Stereospecific Activity of Glutamine Synthetase toward *threo*-β-Methyl-D-glutamic acid*

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ABSTRACT: Highly purified glutamine synthetase from sheep brain acts on both the D and L isomers of glutamic acid, on only the L isomer of α-methylglutamic acid, and on β-glutamic acid (β-aminoglutaric acid) to yield only D-β-glutamine. These observations are explained here by a 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 carboxyl and amino 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 so that the α-hydrogen atom of D-glutamic acid is oriented toward the enzyme. (These points are more clearly seen from color stereophotographs of models given in this paper.) Study of models of the isomers of glutamic acid constructed and oriented in this manner led to the prediction that only one of the four isomers of β-methylglutamic acid (the *threo*-D isomer) would be a substrate. In agreement with this prediction, glutamine synthetase was found to act on *threo*-β-methyl-D-glutamic acid, but not on the other three β-methylglutamic acid isomers. Of the four β-hydroxyglutamic acids, *threo*-β-hydroxy-D-glutamic acid was considerably more active than the other three β-hydroxyglutamic acid isomers. These and other observations reported here support the proposed conformation of the substrates of glutamine synthetase and provide insight into the way in which the substrates combine with the enzyme. An explanation is also presented for the finding that the β-substituted D-glutamic acid derivatives (as well as D-glutamic acid) react less readily with ammonia than with hydroxylamine.

The specificity of glutamine synthetase is remarkable in that both D-glutamic acid and L-glutamic acid are substrates (Levintow and Meister, 1953, 1954; Meister, 1962). In contrast, the enzyme acts stereospecifically toward α-methylglutamic acid, utilizing only the L isomer (Kagan *et al.*, 1965), and it catalyzes the synthesis of only one isomer (the D form) of β-glutamine from β-glutamic acid, which does not have an asymmetric carbon atom (Khedouri and Meister, 1965). Study of models of the optical isomers of glutamic acid in which the carbon chains are in an extended conformation has shown that it is possible to orient the amino and carboxyl groups of these enantiomorphs to virtually the same positions in space (Kagan *et al.*, 1965).[1] Furthermore, the spatial relationships of the amino and carboxyl groups of an extended conformation of D-β-glutamine (but not L-β-glutamine) closely approximate those of L-glutamine (Khedouri and Meister, 1965). These considerations, therefore, seem to offer an explanation for the ability of both D-glutamic acid and L-glutamic acid to serve as substrates, and for the preferential synthesis of D-β-glutamine. Examination of models of L-glutamic acid and D-glutamic acid constructed and oriented as described above reveals that the respective α-hydrogen atoms are located on opposite sides of these molecules; therefore, replacement of the α-hydrogen atoms by methyl groups might be expected to have significantly different effects on enzymatic susceptibility. Since α-methyl-D-glutamic acid is not a substrate, while α-methyl-L-glutamic acid is active, it may be postulated that the α-hydrogen atom of D-glutamic acid is on the side of this substrate that is in contact (or very close) to the active site of the enzyme, while the α-hydrogen atom of L-glutamic acid is on the side of this substrate that is directed away from the active site.

In an effort to examine further the validity of these postulated conformations of L- and D-glutamic acids, we have studied the ability of the isomers of β-methylglutamic acid to serve as substrates for glutamine synthetase. Study of the models indicates that both of the β-hydrogen atoms of L-glutamic acid and the *erythro*-β-hydrogen atom of D-glutamic acid are oriented in approximately the same direction as the α-hydrogen atom of D-glutamic acid. On the other hand, the *threo*-β-hydrogen atom of D-glutamic acid occupies a position almost equivalent to that of the α-hydrogen atom of L-glutamic acid. We therefore predicted that substitution by a methyl group of either of the β-hydrogen atoms of L-glutamic acid or of the *erythro*-β-hydrogen atom of D-glutamic acid would lead to loss (or marked reduction) in enzymatic susceptibility. We also were

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[1] This is considered further under Discussion (see below).

led to the prediction that substitution of the *threo*-β-hydrogen atom of D-glutamic acid by a methyl group would not lead to loss of enzymatic susceptibility. Although β-methylglutamic acid was previously shown to be a substrate for glutamine synthetase (Levintow *et al.*, 1955), the β-methylglutamic acid available for these studies was a mixture of isomers, and experiments designed to determine the identity of the active isomer (or isomers) were not carried out. The present studies show, in agreement with the predictions, that only one of the four isomers of β-methylglutamic acid is a substrate for glutamine synthetase, and that the enzymatically active isomer is *threo*-β-methyl-D-glutamic acid. These observations offer additional support for our hypothesis concerning the conformation of the substrates of glutamine synthetase and provide insight into the way in which the substrates combine with the enzyme.

It seems noteworthy that although glutamine synthetase acts on glutamic acid with relatively low optical specificity, it acts stereospecifically on α- and β-methyl-substituted glutamic acids, exhibiting virtually absolute L-specificity toward the former and D-specificity toward the latter. The unusual optical specificity of this enzyme is also evident from its D-specific action on β-glutamic acid and the finding that it acts on *threo*-γ-methyl-L-glutamic acid, but not on the other three isomers of γ-methylglutamic acid (Kagan and Meister, 1965).[2]

These data are explained here in terms of a hypothesis concerning the steric relationships between the substrates and enzyme.

Experimental Section

Materials. Sheep brain glutamine synthetase was isolated as previously reported (Pamiljans *et al.*, 1962). β-Methylglutamic acid was synthesized as described earlier (Meister *et al.*, 1955); paper electrophoretic studies (see below) showed that this product can be separated into two approximately equal components, thus supporting the previous supposition that the synthetic material is a mixture of four optical isomers. *erythro*-β-Hydroxy-DL-glutamic acid and *threo*-β-hydroxy-DL-glutamic acid were kindly donated by Dr. Karl Pfister of Merck Sharp and Dohme Co., Rahway, N. J. The isomers of isoleucine employed in these studies were prepared by Dr. Jesse P. Greenstein (Greenstein *et al.*, 1951). D-Glutamic acid cyclotransferase was obtained from rat kidney as previously described (Meister *et al.*, 1963); we are indebted to Mr. Peter Polgar for carrying out this preparation. D-Amino acid oxidase prepared from hog kidney was kindly donated by Dr. Daniel Wellner.

Resolution of the Diastereoisomers of β-Hydroxyglutamic Acid with D-Glutamic Acid Cyclotransferase. Milligram quantities of the *threo* and *erythro* forms of racemic β-hydroxyglutamic acid were resolved by treatment with D-glutamic acid cyclotransferase; the procedure followed was essentially that previously used for the resolution of the DL-α-aminotricarballylic acid (Meister *et al.*, 1963). Reaction mixtures containing DL-β-hydroxyglutamic acid and enzyme in 0.1 M Tris–HCl buffer (pH 8.3), 0.03 M magnesium chloride, 0.04 M 2-mercaptoethanol were incubated at 37°. The cyclization reaction was followed by determinations of the decrease in ninhydrin-reacting material (Rosen, 1957). After deproteinization of the reaction mixture with ethanol, the solution was applied to a small column of Dowex 50 (H^+) and the column was eluted with water until the pH of the effluent returned to a value between 6 and 7. The effluent was evaporated to dryness and then dissolved in 5 ml of 3 N HCl. This solution was heated at 100° for 2 hr after which it was repeatedly evaporated *in vacuo* to remove excess hydrochloric acid. The residue was dissolved in water and the D-amino acid concentration was determined by the ninhydrin method. The L-amino acid was eluted from the column with 2 N ammonium hydroxide and the eluate was repeatedly evaporated to dryness to remove excess ammonia. After adjustment to pH 12 with sodium hydroxide, further evaporation was carried out to remove the last traces of ammonia. The final residue was dissolved in water and the amino acid concentration was determined by the ninhydrin method. The D isomers prepared in this manner are considered to be at least 99% optically pure; since the enzymatic cyclization reaction proceeds to about 96% of completion, the L isomers are considered to be about 96% optically pure. Paper chromatography of the isolated products revealed only one ninhydrin-positive compound; this exhibited R_F values that corresponded to those of authentic β-hydroxyglutamic acid.

Methods. DETERMINATION OF GLUTAMINE SYNTHETASE ACTIVITY. Enzymatic activity was followed by determinations of the formation of γ-glutamylhydroxamate or inorganic phosphate using the conditions previously described (Pamiljans *et al.*, 1962). It was assumed that the absorbancies obtained with β-methyl-γ-glutamylhydroxamic acid and β-hydroxy-γ-glutamylhydroxamic acid are the same as that observed for γ-glutamylhydroxamic acid; this assumption is consistent with the observation that determinations of the β-substituted-γ-glutamylhydroxamic acids gave values that were in close agreement with those for inorganic phosphate formation.

SEPARATION OF THE DIASTEREOISOMERS OF β-METHYLGLUTAMIC ACID AND ISOLEUCINE BY PAPER ELECTROPHORESIS. Paper electrophoresis was carried out on 157-cm strips of Whatman 3MM paper at pH 1.85 (7.4% formic acid) at 46° for 90 min at 57 v/cm in a Savant apparatus. After electrophoresis, the paper strips were dried and sprayed with 0.25% ninhydrin in acetone. Under these conditions, synthetic β-methylglutamic acid gave two bands of about equal size, which moved 65 and 67 cm, respectively, from the

[2] Additional work on the activity of glutamine synthetase toward the γ-methylglutamic acids and the γ-hydroxyglutamic acids is in progress and will be published subsequently (Kagan and Meister, unpublished data).

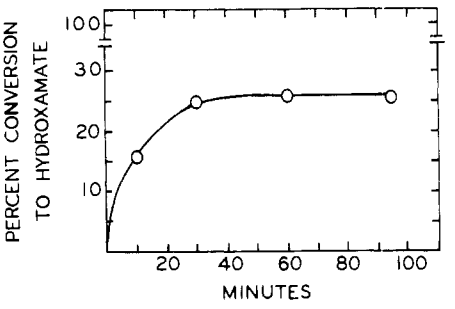


FIGURE 1: Synthesis of β-methyl-γ-glutamylhydroxamate. The reaction mixtures contained imidazole–HCl buffer (pH 7.2; 50 μmoles), $MgCl_2$ (20 μmoles), ATP (10 μmoles), 2-mercaptoethanol (25 μmoles), hydroxylamine hydrochloride (adjusted to pH 7.2 with NaOH; 100 μmoles), synthetic DL-β-methylglutamic acid (mixture of four isomers) (2 μmoles), and enzyme (13.3 units), in a final volume of 1.0 ml; incubated at 37°.

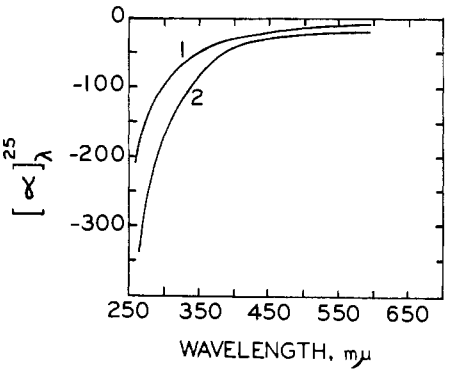


FIGURE 2: Optical rotatory dispersion of *threo*-β-methyl-D-glutamic acid. Curve 1—in water; Curve 2—in 3 N HCl. Concentration: 0.372%. (Other experimental details are given in text.)

origin; alloisoleucine and isoleucine moved 90 and 91.5 cm, respectively.

Results

Enzymatic Synthesis of threo-β-Methyl-D-γ-glutamylhydroxamic Acid. When synthetic β-methylglutamic acid (mixture of four isomers) was incubated with glutamine synthetase, magnesium ions, ATP[3] (adenosine triphosphate), and hydroxylamine, the reaction proceeded rapidly at first, but reached a plateau when close to 25% of the substrate had been converted to the hydroxamate (Figure 1). Determinations of the inorganic phosphate released were in close agreement with the values obtained for hydroxamate formation. Addition of more enzyme did not increase the extent of utilization of substrate, and it was, therefore, tentatively concluded that only one of the four isomers of β-methylglutamic acid present was a substrate for glutamine synthetase. Additional evidence in support of this conclusion was obtained by paper electrophoretic study of the reaction mixture after incubation which revealed that approximately 50% of the more slowly moving diastereoisomer of β-methylglutamic acid had disappeared.

In order to isolate the enzymatically susceptible isomer, a large-scale experiment was carried out as follows: a mixture containing ATP (24.8 mmoles; adjusted to pH 7.2 with NaOH), $MgCl_2$ (50 mmoles), 2-mercaptoethanol (2.5 mmoles), hydroxylamine hydrochloride adjusted to pH 7.0 with NaOH (20 mmoles), β-methylglutamic acid (four isomers; adjusted to pH 7.2 with NaOH; 4 g (24.8 mmoles)), sodium phosphoenolpyruvate (6.2 mmoles), pyruvate kinase (1333 units), and glutamine synthetase (12,000 units) in a final volume of 180 ml was adjusted to pH 7.2 by addition of NaOH and incubated at 37° with gentle shaking. The course of the reaction was followed by the hydroxamate method. Hydroxamate formation leveled off at a value equivalent to about 25% of the initial amino acid concentration after 130 min. The mixture was then placed in a 100° bath for 20 min in order to denature and precipitate the enzymes and to cause cyclization of the β-methyl-γ-glutamylhydroxamic acid to β-methylpyrrolidonecarboxylate. The latter reaction, which occurs rapidly under these conditions (at about the same rate as observed for the cyclization of γ-glutamylhydroxamate (Levintow *et al.*, 1955)), was followed by the $FeCl_3$–hydroxamate reaction. The mixture was cooled and the precipitated protein was removed by centrifugation. The supernatant solution was removed and the precipitate was washed with 30 ml of water. The combined supernatant and wash solutions were concentrated *in vacuo* to about 50 ml, and 100 ml of cold ethanol was added; this resulted in precipitation of considerable ATP. After centrifugation the supernatant solution was concentrated *in vacuo* to an oil, which was dissolved in 80 ml and then added to the top of a Dowex 50 column (H^+; 25 × 3.7 cm; 200–400 mesh). The column was washed with water (about 250 ml) until the effluent was no longer acid. The acid effluent was concentrated *in vacuo* to a thick oil. Attempts to hydrolyze the β-methylpyrrolidonecarboxylate present in this oil to the corresponding amino acid by heating at 100° in 2 N HCl were not successful because considerable destruction of amino acid occurred in association with a browning reaction apparently related to the nucleotide still present. Therefore, the oil was extracted with several volumes of anhydrous ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness, and the residue was dissolved in 20 ml of water; treatment with about 0.5 g of charcoal was carried out to adsorb residual nucleotide. The clear filtrate was evaporated to dryness. The residue was taken up in 10 ml of 2 N HCl and placed at 100° for 2 hr. The water-clear solution was repeatedly evaporated to dryness to remove excess HCl,

[3] Abbreviations used: ATP, adenosine triphosphate.

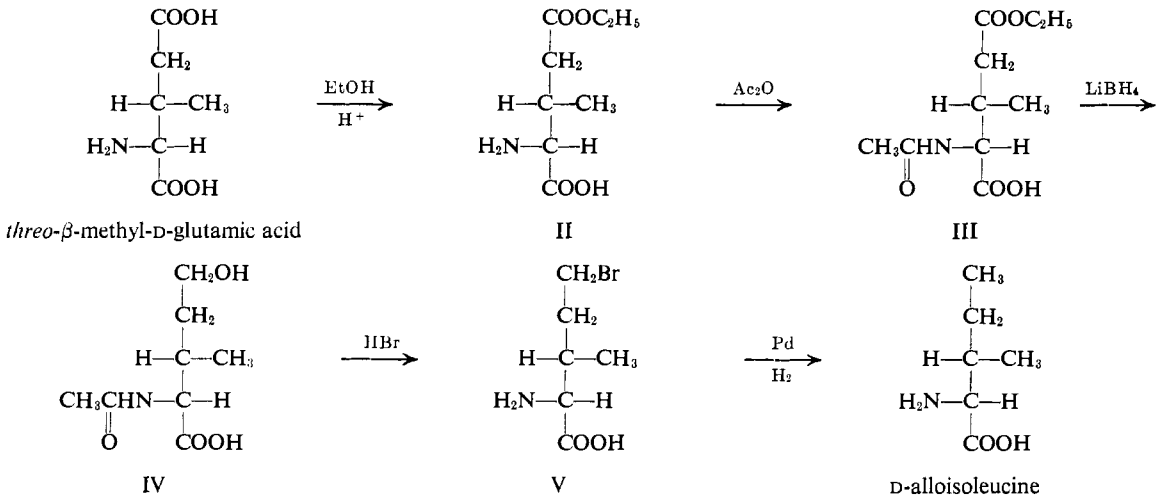


FIGURE 3: Conversion of the enzymatically susceptible isomer of β-methylglutamic acid to D-alloisoleucine (see the text).

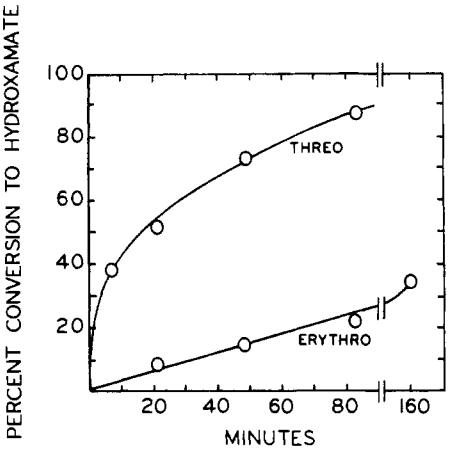
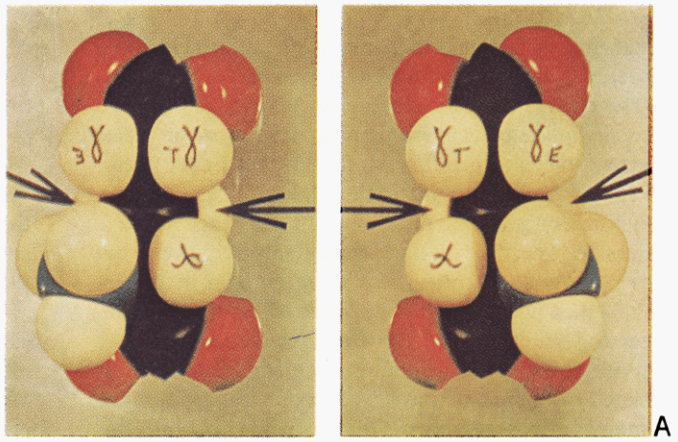


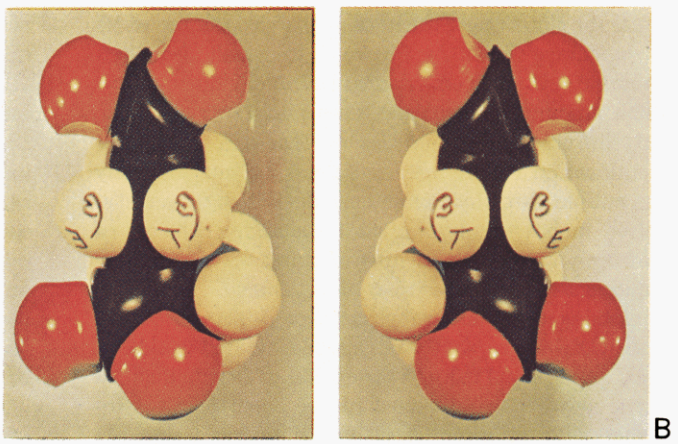
FIGURE 4: Utilization of β-hydroxyglutamic acids by glutamine synthetase. The reaction mixtures contained imidazole–HCl buffer (75 μmoles; pH 7.2), $MgCl_2$ (30 μmoles), ATP (15 μmoles), 2-mercaptoethanol (38 μmoles), $NH_2OH \cdot HCl$ adjusted to pH 7.2 with NaOH (150 μmoles), either *threo* or *erythro*-β-hydroxy-DL-glutamic acid (2.75 μmoles), and enzyme (23.2 units) in a final volume of 1.5 ml; 37°.

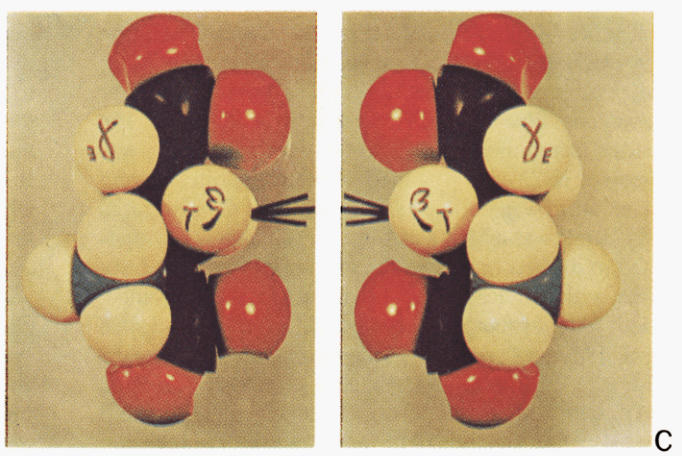
and then dissolved in a small amount of water and applied to a column of Dowex 50 (H^+; 1 × 26 cm). The column was washed with water until the effluent gave a negative test for chloride ion; the β-methylglutamic acid was eluted with 2 N NH_4OH. The ammoniacal effluent was repeatedly concentrated *in vacuo* to remove excess ammonia; the residue was dissolved in the minimal amount of water and this solution was adjusted to pH 3 by addition of acetic acid. Absolute ethanol was added to faint turbidity; after cooling at 4° for 18 hr, the precipitated amino acid was collected by centrifugation. It was washed successively with cold 50% ethanol, absolute ethanol, and ether, and then dried *in vacuo* over P_2O_5. The product weighed 800 mg (80% based on 25% of the β-methylglutamic acid isomers present initially). It did not contain chloride ion or ammonia, and was estimated to be greater than 98% pure by the quantitative ninhydrin procedure. The product moved with synthetic β-methylglutamic acid on ascending paper chromatography in solvents consisting of: (a) 78% ethanol, and (b) 88% phenol–concentrated NH_4OH (99:1). *Anal.* Calcd for $C_6H_{11}NO_4$: N, 8.7. Found: N, 8.7. The product was further identified as described below.

Identification of threo-β-Methyl-D-glutamic Acid. Paper electrophoresis of the product obtained above at pH 1.85 carried out as described under Methods gave only a single band which corresponded in mobility to the more slowly moving of the two diastereoisomers of β-methylglutamic acid. When the product was incubated with glutamine synthetase under the conditions described in Figure 1, the reaction went virtually to completion as judged by hydroxamate and inorganic phosphate formation.

The product was incubated with rat kidney D-glutamic acid cyclotransferase as follows: A reaction mixture consisting of the isolated β-methylglutamic acid (5 μmoles), Tris–HCl buffer (pH 8.3; 100 μmoles), $MgCl_2$ (33 μmoles), 2-mercaptoethanol (100 μmoles), and enzyme (20 mg) in a final volume of 1.0 ml was incubated at 37° for 1 hr. Deproteinization was effected by adding 4 ml of cold absolute ethanol followed by centrifugation. The supernatant solution was evaporated to dryness and the residue was dissolved in water and subjected to paper electrophoresis on 96-cm strips of Whatman 3 MM paper in 0.05 M sodium acetate buffer (pH 5.5) at 47 v/cm at 15° for 35 min. The paper strips were dried and then sprayed with ninhydrin. Under these conditions virtually all of the β-methylglutamic acid disappeared. No such disappearance occurred in controls in which enzyme was omitted. In similar ex-







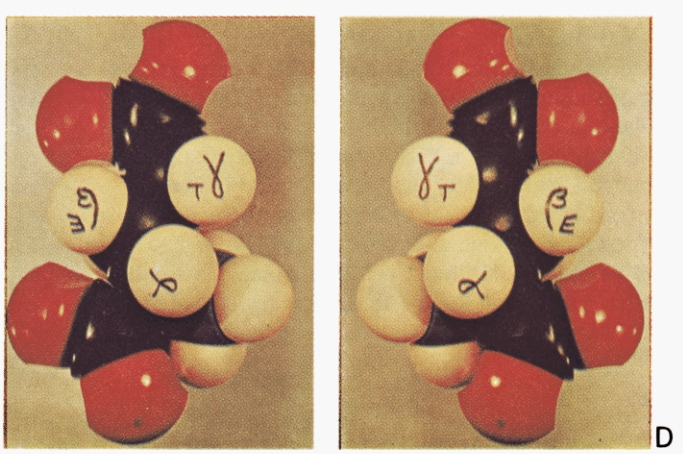


FIGURE 5: Stereophotographs of models of L-glutamic acid (A, B) and D-glutamic acid (C, D). (See the text.) To obtain a three-dimensional effect, place a mirror in the narrow space between each pair of photographs with the mirrored surface facing to the left. Then, look down from the top edge of the 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.)

periments with synthetic β-methylglutamic acid, only about half of the amino acid disappeared.

The optical rotatory dispersion curve of the isolated β-methylglutamic acid isomer was determined in water and in 3 N HCl with a Cary Model 60 spectropolarimeter at 25° with a 1-cm light path (Figure 2). The curves, which are anomalous, indicate a shift to more negative values for specific rotation in acid solution. Although this finding is indicative of a D-amino acid, it cannot be taken as unequivocal proof of configuration (Greenstein and Winitz, 1961). However, the data given above provide strong evidence that the β-methylglutamic acid isolated from the reaction mixture containing glutamine synthetase is a D isomer. In order to establish this definitely and, further, to determine which of the two possible D isomers of β-methylglutamic acid is a substrate for glutamine synthetase, we carried out the series of reactions shown in Figure 3 in which β-methylglutamic acid was converted to isoleucine. Since only a small amount of β-methylglutamic acid was available, only infrared absorption curves were carried out for identification of the intermediates. The enzymatically susceptible β-methylglutamic acid (100 mg) was dissolved in hot absolute ethanol containing 100 mg of hydrogen chloride, and after shaking for 5 min, the solution was evaporated to dryness *in vacuo*. The residue containing the γ-half ethyl ester of β-methylglutamic acid (II) was dissolved in 1 ml of saturated $NaHCO_3$ (pH, 7.5–8.0) and 0.3 ml (5 M excess) of freshly distilled acetic anhydride was added in five equal portions together with sufficient $NaHCO_3$ to maintain the pH in the range 7.5–8.0. The mixture was shaken vigorously between additions, and finally was made acid to Congo red paper by addition of HCl. The acidified solution was extracted with five 5-ml portions of ethyl acetate; the combined ethyl acetate extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue containing the *N*-acetyl derivative (III) was dissolved in 25 ml of 1 M $LiBH_4$ in tetrahydrofuran, and the solution was refluxed gently for 1 hr. Concentrated HCl (5 ml) was added and the acid solution was evaporated to dryness *in vacuo*. The residue was extracted five times with ethyl acetate; the extracts were combined, dried, and evaporated to dryness in a thick-walled 18 × 150 mm Pyrex tube. This residue (IV) was treated with 0.25 ml of glacial acetic acid previously saturated at 0° with anhydrous HBr; the tube was sealed and heated at 130–140° for 10 hr. After cooling, the tube was opened and the solution was repeatedly evaporated to dryness to remove excess HBr. This product (containing V and the corresponding *N*-acetyl derivative) was dissolved in 3 ml of 1.25 M acetic acid containing 100 mg of sodium acetate. After addition of 500 mg of 5% palladium on charcoal, the mixture was hydrogenated at 39 psi for 1 hr in a Parr apparatus. The catalyst was separated by filtration and washed with boiling water. The combined filtrate and washings were evaporated to dryness. The residue was dissolved in 2 ml of 2 N HCl and placed at 100° for 2 hr, after which the solution was repeatedly evaporated to dryness to remove excess HCl. This residue was dissolved in the minimal amount of water; this solution was applied in a line on a sheet of Whatman No. 1 paper. Chromatography was carried out in a solvent consisting of 78% ethanol. The area corresponding to isoleucine was cut out and eluted with water. The over-all yield in the seven step conversion of β-methylglutamic acid to isoleucine was 5%; the amount of pure isoleucine obtained (4.5 mg) is consistent with the yields estimated from infrared data for the individual steps in the sequence of reactions.

The isoleucine product was subjected to paper electrophoresis as described under Methods. Virtually all of the ninhydrin-reactive material corresponded to alloisoleucine; however, a trace of isoleucine was also present.[4] The alloisoleucine was shown to be entirely of the D configuration by studies with D-amino acid oxidase. The reaction mixtures contained D-amino acid oxidase (160 units), flavin-adenine dinucleotide (5 μg), sodium pyrophosphate buffer (pH 8.3; 100 μmoles), and isoleucine sample (3–5 μmoles). After incubation with shaking at 37° for 1 hr, samples of the reaction mixture (and a control in which heat-inactivated enzyme was added) were deproteinized by heating followed by centrifugation in the cold, and then subjected to electrophoresis as described under Methods. Standard reaction mixtures containing authentic samples of the isomers of isoleucine were also employed. These studies showed that all of the alloisoleucine of the product disappeared when incubated with D-amino acid oxidase.

A comparison of the values for K_m and relative maximal velocity for *threo*-β-methyl-D-glutamic acid with those of L- and D-glutamic acids are given in Table I. It is of interest that the K_m value for *threo*-β-methyl-D-glutamic acid with ammonia is appreciably higher than that found with hydroxylamine; this result is similar to that observed with D-glutamic acid. The K_m values for the isomers of glutamic acid and for *threo*-β-methyl-D-glutamic acid with hydroxylamine are similar. It is also noteworthy that the relative maximal velocity with *threo*-β-methyl-D-glutamic acid is considerably lower with ammonia than with hydroxylamine, a finding also noted with D-glutamic acid.

Action of Glutamine Synthetase on the β-Hydroxyglutamic Acids. As shown in Figure 4, both *threo*-β-hydroxy-DL-glutamic acid and *erythro*-β-hydroxy-DL-glutamic acid are substrates for glutamine synthetase. In agreement with earlier work in which the glutamine synthetase from peas was used (Levintow *et al.*, 1955) the *threo* (allo) form is a much better substrate than the *erythro* form. About 50% of the racemic *threo* form was converted to the hydroxamate in less than 20 min, while 90% conversion to hydroxamate was observed in about 80 min. This finding suggests that one isomer of *threo*-β-hydroxy-DL-glutamic acid is utilized more rapidly than the other. Resolution of small amounts of both diastereoisomers was carried out

[4] The isoleucine was not oxidized by D-amino acid oxidase; it therefore appears that this material is L-isoleucine, probably formed by epimerization during the procedures employed.

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

Amino Acid Substrate	Relative Maximal Velocity With NH_2OH	Relative Maximal Velocity With NH_3	K_m ($\times 10^3$) With NH_2OH	K_m ($\times 10^3$) With NH_3
threo-β-Methyl-D-glutamic acid	46	2.2	5.9	25
threo-β-Hydroxy-D-glutamic acid	48	22	6.0	17
L-Glutamic acid	100[b]	100[b]	3.3	3.9
D-Glutamic acid	54	27	3.8	13

[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] Equivalent to a rate of 200 μmoles of L-γ-glutamylhydroxamate or L-glutamine formed per mg of enzyme per 15 min.

with D-glutamic acid cyclotransferase as described under Methods. Studies with the four separate optical isomers thus obtained gave values for initial velocity (relative to L-glutamate (100)) for hydroxamate formation of 30, 6, 3, and 1.5 for the D-*threo*, L-*threo*, L-*erythro*, and D-*erythro* forms, respectively. These comparisons were carried out using the conditions given in Figure 4. It is of interest that the synthesis of hydroxamic acid from *threo*-β-hydroxy-D-glutamic acid takes place at a significantly more rapid rate than that of the corresponding amide (Table I). Thus, the rate of hydroxamate synthesis from *threo*-β-hydroxy-D-glutamic acid was about twice as great as that of amide synthesis. The rates of hydroxamate and amide formation from *erythro*-β-hydroxyglutamic acid were about the same under the conditions given in Figure 4. These findings are in general agreement with earlier data obtained with the glutamine synthetase of peas (Levintow *et al.*, 1955).

Discussion

The major experimental finding reported here is that, of the four optical isomers of β-methylglutamic acid, only *threo*-β-methyl-D-glutamic acid is a substrate for glutamine synthetase. As discussed in the introductory section of this paper, this result was predicted on the basis of our hypothesis concerning the conformation of the enzyme-bound L-glutamic acid and D-glutamic acid molecules. Thus, examination of the model of L-glutamic acid (Figure 5A) shows that both the *threo*- and *erythro*-β-hydrogen atoms are on the undersurface of the molecules (indicated by arrows); these can be seen in Figure 5B, which shows the other side of this model. Figure 5C shows a model of D-glutamic acid oriented in the manner described previously (Kagan *et al.*, 1965; Khedouri and Meister, 1965). Thus, this model was obtained by rotating a mirror image model of L-glutamic acid to the right, thereby bringing its amino group into the same relative position in space as the amino group of the model of L-glutamic acid.[5] The carboxyl carbon atoms were then rotated until the carboxyl groups of the model of D-glutamic acid were in the same plane as those of L-glutamic acid. As seen in Figure 5C the *erythro*-β-hydrogen atom (indicated by an arrow) is on the undersurface of the model; Figure 5D is a photograph of the other side of this model. The *threo*-β-hydrogen atom of D-glutamic acid (Figure 5C) is the only β-hydrogen atom that is directed upwards, and this hydrogen atom occupies a position that is close to that occupied by the α-hydrogen atom of L-glutamic acid. The experimental data indicate that substitution by a methyl group of the α-hydrogen atom or of the *erythro*-β-hydrogen atom of D-glutamic acid leads to the loss of enzymatic susceptibility, while similar substitution of the *threo*-β-hydrogen atom of D-glutamic acid or of the α-hydrogen atom of L-glutamic acid does not. Other studies (Kagan and Meister, 1965, and unpublished data) have shown that substitution by a methyl group of the *threo*-γ-hydrogen atom of L-glutamic acid does not destroy enzymatic susceptibility while the other three γ-methyl derivatives of glutamic acid are enzymatically inactive. It may be seen from the models that the *threo*-γ-hydrogen atom of D-glutamic acid is adjacent to the α-hydrogen atom of this molecule; the *erythro*-γ-hydrogen atoms of both L- and D-glutamic acids lie just between the γ-carboxyl groups and the amino groups of the respective molecules. Thus, the only methyl substitutions that do not lead to loss of enzymatic susceptibility (*i.e.*, L-α-methyl, D-*threo*-β-methyl, L-*threo*-γ-methyl) are located on the same side (the left side of the three-dimensional model as shown in Figures 5A and 5C) of the glutamate molecules. The finding suggests that this side of the substrate is not in close contact with the active site of the enzyme, and therefore that the under-surface (Figure 5B and 5D) and perhaps the right-hand side of the stereomodel (Figure 5A and 5C) of the substrate is in contact with the active site of the enzyme and the metal-nucleotide chelate.

The studies with the four β-hydroxyglutamic acids gave results that were similar to those obtained with the corresponding methyl-substituted derivatives. Thus,

[5] By rotating the model 69° about an axis formed by a straight line intersecting the centers of carbon atoms 1, 3, and 5, the amino group of the model of D-glutamic acid is brought to the same position in space (relative to the axis of rotation of both L- and D-glutamic acids) as that of L-glutamic acid.

threo-β-hydroxy-D-glutamic acid is a considerably better substrate than are the other three isomers. The fact that *erythro*-β-hydroxy-D-glutamic acid and the two β-hydroxy-L-glutamic acids exhibit some activity might be explained by the fact that the hydroxyl group occupies less space than the methyl group; it is also possible that attachment of the hydroxy-substituted glutamic acids to the enzyme is facilitated by hydrogen bonding with the enzyme.

Studies previously carried out on the glutamine synthetase of peas (Levintow and Meister, 1953, 1954) showed that this enzyme catalyzes γ-glutamylhydroxamate synthesis from L- and D-glutamic acids at about the same rate, while amide synthesis from L-glutamic acid was about three times more rapid than amide synthesis from D-glutamic acid. This observation led to the proposal that the glutamine synthetase reaction takes place in two steps, an initial activation followed by a more optically specific reaction of the activated intermediate with ammonia. Subsequent work on the glutamine synthetase from sheep brain has led to substantially similar findings (Krishnaswamy *et al.*, 1962). As shown in Table I, although the rate of γ-glutamylhydroxamate formation from D-glutamic acid is less than that of L-glutamic acid, the formation of D-glutamine is relatively much lower. In contrast, similar rates of amide and hydroxamate formation were observed with L-glutamate (Table I), and with α-methyl-L-glutamate (Kagan and Meister, unpublished data). Hydroxamate formation takes place at a much greater rate than amide formation with *threo*-β-methyl-D-glutamic acid, and similar findings were made with *threo*-β-hydroxy-D-glutamic acid. It thus appears that D-glutamic acid and the susceptible substituted D-glutamic acid derivatives are relatively more active in hydroxamate synthesis than in amide synthesis. It was previously suggested that the relatively great reactivity with ammonia of L-glutamic acid as compared to D-glutamic acid might be due to more favorable orientation of the activated form of L-glutamic acid on the enzyme in relation to the binding site of the enzyme for ammonia (Meister *et al.*, 1962). The present findings are consistent with this view. It may be assumed that ammonia is bound to a specific site on the enzyme and that hydroxylamine may also be bound to the same site, but in addition may directly attack the activated carboxyl carbon atom. Examination of the models (Figure 5) shows that the orientation of the γ-carbon atom of D-glutamic acid is not exactly the same as that of L-glutamic acid and therefore would be in a different (presumably less favorable) position in relation to the binding site on the enzyme for ammonia. The fact that the *threo*-β-methyl derivative of D-glutamic acid is less reactive with ammonia than D-glutamic acid suggests that the methyl group interferes with the attack by ammonia. Study of the models suggests several possible detailed explanations for these effects, the further elucidation of which requires additional study.

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