The Acidic Amino Acids of Tulip: Isolation of γ -Ethylideneglutamic acid

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1. γ -Ethylideneglutamic acid has been isolated from fruit capsules of tulip plants. 2. The assigned structure was indicated by examining the products formed after oxidation and catalytic hydrogenation and was confirmed by nuclear-magnetic-resonance spectroscopy and by synthesis of γ -ethylglutamic acid. 3. The ability of γ -ethylideneglutamic acid to participate in transamination and decarboxylation reactions was examined.

Tulips (*Tulipa gesneriana*) are a source of several γ -substituted glutamic acids. Initially, γ -methyleneglutamic acid and γ -methyleneglutamine were obtained from tulip bulbs (Zacharius, Pollard & Steward, 1954), and identified by comparison with authentic materials previously characterized from peanut plants (Done & Fowden, 1952). Subsequently, in a survey of amino acids of liliaceous plants, Fowden & Steward (1957) concluded that tulip leaves contain smaller quantities of γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid. A small amount of the γ -methyl derivative was isolated later (Blake & Fowden, 1964) from tulip leaves and, in better yield, from the fern *Phyllitis scolopendrium*; it was shown to be the *erythro*-L isomer. The γ -methyl and α -amino groups of γ -hydroxy- γ -methylglutamic acid isolated from the same fern possessed a similar *erythro*-L configuration.

In the survey of liliaceous plants the unidentified ninhydrin-positive compound (UI44) was observed as a major constituent of tulip seeds. Chromatographic comparison showed that this compound probably was identical with an unidentified acidic amino acid isolated as a very minor component from tulip leaves (Blake & Fowden, 1964). The present account gives details of its isolation and characterization as γ -ethylideneglutamic acid (I) from immature fruits (capsules containing immature seeds) of tulip in which it occurs in concentrations intermediate between those of the leaf and the ripe seed.

Isolation and identification

γ -Ethylideneglutamic acid was isolated from immature tulip seed capsules by ion-exchange chromatographic techniques. The pure compound had empirical formula $C_7H_{13}NO_5$, which included a molecule of water of crystallization.

The compound could be catalytically hydrogenated and the reduction product was resolved into two spots of approximately equal intensity when paper chromatograms were developed with a 2-methylbutan-2-ol-acetic acid-water mixture, which is particularly effective for separating diastereoisomeric amino acids (Gray, Blake, Brown & Fowden, 1964).

L-Aspartic acid was the only ninhydrin-reacting product identified after oxidation of γ -ethylideneglutamic acid with acidic permanganate. The increased positive rotation observed for γ -ethylideneglutamic acid in 6N-hydrochloric acid compared with water also indicated that it possessed an L-configuration (Clough, 1918; Lutz & Jirgensons, 1930, 1931). When γ -ethylideneglutamic acid was oxidized with alkaline permanganate followed by periodate (Done & Fowden, 1952), the presence of acetaldehyde in the distillate was indicated by the piperidine-sodium nitroprusside colour reaction (Feigl, 1956). The identification of aspartate as the product of oxidation established that the unsaturated linkage (indicated by catalytic hydrogenation) could not occur in the chain before the γ -carbon atom is reached.

γ -Ethylideneglutamic acid was completely destroyed when solutions, adjusted to pH 3 with acetic acid, were autoclaved. This treatment led to a complete loss of ninhydrin-reacting material but disappearance of γ -ethylideneglutamic acid was associated with the formation, by cyclization, of a lactam (positive starch-iodide-chloroimide test; Rydon & Smith, 1952). By analogy with earlier

studies (Blake & Fowden, 1964) the lactam was presumed to be 3-ethylidenepyrrolid-2-one-5-carboxylate.

The completeness of the cyclization reaction suggested that the main carbon chain of γ -ethylideneglutamic acid contained a glutamate skeleton; a dicarboxylic amino acid containing a linear C₄ chain as in aspartate would not cyclize, and a substituted α -aminoadipic acid would yield an equilibrium mixture of open-chain and cyclized forms. In all these reactions, the behaviour of γ -ethylideneglutamic acid was akin to that of γ -methyleneglutamic acid, and as with this compound, the production of two substances after catalytic hydrogenation suggested that the unsaturated linkage must involve a carbon atom located at the branch point of the carbon skeleton. These conclusions can be rationalized only with the γ -ethylideneglutamic acid structure. The assignment of this structure was supported by the results of nuclear-magnetic-resonance spectroscopy. The spectrum, measured in deuterated water, could be integrated and indicated the correct proton number (excepting the exchangeable carboxyl and amino group protons): the presence of CH₃-CH and -CH₂-CH structures was also confirmed, and other details of the spectrum suggested that the methyl group is probably *cis*- to the γ -carboxyl group.

The γ -ethylideneglutamic acid structure was confirmed partially by an unequivocal synthesis of γ -ethylglutamic acid by a stepwise condensation of dibromomethane first with sodio diethyl ethylmalonate and then with sodio diethyl acetamidomalonic acid. The product obtained after acidic hydrolysis behaved identically with the reduction product of γ -ethylideneglutamic acid in all chromatographic solvents tested: it was separated into *threo*- and *erythro*- isomers after chromatography in 2-methylbutan-2-ol-acetic acid-water (compare above) and by high-voltage ionophoresis at pH 3.6. After racemizing the reduction product of γ -ethylideneglutamic acid by treatment with barium hydroxide, an infrared spectrum was obtained which was identical in all major details with that obtained from synthetic γ -ethylglutamic acid.

Other acidic amino acids

The acidic amino acid fraction retained by Dowex 1 was separated into more than 20 components during subsequent elution with acetic acid. In addition to γ -ethylideneglutamic acid, aspartic acid, glutamic acid, α -aminoadipic acid, γ -methylene-, γ -methyl- and γ -hydroxy- γ -methyl-glutamic acid were characterized by isolation and comparison with authentic samples. Many of the remaining compounds were unstable when treated with N-

hydrochloric acid at 100° for a few hours and chromatographic identification of the hydrolysis products suggested that several were γ -glutamyl peptides, e.g. γ -glutamylvaline, γ -glutamylleucine and γ -glutamylphenylalanine (previously identified in the Family Liliaceae; Virtanen & Matikkala, 1961) and possibly γ -glutamylalanine, γ -glutamylserine, γ -glutamylglycine and γ -glutamylthreonine. γ -Ethylglutamic acid was not identified among the spots stable to hydrochloric acid treatment. Similarly, an attempt to identify γ -hydroxy- γ -ethylglutamic acid among this group of compounds also proved negative: substances moving close to the position that the hydroxyamino acid might be expected to occupy on two-dimensional chromatograms were treated with hydriodic acid and red phosphorus under conditions that would lead to the replacement of hydroxyl by hydrogen (Blake & Fowden, 1964), but in no case was γ -ethylglutamic acid formed.

Enzymic degradation of γ -ethylideneglutamic acid

γ -Methylene- and γ -methyl-glutamic acids were first observed to participate in transamination reactions with various keto acids by using peanut seedlings as a source of transaminases (Fowden & Done, 1953). Subsequently, a purified glutamate-aspartate transaminase from cauliflower buds was shown to utilize both C₆ acids as alternative, but relatively poor, substrates in place of glutamate (Ellis & Davies, 1961). In new experiments with enzyme preparations from peanut seedling mitochondria γ -ethylideneglutamic acid has been shown to undergo transamination with oxaloacetate at a rate comparable with that observed with γ -methyleneglutamic acid.

Glutamate decarboxylase, present in a variety of plant tissues (Fowden, 1954), is able to utilize γ -methyleneglutamic acid at about 40% of the rate of the normal substrate, glutamic acid. Now γ -ethylideneglutamic acid has been tested as a substrate for the glutamic decarboxylase of pepper fruits and tulip leaves (two of the original plant sources tested), but no decarboxylation was observed.

DISCUSSION

The identification of a further γ -substituted glutamic acid in plants possessing a range of such branched C₆ amino acids leads immediately to speculation concerning biogenetic relationships. Early experiments with peanut seedlings indicated that at least one molecule of pyruvate was incorporated into γ -methyleneglutamic acid as an intact C₃ unit (Fowden & Webb, 1958), and the possibility

has been outlined that biosynthesis involves a type of aldol condensation between two molecules of pyruvate (or some activated derivative) with the initial formation of γ -hydroxy- γ -methyl- α -oxo-glutaric acid (Fowden, 1958; Marcus & Shannon, 1962). By utilizing analogous reactions, γ -ethylideneglutamic acid might result by the initial condensation of pyruvate with α -oxobutyrate although the corresponding γ -hydroxy- γ -ethylglutamate has not been detected in tulip extracts. This view would place γ -methyleneglutamic acid and γ -ethylideneglutamic acid in a similar biogenetic relationship to that existing between valine and isoleucine: an early step in the pathways leading to these two protein constituents involves the condensation of an activated acetaldehyde moiety with either pyruvate or α -oxobutyrate respectively. Unfortunately the limited distribution and low concentration of γ -ethylideneglutamic acid will make biosynthetic studies difficult even when labelled precursors are used.

Biogenetic relationships are seen also between dicarboxylic amino acids and cyclic imino acids. The conversion of glutamate into proline is the most familiar, but *erythro*- γ -hydroxy-L-glutamate is reversibly converted into 4-hydroxy-L-proline in animal tissues (Goldstone & Adams, 1963). Other pairs of compounds showing analogous structural relationships exist in plants, e.g. *erythro*- γ -methyl-L-glutamate and *trans*-4-methyl-L-proline, and γ -methylene-L-glutamic acid and 4-methylene-DL-proline (Fowden, 1964), but no imino acid comparable with γ -ethylideneglutamic acid is known in higher plants. Recently an *N*-methyl-4-propylproline moiety has been shown to be a component of an antibiotic, lincomycin, produced by *Streptomyces lincolnensis* (Hoeksema *et al.* 1964), while the 4-ethylhygric acid (4-ethyl-N-methyl-L-proline) was identified subsequently in another lincomycin-type antibiotic present in the same culture filtrates (Argoudelis, Fox, Mason & Eble, 1964).

EXPERIMENTAL

Chromatographic and ionophoretic techniques. Solvents used for paper chromatography were: 75% (w/w) phenol solution in the presence of NH_3 ; a one-phase mixture of butan-1-ol-acetic acid-water (90:10:29, by vol.); the upper phase of 2-methylbutan-2-ol (technical grade; British Drug Houses Ltd.)-acetic acid-water (10:1:10, by vol.). Amino acids were detected by spraying the chromatograms with a 0.1% solution of ninhydrin in 95% (v/v) ethanol while the starch-iodide-chloroimide technique of Rydon & Smith (1952) was used to locate the pyrrolidone-carboxylic acids (lactams) obtained after cyclization of the γ -substituted acids.

Paper-ionophoretic separations were performed in buffers at pH 2.0 (formic acid-acetic acid-water, 61:97:1842,

by vol.) and pH 3.6 (formic acid-pyridine-water, 10:1:189, by vol.) by using a horizontal method based on that of Gross (1961).

Isolation of γ -ethylideneglutamic acid (I). Fruit capsules (40 kg.) were collected, deep-frozen and minced. After thawing the mush was pressed (6000 lb./in.²) and the juice adjusted to pH 4.6. Protein was coagulated by warming to 60° and after stirring in absorbent charcoal the mixture was filtered. The clarified extract (30 l.) was applied to a squat column containing 20 lb. of Zeo-Karb 225 (H^+ form; mesh 52-100). All ninhydrin-positive material was held by the resin which was then thoroughly washed with 60 l. of water. The amino acids were displaced from the column by 2N- NH_3 (35 l.) without fractionation and the eluate was concentrated by evaporation *in vacuo* at 40° to remove NH_3 .

This eluate fraction (2.5 l., pH 5.8) was applied to a column (80 cm. $\times$ 5.5 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100-200) to retain acidic amino acids. Neutral and basic amino acids were washed through with CO_2 -free water (15 l.) before the acidic substances were displaced by gradient elution with 0.25-2N-acetic acid. Fractions (50 ml.) were collected after the breakthrough of the first acid, glutamic acid. γ -Ethylideneglutamate (initial colour brown with ninhydrin) was present in fractions 2-14 together with an approximately equal amount of glutamic acid. Small amounts of γ -methyleneglutamic acid and γ -methylglutamic acid also were present, especially in later fractions of this range. Aspartic acid was found in low concentration in fractions 19-49 and γ -hydroxy- γ -methylglutamic acid in fractions 60-85. This γ -hydroxy-amino acid crystallized (0.12 g.) when fractions 62-82 were reduced in volume to 25 ml. Altogether 220 fractions containing ninhydrin-positive material were collected: many of the substances tentatively identified as γ -glutamyl peptides occurred in discrete groups of fractions between nos. 92 and 220.

Fractions 2-13 were combined and evaporated to dryness *in vacuo* below 50° to remove acetic acid. The residue was redissolved in 250 ml. of water and adjusted to pH 6.0 by addition of dilute NH_3 . This solution was fractionated on a second column (125 cm. $\times$ 2 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100-200), 0.25N-acetic acid being used for elution. Fractions (5 ml.) were collected as glutamic acid appeared in the eluate. γ -Ethylideneglutamic acid was present in fractions 44-151; glutamic acid was the only other component of these fractions. The residue (4.6 g.) obtained after evaporating combined fractions 55-142 was redissolved in 50 ml. of hot water, and the solution deposited crystals of γ -ethylideneglutamic acid (0.44 g.) when stored overnight at 2°. Ethanol (1 vol.) was added to the mother liquor (3 vol.), when a second batch of crystals (0.58 g.) was collected. The two samples were combined and recrystallized from water (10 ml.) to yield 0.70 g. of pure γ -ethylideneglutamic acid (Found: C, 43.9; H, 6.8; N, 7.1; loss at 105°, 9.2. $\text{C}_7\text{H}_{11}\text{NO}_4 \cdot \text{H}_2\text{O}$ requires C, 44.0; H, 6.8; N, 7.3; H_2O , 9.4%). The amino acid had $[\alpha]_D^{20} + 21^\circ$ (c 2.8 in water) and $+ 38.3^\circ$ (c 1.4 in 6N-HCl).

Synthesis of γ -ethyl-DL-glutamic acid. Sodium (2.4 g.) was added to dry ethanol (45 ml.) and then diethyl ethylmalonate (19 ml.) and excess of dibromomethane (20 ml.) were slowly added. The mixture was refluxed for 3 hr. and then insoluble sodium bromide was removed by filtration. Ethanol and excess of dibromomethane were removed on an

oil bath at 120°, leaving crude diethyl 2-bromomethyl-2-ethylmalonate. This intermediate was redissolved in dry ethanol (80 ml.) containing sodium (2.3 g.) and diethyl acetamidomalonate (22 g.). The mixture was now refluxed for 8 hr., precipitated sodium bromide was again removed by filtration, and most of the ethanol was distilled off. The residue was hydrolysed by refluxing with 6*N*-HCl (200 ml.) for 5 hr.: crude γ -ethylglutamic acid hydrochloride remained after removal of HCl by evaporation *in vacuo*.

γ -Ethylglutamic acid was separated from other reaction products by using anion-exchange chromatography. The crude γ -ethylglutamic acid hydrochloride was redissolved in water (200 ml.) and adjusted to pH 6.0 by addition of NH₃. The solution was applied to a column (25 cm. $\times$ 5 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100–200) when only γ -ethylglutamic acid was held, other ninhydrin-positive by-products being washed through with water. γ -Ethylglutamic acid was displaced with 0.5*N*-acetic acid and was present in ten 50 ml. fractions. Paper-chromatographic examination of these with 2-methylbutan-2-ol-acetic acid-water as solvent showed that the first seven fractions contained both diastereoisomers (total yield, 2.8 g.) but the last three (yield 0.34 g.) contained only the slower-moving isomer; by analogy with the behaviour of the diastereoisomers of γ -methylglutamic acid (Blake & Fowden, 1964), the slower-moving isomer was probably the *erythro* form (Found for the mixed diastereoisomers: C, 47.6; H, 7.5; N, 8.2. C₇H₁₃NO₄ requires C, 48.0; H, 7.4; N, 8.0%).

Reduction of γ -ethylideneglutamic acid. Hydrogen was bubbled through a solution of γ -ethylideneglutamic acid (2 mg./ml.) in 2% (v/v) acetic acid in the presence of platinum oxide (5 mg./ml.). Reduction was complete in 30 min. at 20°. The reduction product gave a single spot (blue-purple) on chromatograms developed in phenol-NH₃ (*R_F* 0.41) and in butan-1-ol-acetic acid-water (*R_{Leu}* 0.72), but it was resolved into two compounds (*R_{Leu}* 0.71 and 0.77) when 2-methylbutan-2-ol-acetic acid-water was used as solvent. The reduction product could not be separated from synthetic γ -ethylglutamic acid when the two were co-chromatographed in each of these solvents.

The diastereoisomeric reduction product was separated by paper ionophoresis in the pH 3.6 buffer during 3 hr. at 60 v/cm.; under these conditions the slower and faster isomers in 2-methylbutan-2-ol-acetic acid-water travelled 4.6 and 2.6 cm. respectively towards the anode, and glutamic acid moved 3.9 and aspartic acid 15.6 cm. The synthetic γ -ethylglutamic acid (mixed diastereoisomers) again behaved like reduced γ -ethylideneglutamic acid. Ionophoresis at pH 1.9 failed to resolve the diastereoisomeric mixture.

Oxidation of γ -ethylideneglutamic acid. The amino acid (200 mg.) was dissolved in 5% (w/v) H₂SO₄ and maintained at 50°. A 1% KMnO₄ solution in 2% (w/v) H₂SO₄ was added dropwise over a period of 2–3 hr. until a pink colour persisted. Sulphate was precipitated with barium and removed with some MnO₂ by filtration. Aspartic acid was the predominant ninhydrin-positive compound in the filtrate. It was separated by adsorption on a small Dowex 1 column eluted with 0.5*N*-acetic acid and obtained as a residue (39 mg.) on evaporation. It had [α]_D²⁰ + 22° (c 2 in 6*N*-HCl); authentic L-aspartic acid has [α]_D²⁰ + 24.6° in 6*N*-HCl.

γ -Ethylideneglutamic acid (12 mg.) was dissolved in 0.1*N*-NaOH to give a solution of pH 7.0. The solution was maintained at 5° and 1% KMnO₄ (1 ml.) was added dropwise over 30 min. Now 0.05 g. of HIO₄·2H₂O was added and the

mixture stood at laboratory temperature for 1 hr. Residual periodate was precipitated by addition of Ba(OH)₂ and was removed by centrifugation. The supernatant was made slightly acid with H₂SO₄ and then distilled in a stream of N₂. The distillate when passed into 1 ml. of the piperidine-sodium nitroprusside reagent of Feigl (1956) gave a strong blue colour indicative of the presence of acetaldehyde. However, other aldehydes may react less sensitively with this reagent.

Cyclization of γ -ethylideneglutamic acid. A solution of the amino acid (5 mg./ml.) in acetic acid at pH 3.0 was autoclaved at 120° for 3 hr.: at the end of this time no ninhydrin-positive material remained but paper chromatography indicated the presence of an organic acid (aniline-xylose reagent) which also reacted as a lactam (starch-iodide-chloroimide test). By analogy with the behaviour of glutamic acid and γ -methyleneglutamic acid (Blake & Fowden, 1964), which cyclized slightly more slowly under these conditions, this lactam was presumably 3-ethylidenepyrrolid-2-one-5-carboxylic acid. It had *R_{Leu}* 1.23 on chromatograms run in butan-1-ol-acetic acid-water (pyrrolid-2-one-5-carboxylic acid obtained from glutamic acid had *R_{Leu}* 0.96).

Other compounds. Tentative evidence for the presence of various γ -glutamyl peptides in fractions eluted from the Dowex 1 column was obtained by showing that the original substances were hydrolysed by *N*-HCl during 2–4 hr. at 100° to give glutamic acid and the appropriate neutral amino acid in the approximately equivalent amounts. The assessment of a γ -peptide link rests only upon the ease with which the compounds were hydrolysed (an α -peptide link normally splits much more slowly under the conditions described).

Infrared spectroscopy. The product obtained after catalytic hydrogenation of γ -ethylideneglutamic acid was racemized by treatment with 5*N*-Ba(OH)₂ for 48 hr. at 100°. The i.r.-absorption spectrum of the racemized material (consisting of a mixture of approximately equal quantities of the diastereoisomeric DL-forms) was determined in Nujol (liquid paraffin) and compared with the spectrum given by synthetic γ -ethylglutamic acid. The two spectra were identical in respect to all major absorption peaks.

Nuclear-magnetic-resonance spectroscopy. The spectra of γ -ethylideneglutamic acid and γ -methyleneglutamic acid were determined in D₂O, τ values (± 0.02 p.p.m. except where stated) being obtained by reference to the methyl resonance peak of 2-methylpropan-2-ol used as internal standard. The two spectra possess many points of similarity: the α - and β -protons of both compounds give rise to the ABX type of spectrum (the two β -protons differ slightly in their respective environments) and τ_α and τ_β values are almost identical in each case. In γ -methyleneglutamic acid, the high field methylene δ -proton ($\tau - 4.38$) is markedly broadened, probably through coupling to one of the H β , and is probably *trans*- to C β . The coupling constant between the two δ -protons is small (1.0 cyc./sec.). In γ -ethylideneglutamic acid the methine δ -proton is not significantly broadened, indicating that the methyl group is probably *trans*- to the β -carbon atom. This spectrum shows the expected coupling ($J_{\delta\epsilon} = 7.6$ cyc./sec.) between the methyl ϵ - and methine δ -protons and the low field shift of the methine δ -proton is due to the influence of the adjacent methyl group. τ values (p.p.m.) are as follows: H α - 2.70; the two

$H_\beta - 1.59 \pm 0.08$; $H_\delta - 4.38$ and -4.82 for γ -methylene-glutamic acid, and $H_\alpha - 2.60$; the two $H_\beta - 1.61 \pm 0.08$; $H_\delta - 5.69$ and $H_\epsilon - 0.59$ for γ -ethylideneglutamic acid.

Transamination and decarboxylation studies. Transamination was studied with a mitochondrial preparation from 3-day-old peanut seedlings (grown at 30°), which contain the branched C_6 γ -substituted glutamic acids. The mitochondrial fraction was isolated from seedlings (100 g.) by following the procedure of Blake & Fowden (1964); it was resuspended in 0.05 M-potassium phosphate buffer, pH 8.0, final vol. 2.5 ml., and used as a source of transaminases. Reaction mixtures, which were maintained at 30°, contained 0.03 M-oxaloacetate (pH 8.0), 0.03 M-amino acid (pH 8.0, glutamic acid, γ -methyleneglutamic acid or γ -ethylideneglutamic acid) and 0.3 ml. of transaminase preparation in a final volume 0.5 ml. The relative amounts of transamination occurring with the three amino acids after various time-intervals were estimated after chromatographic separation of the common product, aspartic acid. At 15 and 30 min. considerably more transamination had occurred with glutamic acid than with either of the unsaturated acids; the latter compounds reacted at approximately the same rate. After periods of 1 hr. or longer, an equal amount of aspartic acid had been formed from each of the three substrates, indicating the attainment of a similar equilibrium in each case.

Decarboxylation studies were performed with extracts of *Capsicum* fruits and tulip leaves by following the procedure of Fowden (1954). Although decarboxylation of glutamic acid proceeded to more than 90% completion and that of γ -methyleneglutamic acid to about 70% completion in 4 hr. at 27°, no reaction could be detected with either γ -ethylideneglutamic acid or synthetic γ -ethylglutamic acid.

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