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IDENTIFICATION OF γ-METHYLGLUTAMIC ACID IN *LATHYRUS MARITIMUS*

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Abstract—From *Lathyrus maritimus* seeds a non-protein amino acid has been isolated by ion-exchange chromatography and identified as *erythro*-γ-methyl-L-glutamic acid. The compound is estimated to constitute about 0·5 and 1 per cent of the dry matter of *L. maritimus* and *L. aphaca* seeds, respectively.

INTRODUCTION

ANALYSIS of a number of *Lathyrus* species for new amino acids revealed the presence in *Lathyrus maritimus* and *L. aphaca* of a ninhydrin-positive compound similar to, but distinct from, glutamic acid in chemical stability and chromatographic behaviour.[1] The present paper describes the isolation of this compound (designated[1] "y_6") in crystalline form and its identification as *erythro*-γ-methyl-L-glutamic acid.

RESULTS

The isolated material melted at 158–161° and analyzed correctly for $C_6H_{11}O_4N$. Preparations of "y_6" which had previously been separated from glutamic acid by preparative paper chromatography but not crystallized[1] were subjected to the following treatments: 3 N HCl hydrolysis at 120° in a sealed glass ampule for 24 hr; oxidation with 1 per cent (w/v) $KMnO_4$ in 20 per cent (v/v) H_2SO_4 at 12°; reduction with HI ($d=1·7$) in the presence of red phosphorus at 124° for 4 hr. Paper electrophoresis and circular paper chromatography (in four solvent systems) of the treated samples showed no significant losses, and no other ninhydrin-positive compounds were formed. That the unknown compound was an α-amino acid was strongly suggested by a negative ninhydrin test after treatment (on paper) with a cupric nitrate reagent.[2]

Paper chromatography of an aqueous solution of "y_6" (5 mg/ml) which had been heated in a sealed glass ampule at 120° for 4 hr revealed that the ninhydrin-positive substance had disappeared and that an acidic substance, detected by the starch-iodide-chlorimide technique of Rydon and Smith,[3] had been generated.

The above characteristics, the $C_6H_{11}O_4N$ formula, and the acid character of "y_6" shown by paper electrophoresis and ion-exchange chromatography, suggested that the isolated compound was a methylglutamic acid and that the acid produced on heating was the corresponding pyrrolidone carboxylic acid.

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Since α-methylglutamic acid exhibited markedly slower and less intense development of ninhydrin color than "y_6",[4] and since samples of γ-methyl-L-glutamic acid, an amino acid known to occur in nature, were available, a direct comparison of the latter with "y_6" was

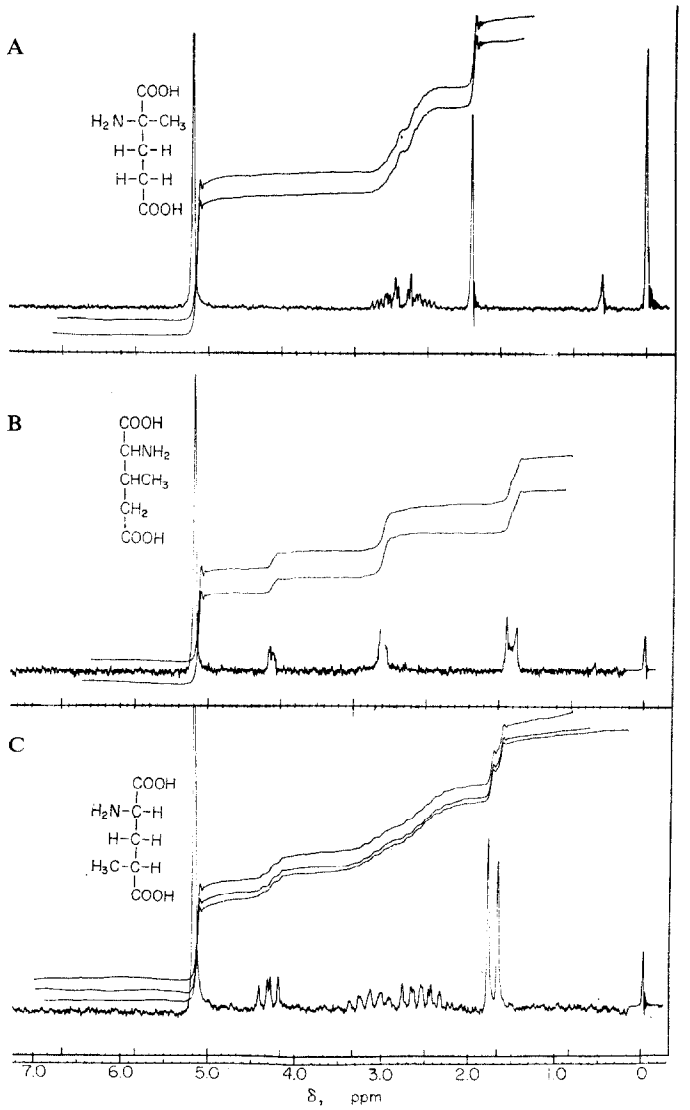

FIG. 1. NMR SPECTRA: A: D,L-α-METHYLGLUTAMIC ACID; B: β-METHYLGLUTAMIC ACID (SYNTHETIC MIXTURE OF FOUR STEREOISOMERS); C: *erythro*-γ-METHYL-L-GLUTAMIC ACID ("y_6" FROM *L. maritimus* SEEDS). SPECTRA MEASURED IN D_2O WITH TETRAMETHYL SILANE (TMS) AS INTERNAL STANDARD. THE PEAK AT δ0·5 IN SPECTRUM A IS A SPINNING SIDE BAND OF TMS.

carried out. Both the *erythro* and *threo* forms of γ-methyl-L-glutamic acid were utilized for this comparison. Both showed identical color reactions with ninhydrin reagent and the same

[4] J. PRZYBYLSKA, unpublished.

R_f values on circular paper chromatograms run in four different solvent systems as did "y_6". When subjected to paper electrophoresis at pH 4·4, the *threo* form moved distinctly more slowly toward the anode than the *erythro* form, whereas the ionic mobility of the isolated compound corresponded exactly to that of the *erythro* isomer. The infrared spectrum (KBr pellet) of preparation 1 and that of *erythro*-γ-methyl-L-glutamic acid showed no significant differences. Optical rotation values for the isolated compound likewise corresponded very closely with those reported[5] for the *erythro* form.

Further support for the γ-methylglutamic acid structure of "y_6" came from the NMR spectrum (Fig. 1C). The spectrum shows a 3-proton doublet at δ1·7 ppm (J = 7·5 c/s) due to the C-methyl group. A quartet at δ4·29 (the X part of an ABX) with coupling constants of 8 and 6 c/s, respectively, is assigned to the α-proton. The β-methylene protons (the AB part of the ABX system) appear as a complex multiplet with the center at δ2·5. Another multiplet with the center at δ3·12 is due to the γ-proton.

We have also obtained the NMR spectra of α- and β-methylglutamic acids (Fig. 1A, B). The spectrum of α-methylglutamic acid (singlet at δ2·02, complex A_2B_2 at δ2·7) is quite different from that obtained on our isolate. The spectrum of β-methylglutamic acid cannot be compared directly with that of "y_6" because the sample used was a mixture of stereoisomers. The former is characterized by a broad methyl doublet at δ1·56, a broad signal for three protons at δ3·0 and an unresolved doublet at δ4·3.

The NMR spectrum of "y_6" is thus consistent only with the γ-methylglutamic acid structure; in particular the appearance of the quartet at δ4·29 is not consistent with the structure of β-methylglutamic acid.

DISCUSSION

γ-Methylglutamic acid was first synthesized by Done and Fowden[6] to prove the structures of γ-methyleneglutamine and γ-methyleneglutamic acid isolated from *Arachis hypogea*. It was not found, however, in ethanolic extracts of any of the analyzed organs of this plant. In 1955, Virtanen and Berg[7] reported the isolation of γ-methylglutamic acid from fern, *Phyllitis scolopendrium*. It was identified mainly by cochromatography with Done and Fowden's[6] material. Subsequently, γ-methylglutamic acid was found in different species of *Liliaceae*[8] and in *Polygala vulgaris*,[9] the identification being based on paper chromatographic analysis.

Unequivocal confirmation of the structure of natural γ-methylglutamic acid was obtained by Blake and Fowden[10] on material isolated from fern fronds (*Phyllitis*). By comparison with the *erythro*- and *threo*-isomers and by conversion into D(+)-α-methylsuccinic acid, the natural product was shown to have the *erythro*-L configuration.

The reported occurrence of the same diastereoisomeric form of γ-methylglutamic acid in the leguminous plant, *Lathyrus maritimus*, is quite interesting. Although the free amino acids in many species of *Papilionaceae* have been carefully studied, so far as we know, no evidence has been obtained indicating the presence of this particular compound. According to paper chromatographic analysis, γ-methylglutamic acid (designated as "y_6") occurs also in other *Lathyrus* species, e.g. *L. aphaca*.[1]

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[10] J. BLAKE and L. FOWDEN, *Biochem. J.* **92**, 136 (1964).

Besides γ-methylglutamic acid, there occur in plants such related compounds as γ-methyleneglutamine, γ-methyleneglutamic acid and γ-methyl-γ-hydroxyglutamic acid.[11–14] Some species of *Liliaceae* contain all four amino acids.[8] Such coexistence suggests that interconversion among these acids may occur *in vivo* by reversible oxidation–reduction and hydration–dehydration reactions. Although none of the related acids were detected in ethanolic extracts of *L. maritimus* seeds, it cannot be excluded that they occur in very small amounts. The presence of γ-methyl-γ-hydroxyglutamic acid in *Phlox decussata*[15] and in *Arachis hypogea*[16] was not demonstrated until isotopic techniques were used.

If interconversion reactions between the compounds in question really exist, γ-methylglutamic acid may be expected to occur in other members of *Papilionaceae*, namely in *Arachis hypogea* and in *Amorpha* species which are known to accumulate the corresponding γ-methylene compounds.[6,17] α-Keto-γ-methylglutaric acid found in the fern, *Phyllitis scolopendrium*, by Virtanen *et al.*,[7,18] may be a precursor of γ-methylglutamic acid since Fowden and Done[19] have shown that the latter participates actively in transamination reactions.

Kagan and Meister[5] have shown that only one of the four isomers of γ-methylglutamic acid, namely *threo*-γ-methyl-L-glutamic acid is a substrate for the glutamine synthetases of sheep brain and peas. This finding agrees with the fact that no natural occurrence of γ-methylglutamine has been reported in plants, where probably γ-methylglutamic acid exists only as the *erythro*-L-isomer.

According to a rough estimation based on the color intensity of the spots obtained on paper chromatograms, γ-methylglutamic acid represents about 0·5 per cent and 1 per cent of the dry matter of *L. maritimus* and *L. aphaca* seeds respectively. The accumulation of this compound in mature seeds suggests that it may be of significance in nitrogen storage.

EXPERIMENTAL[20]

Materials

Lathyrus maritimus seeds were obtained from the collection of the Institute of Plant Genetics of the Polish Academy of Sciences, Poznań. They were harvested in 1964–1966. As shown by two-dimensional paper chromatography, the free amino acid composition of the seeds harvested in different years was nearly the same.

D,L-α-Methylglutamic acid was obtained from the California Biochemical Research Corporation, Los Angeles. β-Methylglutamic acid was a synthetic sample containing the four stereoisomers. D,L-*Erythro*- and *threo*-γ-methylglutamic acids were samples that had been prepared by synthesis and separated into the two diastereoisomeric mixtures.[10]

Isolation

800 g of *L. maritimus* seeds were finely ground in a ball mill and percolated with light petroleum for 8 hr. The air-dried residue was extracted three times with 3-l. portions of cold 75 per cent ethanol and once with 3 l. of hot 75 per cent ethanol. Finally, the insoluble material was boiled with 600 ml of water for 15 min, 2 l. of 96 per cent ethanol added, the mixture allowed to stand for 3 to 4 hr at room temperature and then

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[18] A. I. VIRTANEN and M. ALFTHAN, *Acta Chem. Scand.* **9**, 188 (1955).
[19] L. FOWDEN and J. DONE, *Nature* **171**, 1068 (1953).
[20] Melting points were determined on the hot stage heated 1·5° per min. Infrared spectra were measured with a Beckman Model 5 spectrophotometer. NMR spectra were taken on a Varian A-60A instrument. Microanalyses were by Micro-Tech Laboratories, Skokie, Illinois.

filtered. The combined extracts were evaporated in a rotary evaporator at 40°. The residue was dissolved in water (0·5 l.) and the total amino acid fraction was bound on a strongly acid ion-exchange resin (Amberlite CG-120, 100–200 mesh), in the acid form. For this purpose the total extract was divided into eight equal portions which were applied to separate columns (each 2 × 30 cm). After washing the columns with water, the amino acids were eluted with 0·5 N ammonia (0·5 l. for each column), 20-ml fractions being collected. Fractions showing a ninhydrin-positive reaction were combined, evaporated in a rotary evaporator at 40° to a dark syrup which was dissolved in water (50 ml), and applied to a strongly basic ion-exchange resin (Dowex 1-X8, 200–400 mesh) in the acetate form. The total was applied on four columns (each 2 × 30 cm). Each column was washed with 400 ml of CO_2-free water, and the effluent and washings collected in 20-ml fractions. As shown by paper electrophoresis, they contained only neutral and basic amino acids and were discarded. Acidic amino acids were eluted subsequently with 0·25 M CH_3COOH, twelve fractions of about 20 ml each being collected from each column. The "y_6" accompanied by glutamic acid was found in fractions 3–4. These fractions were evaporated to dryness in a rotary evaporator at 40°, and acetic acid was removed by repeated evaporation with water. The total yield of "y_6" plus glutamic acid was 3·2 g.

Separation of "y_6" from glutamic acid was achieved with the amino acid analyzer[21] (Beckman and Spinco Model 120 amino acid analyzer; Spinco 150 A sulfonated polystyrene resin; column 3·9 × 150 cm; citrate buffer pH 3·28; flow rate 480 ml/hr). 100 mg of the mixture of "y_6" and glutamic acid was separated at one time and two such runs were made. Glutamic acid emerged between 7 and 8 hr and "y_6" between 9 and 10 hr. To remove buffer components the combined fractions containing "y_6" were passed through a Dowex 50-X8 (200–400 mesh) column in the acid form. The column was washed with water and "y_6" then eluted with 1 N ammonia. The eluate was lyophilized since evaporation was difficult because of foaming, probably due to detergent.[21] The white solid obtained (140 mg) when examined by circular paper chromatography showed only one ninhydrin-positive substance. Final purification of "y_6" was accomplished by application to a strongly basic resin column (Dowex 1-X8, 200–400 mesh, 1·2 × 20 cm) in the acetate form followed by elution with 0·25 N acetic acid. Fractions giving a ninhydrin-positive reaction were evaporated to dryness and the acetic acid was removed by repeated evaporation with water. "y_6" crystallized from water when the solution was being evaporated to a small volume. The crystalline substance (preparation 1) was centrifuged, washed with water:acetone (50:50, v/v), centrifuged again and dried *in vacuo*. The yield was 34 mg. The combined supernatants were evaporated to 1–2 ml, treated with 0·1–0·2 ml acetone and left overnight in the refrigerator. 17 mg of colorless plates (preparation 2) were obtained. (Found for preparation 1: C, 44·70; H, 6·91; N, 8·91. Found for preparation 2: C, 44·82; H, 6·92; N, 8·78. Calc. for $C_6H_{11}O_4N$: C, 44·71; H, 6·88; N, 8·69 per cent); $[\alpha]_D^{25°} = -2·1°$ (*c* 0·6 in water), 22·2° (*c* 0·6 in 5 N HCl), $[\alpha]_{365\,nm}^{25°} = 8·8°$ (*c* 0·6 in water). Kagan and Meister[5] report −2·03°, 22·2° and 8°, respectively (the last value being read from a plot of their data).

Chromatographic and Ionophoretic Techniques

Two-dimensional paper chromatography was performed after Wolfe[22] as modified by Przybylska.[23] Circular chromatograms (Whatman No. 3 paper) were run in four solvent systems:

1. n-butanol:acetic acid:water; 12:3:5, by volume,
2. n-butanol:propionic acid:water; 1264:620:974, by volume,
3. phenol:ethanol:water; 120:40:40, by volume,
4. n-butanol:methyl ethyl ketone:ammonia (sp. gr. 0·88):water; 5:3:1:1, by volume.

Paper electrophoresis was carried out in pyridinium acetate buffer, pH 4·4 (acetic acid:pyridine:water, 25:15:960)[24] on strips of Whatman No. 3 paper (58·5 × 6·5 cm) for 4 hr at 1000 V. Under these conditions a significant difference was observed in the mobilities of the stereoisomers of γ-methylglutamic acid (*threo*, 11·9 cm; *erythro*, 13·5 cm). The isolated compound moved together with the *erythro* form.

Chromatograms and electropherograms were developed with 0·4 per cent solution of ninhydrin in acetone.

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