yield a clear oil, which was dissolved in water; the amino acid concentration was determined by the quantitative ninhydrin method (Rosen, 1957). Paper electrophoresis (see below) indicated the presence of a single ninhydrin-positive compound corresponding to authentic γ-methylglutamic acid; no γ-methyleneglutamic acid was found. *Preparation 6* was obtained by catalytic reduction of γ-methylene-L-glutamine followed by

[1] Abbreviation: ATP, adenosine 5′-triphosphate.

[2] This product was isolated from the fronds of the fern *Phyllitis scolopendrium* (personal communication from Dr. J. K. Pollard).

hydrolysis. Hydrogenation of 200 mg of the γ-methylene compound was carried out in 8 ml of solution containing 500 mg of 10% palladium on charcoal in a Parr apparatus under 27.5 psi for 2 hr at 26°. After hydrogenation, the catalyst was removed by filtration and washed with hot water. The combined filtrate and the washings were evaporated to about 10 ml, and the solution was brought to 2 N with respect to HCl and placed at 100° for 2 hr. After repeated evaporation to remove HCl, the residue was dissolved in water and the pH was adjusted to about 11.5 by addition of NaOH. This solution was repeatedly evaporated to dryness to remove free ammonia; the residue was then dissolved in water and the pH was adjusted to 7 by addition of HCl; the concentration of amino acid was determined by the ninhydrin method (Rosen, 1957). Paper electrophoresis revealed a single ninhydrin-positive component which was indistinguishable from authentic γ-methylglutamic acid. *Preparation 7* was that previously described (Meister, 1954); this was obtained by converting preparation 2 to the corresponding *N*-carbobenzoxy-γ-methylglutamic acid diamide followed by incubation of this derivative with papain at pH 5 for 24 hr. The residual *N*-carbobenzoxy-γ-methyl-D-glutamic acid diamide was recrystallized from ethanol and converted to the free amino acid by catalytic hydrogenolysis and acid hydrolysis. *Preparation 8* was obtained from Calbiochem.[2] This material contained about 10% of a ninhydrin-positive impurity which moved with authentic glutamic acid on paper chromatography in 1-butanol–acetic acid–water (4:1:1). Small amounts of this material were purified by thin layer chromatography on silica gel G (Merck) using the same solvent; the section containing γ-methylglutamic acid was separately eluted with water; the amino acid concentration of this solution was determined by the ninhydrin method (Rosen, 1957). Rechromatography of this material indicated that it was free of the impurity present in the original sample. *Preparation 9* was obtained by acid hydrolysis of the γ-methylglutamine formed by glutamine synthetase (see below).

Larger amounts of preparation 8 (*erythro*-γ-methyl-L-glutamic acid, see below) were obtained from the commercially available material by incubation with glutamine synthetase followed by chromatographic purification. As stated below, this isomer of γ-methylglutamic acid is not a substrate of glutamine synthetase. The commercially available preparation was incubated with glutamine synthetase, ATP, and NH_2OH to convert the contaminating glutamate to γ-glutamylhydroxamic acid; the mixture was then heated to convert the latter compound to pyrrolidone carboxylic acid, which was readily removed from the remaining *erythro*-γ-methyl-L-glutamic acid.

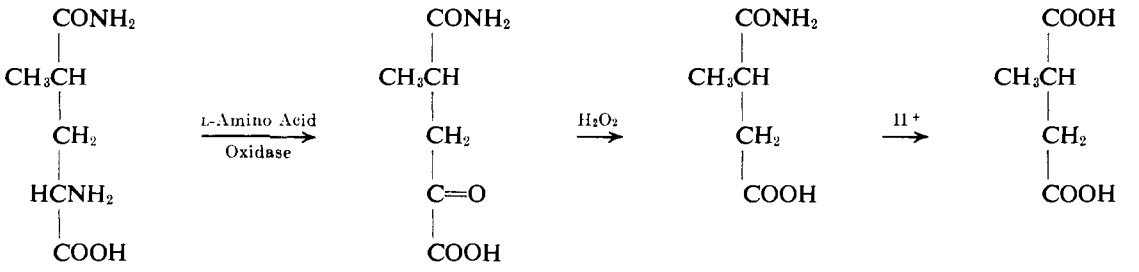
A reaction mixture containing 546 mg of the γ-methyl-L-glutamic acid product (containing about 11% of glutamic acid), ATP (2 mmoles), $MgCl_2$ (2 mmoles), hydroxylamine hydrochloride adjusted to pH 7.2 with NaOH (10 mmoles), 2-mercaptoethanol (2.5 mmoles), and sheep brain glutamine synthetase (2160 units) in a final volume of 100 ml was incubated at 37° until the formation of hydroxamate had reached completion (2 hr). The reaction mixture was then placed at 100° for 20 min in order to effect cyclization of the enzymatically formed γ-glutamylhydroxamate to pyrrolidonecarboxylate. After removal of precipitated protein by centrifugation, the supernatant solution was evaporated to about 10 ml and was then added to the top of a column of Dowex 50 (H^+, 63 × 2 cm). The column was thoroughly washed with water until the effluent no longer contained chloride ion and nucleotide and the pH had returned to 6–7. The γ-methyl-L-glutamic acid was then eluted with 2 N ammonium hydroxide. The ammoniacal effluent was repeatedly evaporated to dryness and the residue was then dissolved in 10 ml of water. This solution was added to the top of a column of Dowex 1-acetate (30 × 2 cm) and this column was washed with water until the effluent became free of ammonia. The amino acid was eluted from the column with 0.5 N acetic acid; evaporation of the effluent gave an amorphous product that weighed 321 mg. The amino acid was crystallized from ethanol–water. Paper chromatography showed that the product contained less than 1% glutamic acid. *Anal.* Calcd for $C_6H_{11}NO_4$: N, 8.7%. Found: N, 8.7%. Values for the optical rotation of *erythro*-γ-methyl-L-glutamic acid are given in Table II.

Methods. Glutamine synthetase activity was followed by determinations of the formation of γ-glutamylhydroxamate or of inorganic phosphate using the conditions previously described (Pamiljans *et al.*, 1962). The absorbancies of the various derivatives of γ-glutamylhydroxamic acid are close to that of γ-glutamylhydroxamic acid; this follows from the observation that determinations of the hydroxamic acids gave values that were in close agreement with those for inorganic phosphate formation.

Paper electrophoresis was carried out in 0.05 M sodium acetate buffer (pH 5.5) at 25° and 40 v/cm for 2 hr on 97-cm strips of Whatman No. 3MM paper. Under these conditions, γ-methylglutamic acid and γ-methyleneglutamic acid moved 33.9 and 36.5 cm, respectively, toward the cathode; γ-methylglutamine moved 1 cm toward the anode. Paper electrophoresis carried out as described previously (Kagan and Meister, 1966) at pH 1.9 resolved *threo*-γ-hydroxyglutamic acid (45 cm) from the corresponding *erythro* form (50.5 cm) as well as the stereoisomers of β-methylglutamic acid (*threo*, 52 cm; *erythro*, 54.3 cm); under these conditions, a significant difference was observed in the mobilities of the stereoisomers of γ-methylglutamic acid (*threo*, 56.4 cm; *erythro*, 57.4 cm).

Results

Activity of Glutamine Synthetase toward Various Preparations of γ-Methylglutamic Acid. Preparations of γ-methylglutamic acid obtained by synthesis from methyl methacrylate and diethylacetamidomalonate and by catalytic reduction of γ-methylene-DL-glutamic acid were utilized by the sheep brain enzyme to the



threo-γ-methyl-L-glutamine | D-γ-methyl-α-keto-glutaramic acid | D-β-methyl-succinamic acid | L-α-methyl-succinic acid

FIGURE 1: Conversion of enzymatically synthesized γ-methyl-L-glutamine to L-α-methylsuccinic acid.

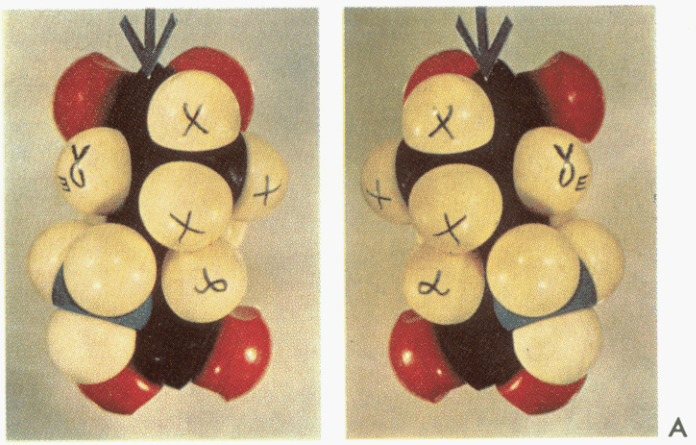
TABLE II: Optical Rotation of *threo*-γ-Methyl-L-glutamine and the γ-Methyl-L-glutamic Acids.

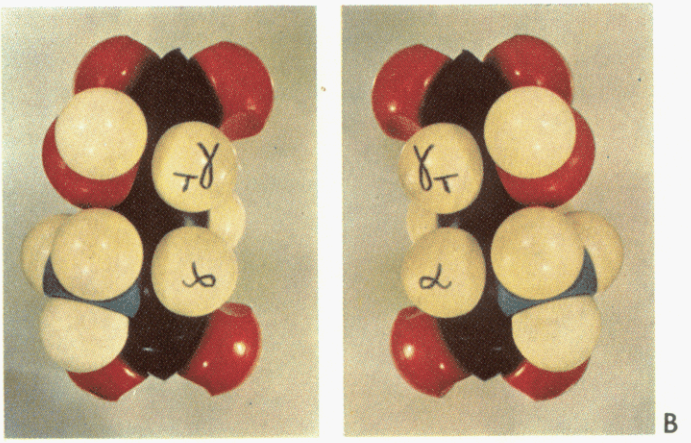
Wavelength (mμ)	Specific Optical Rotation, $[\alpha]^{25}$ (deg)					
			γ-Methyl-L-glutamic Acids			
	threo-γ-Methyl-L-glutamine		*threo*		*erythro*	
	H_2O[a]	3 N HCl[b]	H_2O[c]	5 N HCl[d]	H_2O[e]	5 N HCl[f]
250	+280	+729	+214	+442	+283	+749
275	+216	+399	+136	+251	+101	+350
300	+127	+274	+95.4	+177	+45.3	+209
325	+97.8	+201	+73.0	+136	+23.5	+144
350	+81.3	+157	+59.5	+109	+11.8	+106
375	+68.0	+126	+51.0	+91.5	+6.34	+80.9
400	+57.5	+102	+42.5	+76.5	+2.57	+64.3
425	+48.8	+82.6	+35.7	+63.8	+0.45	+53.0
450	+41.3	+68.3	+32.4	+55.3	−0.67	+45.3
475	+37.5	+58.3	+27.5	+48.9	−0.97	+38.6
500	+31.5	+48.6	+24.6	+43.8	−1.21	+33.0
550	+25.0	+35.6	+18.4	+32.5	−1.81	+25.7
589	+23.4	+31.4	+17.7	+30.2	−2.03	+22.2

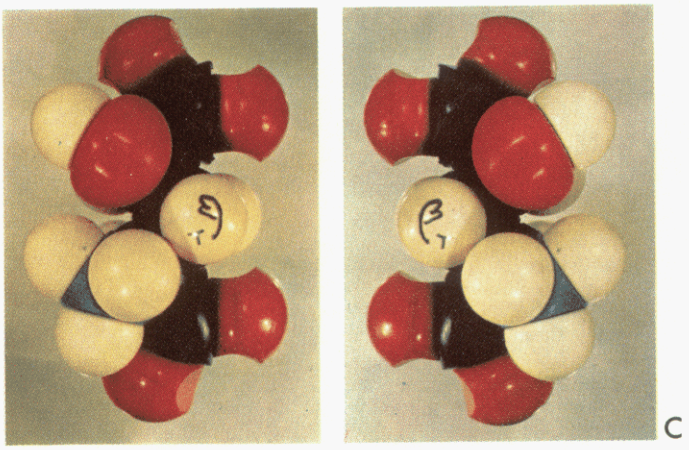
[a] 0.400%. [b] 0.309%. [c] 0.294%. [d] 0.235%. [e] 1.323%. [f] 0.661%.

extent of 20–25% (preparations 1, 2, and 5, Table III). Crystallization of preparation 2 led to a product that was only 12% utilized by the enzyme, and further crystallization gave a product (preparation 4) which was not a substrate for glutamine synthetase. These findings indicate that only one of the four isomers of γ-methylglutamic acid is a substrate for the enzyme. Preparation 6 was obtained by catalytic reduction and hydrolysis of γ-methylene-L-glutamine, and would therefore be expected to contain both L isomers of γ-methylglutamic acid; this preparation was 50% utilized, while a preparation of γ-methyl-D-glutamic acid (preparation 7) was inactive. The product isolated from plants (preparation 8) was inactive. It may be concluded that the susceptible isomer of γ-methylglutamic acid is an L form. Isolation and identification of the enzymatically active isomer of γ-methylglutamic acid were carried out as described below.

Isolation of Enzymatically Synthesized γ-Methylglutamine. A reaction mixture containing γ-methylglutamic acid (mixture of four isomers; preparation 1, Table III, 8 g, 49.7 mmoles), ATP (1 mmole), $MgCl_2$ (2 mmoles), sodium phosphoenolpyruvate (13.9 mmoles), pyruvate kinase (1330 units), ammonium chloride (20 mmoles), 2-mercaptoethanol (3 mmoles), and sheep brain glutamine synthetase (5520 units), in a final volume of 150 ml (adjusted to pH 7.2 by addition of NaOH prior to addition of the enzymes), was incubated at 37°. The course of the reaction was followed by determinations of inorganic phosphate. The reaction stopped after 250 min of incubation at which time 24.3% of the added amino acid had been utilized as judged by determinations of inorganic phosphate. An equal volume of cold ethanol was added and the precipitated protein was removed by centrifugation. The precipitate was washed with 80 ml of 50% ethanol







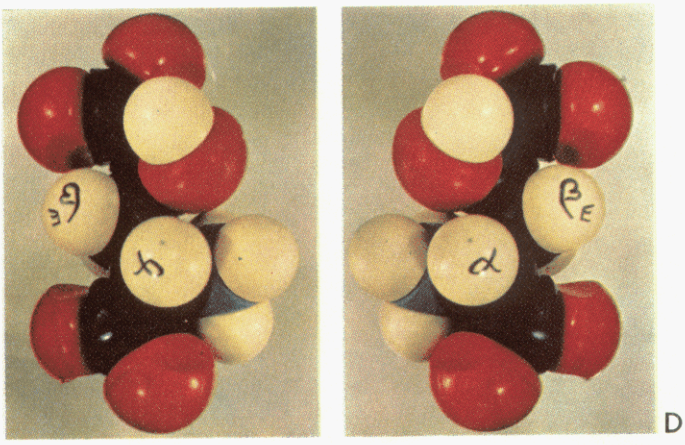


FIGURE 2: Stereophotographs of models of *threo*-γ-methyl-L-glutamic acid (A), *erythro*-γ-hydroxy-L-glutamic acid (B), *erythro*-γ-hydroxy-D-glutamic acid (C), and *threo*-γ-hydroxy-D-glutamic acid [undersurface (D), see text]. To obtain a three-dimensional effect, place a mirror in the narrow space between each pair of photographs with the mirrored surface facing to the left. Then, look down from the top edge of the mirror (tilting the mirror toward the right as needed) so that the reflection of the photograph on the left is seen by the left eye and the photograph on the right is seen by the right eye. A three-dimensional view is seen when the two images coincide. (Both photographs should be about equally illuminated. A slightly sharper picture is obtained with a front-surfaced mirror than with the usual type of glass mirror.)

TABLE III: Utilization of Various Preparations of γ-Methylglutamic Acid by Glutamine Synthetase.[a]

Prepn No.	Method of Prepn[b]	Max Utilzn (%[c])
1	By synthesis from methyl methacrylate and diethylacetamidomalonate	24
2	By synthesis from methyl methacrylate and diethylacetamidomalonate	20
3	Obtained from prepn 2 by crystallization *via* the *N*-chloroacetyl derivative	12
4	Obtained by further crystallization of prepn 3	0
5	By catalytic reduction of γ-methylene-DL-glutamic acid	25
6	By catalytic reduction and hydrolysis of γ-methylene-L-glutamine	50
7	(D Isomers) obtained from the residue obtained after exhaustive papain treatment of the *N*-carbobenzoxydiamide derivative of prepn 2	0
8	Isolation from plants (purified from commercial product)	0
9	By acid hydrolysis of the product formed by glutamine synthetase	100

[a] The reaction mixtures consisted of imidazole–HCl buffer (50 μmoles, pH 7.2), 2-mercaptoethanol (25 μmoles), $MgCl_2$ (20 μmoles), ATP (10 μmoles), $NH_2OH \cdot HCl$ adjusted to pH 7.2 with NaOH (100 μmoles), γ-methylglutamic acid (2–10 μmoles), and glutamine synthetase (5–20 units) in a final volume of 1.0 ml; incubated at 37°. [b] See text for details. [c] ±2%.

and the combined supernatant solution and wash solution were evaporated under reduced pressure at 30° to a thick oil. The residue was diluted to 75 ml with water and this solution was added at 4° to the top of a column of Dowex 50 (H^+, 30 × 3.7 cm) in a cold room. The column was washed with water until the eluate no longer contained chloride ion or nucleotide and the pH had returned to 6–7. The column was then washed with 2 N ammonium hydroxide until the eluate gave a negative ninhydrin spot test on paper. The effluent (containing γ-methylglutamic acid and γ-methylglutamine) was repeatedly evaporated under reduced pressure at 30° to remove excess ammonia. The amorphous residue obtained in this manner was dissolved in 30 ml of water and added at 4° to the top of a column of Dowex 1-acetate (35 × 3.7 cm) in a cold room. Water was passed through this column to elute the amide. The aqueous effluent was evaporated at 30° under reduced pressure to yield an oily residue, which was dissolved in 5 ml of water; absolute ethanol was added gradually to precipitate the γ-methylglutamine. The amide was collected by filtration and washed successively with 90% ethanol, absolute ethanol, and dry ether. The yield was 1 g (50%). The product was homogeneous on paper electrophoresis (see Methods) and on paper chromatography (88% aqueous phenol–concentrated NH_4OH, 99:1), and moved with authentic γ-methylglutamine. *Anal.* Calcd for $C_6H_{12}N_2O_3$: N, 17.5. Found: N, 17.5. Values for the optical rotation of this product are given in Table II.

The isolated γ-methylglutamine was converted to the corresponding dicarboxylic acid by hydrolysis (3 N HCl, 100°, 2 hr). After repeated evaporation *in vacuo* to remove excess HCl, the residue was dissolved in the minimal amount of water and adjusted to pH 3.2 by addition of triethylamine. The amino acid was crystallized by adding ethanol. *Anal.* Calcd for $C_6H_{11}NO_4$: N, 8.7. Found: N, 8.7. Values for the optical rotation of this amino acid (*threo* γ-methyl-L-glutamic acid) are given in Table II.

Conversion of Enzymatically Synthesized γ-Methylglutamine to α-Methylsuccinic Acid. In support of the conclusion (see above) that the enzymatically susceptible isomer of γ-methylglutamic acid is an L isomer, it was found that the γ-methylglutamine synthesized by glutamine synthetase was completely oxidatively deaminated by L-amino acid oxidase. The sequence of reactions described in Figure 1 was therefore carried out. The oxidation (first step) was carried out in the presence of catalase to permit isolation of the optically active γ-methyl-α-ketoglutaramic acid (Meister, 1952, 1953).

A mixture consisting of enzymatically synthesized γ-methylglutamine (450 mg), crystalline beef liver catalase (10,000 units), and *C. adamanteus* venom (2 g, dialyzed against water at 4° for 18 hr prior to use), in a final volume of 38 ml, was adjusted to pH 7.2 by addition of KOH. The mixture was placed at 37° and oxygen was bubbled vigorously into the solution; foaming was controlled by addition of caprylic alcohol. The reaction, which was followed by determinations of ammonia, reached completion after 1 hr, and the product was then separated from the enzymes by dialysis (Meister, 1952). The protein-free solution was concentrated by evaporation *in vacuo* to approximately 10 ml and this solution was added to the top of a column of Dowex 50 (H^+, 2 × 15 cm). The column was washed with water until the effluent was no longer acid. Evaporation of the effluent gave an oil which failed to crystallize after storage *in vacuo* over P_2O_5 at −10° for 3 days. The oil was dissolved in water

TABLE IV: Optical Rotations of Compounds Derived from Enzymatically Synthesized γ-Methylglutamine.

Wavelength (mμ)	Specific Optical Rotation, $[\alpha]_{\lambda}^{25}$ (deg)				
	D-γ-Methyl-α-ketoglutaramic Acid		D-β-Methylsuccinamic Acid	L-α-Methylsuccinic Acid	
	H_2O[a]	2 N HCl[b]	H_2O[c]	H_2O[d]	Ethanol[e]
250	−598	−615	−234	−255	−268
300	−175	−129	−78.4	−82.0	−110
350	−90.1	−64.0	−38.5	−40.6	−62.0
400	−56.2	−39.4	−24.7	−27.8	−41.5
450	−37.6	−25.8	−14.6	−16.8	−29.2
500	−27.8	−19.5	−12.1	−13.0	−23.0
550	−22.4	−15.5	−9.90	−10.3	−18.5
589	−18.9	−14.1	−9.25	−9.20	−16.5

[a] 0.571%. [b] 0.523%. [c] 0.364%. [d] 1.000%. [e] 1.000%.

and adjusted to pH 4.9 by addition of barium hydroxide solution. The solution was then evaporated to low volume and on addition of absolute ethanol crystals appeared. The yield of barium γ-methyl-α-ketoglutaramate was 564 mg (88%).[3] *Anal.* Calcd for $C_6H_8Ba_{1/2}NO_4$: N, 6.2. Found: N, 6.2. An infrared spectrum (KBr pellet) of the product was identical with that obtained with the racemic product previously prepared (Meister, 1954). The optical rotation of the product is given in Table IV.

γ-Methyl-α-ketoglutaramic acid was converted to β-methylsuccinamic acid by the following procedure. Barium γ-methyl-α-ketoglutaramate (200 mg) was dissolved in 8 ml of 1 N KOH and 8 ml of 50% hydrogen peroxide was added. An immediate precipitate of barium carbonate appeared. After standing for 30 min at 26°, a small amount of crystalline catalase was added to destroy the peroxide. Residual peroxide was decomposed by flash evaporation to dryness at 40°. The residue was dissolved in 10 ml of water and this solution was added to the top of a column of Dowex 50 (H^+, 2 × 15 cm); the column was washed with water until the pH returned to about 7. The aqueous effluent was evaporated *in vacuo* to an oil which was dissolved in a few milliliters of absolute ethanol; ether was added to faint turbidity. After storage at −10° for 3 days, the product crystallized; yield, 55 mg (48%); mp 131–133°. *Anal.* Calcd for $C_5H_9NO_3$: N, 10.7. Found: N, 10.8. Additional material was obtained as the barium salt by adding a solution of barium hydroxide to the supernatant solution obtained from the crystals. Data on the optical rotation of β-methylsuccinamic acid are given in Table IV.

β-Methylsuccinamic acid was converted to α-methylsuccinic acid as follows. β-Methylsuccinamic acid (40 mg) was dissolved in 35 ml of water and 10 g of dry Dowex 50 (H^+) was then added. The suspension was stirred vigorously at 100° for 45 min after which it was cooled and the solution was filtered. The resin was washed repeatedly with water and the combined washings and filtrate were evaporated *in vacuo* to dryness. The residue was dissolved in dry ether and dry petroleum ether was added to faint turbidity. After storage at −10° for 18 hr, the crystals which formed were collected by centrifugation, washed with petroleum ether, and dried over P_2O_5; yield, 34 mg (81%); mp 114° [lit. (Fredga, 1942) mp 114.5°]. *Anal.* Calcd for $C_5H_8O_4$: C, 45.5, H, 6.1. Found: C, 45.7; H, 6.1. The infrared absorption spectrum of the product (KBr pellet) was identical with that of authentic DL-α-methylsuccinic acid. The optical rotation of the α-methylsuccinic acid product (Table IV) agrees with that of L-α-methylsuccinic acid (Fredga, 1942); it may therefore be concluded that the product synthesized by glutamine synthetase is *threo*-γ-methyl-L-glutamine.

Activity of Sheep Brain Glutamine Synthetase Toward Various γ-Substituted Derivatives of Glutamic Acid. A comparison of the values for K_m and relative maximal velocity for *threo*-γ-methyl-L-glutamic acid with those for L- and D-glutamic acid and other substrates of the enzyme are given in Table V. The K_m values for *threo*-γ-methyl-L-glutamic acid with ammonia and hydroxylamine are about the same and are similar to those for L-glutamic acid. The value for relative maximal velocity with *threo*-γ-methyl-L-glutamic acid for amide synthesis is substantially lower than that for synthesis of hydroxamate. A possible explanation of this result is considered below. It is of interest that the K_m and velocity values for *threo*-γ-hydroxy-L-glutamic acid and *erythro*-γ-hydroxy-L-glutamic acid are similar and are not markedly different from the corresponding values for L-glutamic acid. *erythro*-γ-Hydroxy-D-glutamic acid was appreciably active, but *threo*-γ-

[3] The isolated compound probably exists predominantly in the keto lactam form (Otani and Meister, 1957).

TABLE V: Values for Relative Maximal Velocity and K_m.[a]

Amino Acid Substrate	Rel Max Velocity with NH_2OH	Rel Max Velocity with NH_3	$K_m \times 10^3$ (M) with NH_2OH	$K_m \times 10^3$ (M) with NH_3
L-Glutamic acid	100[b]	100[b]	3.3	3.9
D-Glutamic acid	54	27	3.8	13
α-Methyl-L-glutamic acid	67	75	6.4	6.7
threo-β-Methyl-D-glutamic acid	46	2.2	5.9	25
threo-γ-Methyl-L-glutamic acid	63	27	2.6	3.6
threo-γ-Hydroxy-L-glutamic acid	89	100	1.7	2.4
threo-γ-Hydroxy-D-glutamic acid	1.6	<0.08	22	...
erythro-γ-Hydroxy-L-glutamic acid	64	81	4.0	5.6
erythro-γ-Hydroxy-D-glutamic acid	29	38	13	22

[a] Obtained by the method of Lineweaver and Burk (1934). The reaction mixtures consisted of imidazole–HCl buffer (50 μmoles, pH 7.2), 2-mercaptoethanol (25 μmoles), $MgCl_2$ (20 μmoles), ATP (10 μmoles), $NH_2OH \cdot HCl$ adjusted to pH 7.2 with NaOH, or NH_4Cl (100 μmoles), amino acid, and enzyme in a final volume of 1 ml; 37°. [b] All values are relative to that of L-glutamic acid with NH_2OH (200 μmoles of L-γ-glutamylhydroxamate formed/mg of enzyme per 15 min).

hydroxy-D-glutamic acid was only slightly active.[4]

Specificity of Glutamine Synthetase from Peas. Although the available data indicate that there is a marked similarity between the specificity (and other properties) of the enzyme from sheep brain and that from peas (Levintow and Meister, 1954; Meister, 1962), it appeared desirable to study the latter enzyme with the individual isomers that have recently become available. The relative rates of reaction observed with the various glutamic acid derivatives are summarized in Table VI; in general these are similar to the values observed with the sheep brain enzyme.

Discussion

The data indicate that only one of the four optical isomers of γ-methylglutamic acid is a substrate for glutamine synthetase and that the susceptible isomer is *threo*-γ-methyl-L-glutamic acid. Examination of the models previously constructed (Figure 5, Kagan and Meister, 1966) indicates that the γ-*threo*-hydrogen atom of L-glutamic acid occupies a unique position in space as compared to the other three γ-hydrogen atoms of L- and D-glutamic acid. On the other hand, the *erythro*-γ-hydrogen atoms of L- and D-glutamic acids are located in about the same relative position lying between the respective amino and γ-carboxyl groups of these molecules. The γ-*threo*-hydrogen atom of D-glutamic acid occupies a position very close to those of the α-hydrogen atom of D-glutamic acid and the *threo*-β-hydrogen atom of L-glutamic acid. The present findings are therefore in accord with the hypothesis which has developed from the earlier studies. Thus, it appears that the only methyl substitutions that do not lead to loss of enzymatic susceptibility are located on the same side (relative to the enzyme) of the glutamate molecules. Figure 2A is a stereophotograph of a model of *threo*-γ-methyl-L-glutamic acid oriented in the same manner as that of L-glutamic acid in our previous publication (Figure 5A, Kagan and Meister, 1966); the findings indicate that the right-hand side and undersurface of the stereomodel of the substrate are in close contact with the enzyme or nucleotide.

The results obtained with the γ-hydroxyglutamic acids permit additional considerations. Although *threo*-γ-hydroxy-L-glutamic acid is the most active of the four γ-hydroxyglutamic acids, substantial rates of reaction were observed with *erythro*-γ-hydroxy-L-glutamic acid (Figure 2B). [It is of interest that Adams and Goldstone (1965) reported similar rates of reaction for the two γ-hydroxy-L-glutamic acids with the glutamine synthetase of rat liver.] It is of note that *erythro*-γ-hydroxy-D-glutamic acid (Figure 2C) is also appreciably active with sheep brain glutamine synthetase. As shown in Figure 2B and C the hydroxyl groups of L and D isomers of *erythro*-γ-hydroxyglutamic acid are similarly located. However, the *threo*-γ-hydrogen atom of D-glutamic acid is on the opposite

[4] Two synthetic preparations of β,γ-dihydroxyglutamic acid were kindly provided by Dr. E. E. Dekker; one of these (A) was isolated as the hydrochloride and the other (B) as the ammonium salt. These were tested in reaction mixtures consisting of sheep brain glutamine synthetase (7 units), amino acid (4 μmoles, and other components as given in Table V. The initial rates of reaction with hydroxylamine were 12 and 5%, respectively, for preparations A and B, of the corresponding rate with L-glutamic acid. Both preparations were about twice as active with hydroxylamine as with ammonia. The reactions stopped after 200 min when about 30% (with ammonia) and 40% (with hydroxylamine) of the substrate was utilized. The data indicate that one or more of the isomers of this amino acid are substrates.

TABLE VI: Relative Rates of Reactions Catalyzed by Glutamine Synthetase of Peas.[a]

Substrate	Rel Rate with NH_2OH	Rel Rate with NH_3
L-Glutamic acid	100[b]	100
D-Glutamic acid	72	30
α-Methyl-L-glutamic acid	45	56
α-Methyl-D-glutamic acid	0	0
threo-β-Methyl-D-glutamic acid[c]	50	10
threo-γ-Methyl-L-glutamic acid[c]	51	40
erythro-γ-Methyl-L-glutamic acid	0	0
β-Glutamic acid	18	13

[a] The reaction mixtures contained imidazole buffer, pH 7.2 (50 μmoles), ATP (10 μmoles), $MgCl_2$ (20 μmoles), 2-mercaptoethanol (25 μmoles), $NH_2OH \cdot HCl$ adjusted to pH 7.2 with NaOH (100 μmoles) or NH_4Cl (100 μmoles), amino acid [50 μmoles for all except *threo*-β-methyl-D-glutamic acid (100 μmoles) in amide synthesis], and enzyme in a final volume of 1.0 ml. Hydroxamate synthesis was followed by the ferric chloride method and synthesis in the presence of ammonia was followed by determinations of the liberated inorganic phosphate; 37°. [b] All values are relative to that for L-glutamic acid with NH_2OH (1.2 μmoles of product formed/15 min.). [c] It was established in separate experiments similar to those reported previously with the sheep brain enzyme that only one of the four isomers of β-methylglutamic acid and only one of the four isomers of γ-methylglutamic acid are substrates for pea glutamine synthetase.

side of the molecule. Figure 2D is a stereophotograph of the undersurface of *threo*-γ-hydroxy-D-glutamic acid. We may therefore tentatively conclude that there is sufficient space in the region of the *erythro*-γ-hydrogen atom of L-glutamic acid for a hydroxyl group but that a methyl group is of such size as to provide steric hindrance inconsistent with enzymatic susceptibility. (The possibility that the binding of the γ-hydroxyglutamic acids to the enzyme is influenced by hydrogen bonds involving the hydroxyl group must also be considered.) Sure substitution of the *threo*-γ-hydrogen atom of D-glutamic acid by either a methyl group or a hydroxyl group leads to inactivity or to a substrate of very low activity, the "available" space in this region must be quite limited and the substrate must therefore be very close to the enzyme at this point.

It was previously noted that the reactivity of D-glutamic acid with hydroxylamine is significantly greater than with ammonia and that *threo*-β-methyl-D-glutamic acid reacts even less readily with ammonia than with hydroxylamine (Table V). An explanation for these effects was previously considered in which it was postulated that ammonia is bound to a specific site on the enzyme and that although hydroxylamine may bind at this site, it may also directly attack the activated carboxyl carbon atom. Study of the models shows that the γ-carboxyl carbon atom of D-glutamic acid is oriented somewhat differently than that of L-glutamic acid; it might therefore be in a less favorable location for reaction with enzyme-bound ammonia. The much lower reactivity of *threo*-β-methyl-D-glutamic acid with ammonia may in addition reflect steric hindrance offered by the methyl group. It is of interest that *threo*-γ-methyl-L-glutamic acid reacts about twice as readily with hydroxylamine as with ammonia; the methyl group of this substrate is located in a position similar to that of *threo*-β-methyl-D-glutamic acid and it seems possible that the methyl group of *threo*-γ-methyl-L-glutamic acid might also offer steric hindrance to the attack by ammonia. Thus, if the ammonia binding site on the enzyme lies just above the position of the γ-carbon atom of L-glutamic acid (indicated by an arrow, Figure 2A), an unfavorable orientation of the γ-carboxyl carbon atom (as in D-glutamic acid) or the presence of a γ-methyl group (as in *threo*-γ-methyl-L-glutamic acid) might hinder the attack of enzyme-bound ammonia on the γ-carboxyl carbon atom. The very low reactivity of *threo*-β-methyl-D-glutamic acid with ammonia might reflect the combined effect of both phenomena. Although this line of reasoning appears worthwhile as a working hypothesis at this time, additional data are required.

It is of interest that *erythro*-γ-methyl-L-glutamic acid is not a substrate for the glutamine synthetases of sheep brain and peas. This isomer of γ-methylglutamic acid has been isolated from plants (Virtanen and Berg, 1955; Blake and Fowden, 1964). Although γ-methylene-L-glutamine and γ-hydroxyglutamine have been found in various plants [Brandner and Virtanen (1963); see Meister (1965) for additional literature], the natural occurrence of γ-methylglutamine has apparently not been reported.

The present and earlier suggestion that the amino acid substrate of glutamine synthetase is in an extended conformation and that the side of the molecule bearing the amino group (Figure 2A–C, right-hand side) and the undersurface of this molecule are in close contact with the enzyme have led us to consider the possible steric relationships between substrate and ATP on the enzyme. Assuming that a γ-glutamyl phosphate linkage is formed (Meister *et al.*, 1962) and that the α-amino nitrogen atom is involved in a metal chelate–nucleotide complex, plausible models can be constructed involving amino acid, metal ion, and the β- and γ-phosphate moieties of ATP. Additional studies along these lines will be presented subsequently.

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