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γ -Methyleneglutamic Acid and Related Compounds from Plants

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(Received 23 December 1963)

Many amino and imino acids, additional to those normally present in protein molecules, are now known as plant constituents (for reviews see Fowden, 1958, 1962). Several of these newly recognized compounds are dicarboxylic acids possessing a common branched C_6 carbon skeleton.

γ -Methyleneglutamic acid and γ -methyleneglutamine, first isolated from peanut plants, form the most rigorously characterized members of the group (Done & Fowden, 1952; Wailes, Whiting & Fowden, 1954). These two substances have been isolated subsequently from tulips (Zacharius, Pollard & Steward, 1954) and *Amorpha fruticosa* (Tschiersch, 1962) and identified as constituents of several other species on paper-chromatographic evidence (see Fowden, 1958). Chemically synthesized γ -methylglutamic acid has been resolved via the action of brucine or of cinchonine on its *N*-formyl derivative, and the configurations of the *L*- and *D*-isomers were established by degradation to the corresponding isomers of aspartic acid (Nakayama & Kaneko, 1957). However, the stereochemistry of the natural form has remained in doubt because its optical rotation in mineral acid has never been recorded: γ -methylene-*L*-glutamic acid has $[\alpha]_D^{20}$ 0° in water (Nakayama & Kaneko, 1957). The configuration of natural γ -methyleneglutamine is likewise uncertain.

In several species, substances identified as γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid coexist with the γ -methylene compounds (Fowden, 1958). γ -Methylglutamic acid was isolated from *Phyllitis scolopendrium* (Virtanen & Berg, 1955): the minute amount obtained permitted only the determination of C and H content, and identification of the isolate was dependent entirely on chromatographic comparison with synthetic material (Done & Fowden, 1952).

γ -Hydroxy- γ -methylglutamic acid was obtained from *Adiantum pedatum*, the maidenhair fern (Steward, Wetmore & Pollard, 1955; Grobbelaar, Pollard & Steward, 1955), in slightly larger amount (37 mg.); analysis of crystalline material indicated that it was a hemihydrate, but no further evidence was given to confirm this view.

The present paper describes the isolation of larger quantities of all four compounds from either tulips or *Phyllitis*. In addition, some properties of a further unidentified dicarboxylic amino acid from tulips are given. The materials isolated have been used to establish with certainty the identities of γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid and to study the stereochemistry of the natural isomers. The possibility that the compounds cyclize under conditions which yield pyrrolid-2-one-5-carboxylic acid from glutamic acid and glutamine has been tested. An attempted demonstration of the presence in peanut seedlings of an enzyme similar to glutamine synthetase but utilizing γ -methyleneglutamine and γ -methylene-glutamic acid as substrates was unconvincing.

Extractions

Extraction from tulips. An extract of 55 kg. of mature tulip leaves was fractionated by ion-exchange chromatographic techniques to yield γ -methyleneglutamine (16.7 g.) and γ -methylene-glutamic acid (1.1 g.). Certain mixed acid fractions eluted from Dowex 1 resin were separated further by countercurrent and paper-electrophoretic methods to give a small quantity of crude γ -methylglutamic acid and the unidentified substance (72 mg.).

Extraction from Phyllitis. The amino acid fraction of an extract of 18 kg. of fern fronds was separated by anion-exchange resin chromatography to give γ -methylglutamic acid (9.3 g.) and γ -

hydroxy- γ -methylglutamic acid (7 g.). γ -Methyleneglutamic acid was present in the extract in smaller amount, but was not isolated.

Identification and stereochemistry

The identities of γ -methyleneglutamic acid and γ -methyleneglutamine were checked by comparison with authentic samples (Done & Fowden, 1952). Optical-rotation measurements gave $[\alpha]_D^{18} + 12.8^\circ$ in 11% (w/v) HCl for γ -methyleneglutamic acid, so establishing it as the L-form: Nakayama & Kaneko (1957) recorded $[\alpha]_D + 14^\circ$ for the L-isomer obtained by resolution of racemic material. γ -Methylene-glutamine had $[\alpha]_D^{18} 0^\circ$ in water and $+21^\circ$ in 6N-HCl, and after hydrolysis to γ -methyleneglutamic acid gave $[\alpha]_D + 12.1^\circ$ in 11% (w/v) HCl. Therefore the natural isomer of the amide also had an L-configuration. The more positive rotations observed in acid compared with those measured in water are in themselves indicative of L-configurations (Greenstein & Winitz, 1961, vol. 1, p. 85).

The isolated γ -methylglutamic acid, $[\alpha]_D^{18} - 2^\circ$ in water and $+21^\circ$ in 6N-HCl, behaved identically with synthetic material (Done & Fowden, 1952) on paper chromatograms developed in phenol-NH₃ and butan-1-ol-acetic acid-water. However, when the synthetic substance, which consisted of a mixture of four diastereoisomers, was run on chromatograms developed in the 2-methylbutan-2-ol-acetic acid-water mixture it separated into two racemic components; natural γ -methylglutamic acid moved together with the slower component. A mixture of diastereoisomeric L-forms was prepared by catalytic hydrogenation of natural γ -methylene-L-glutamic acid; this mixture separated similarly in the latter solvent system. Pure samples of these two α -L-diastereoisomers were obtained from a larger-scale reduction after fractionation on a Dowex 1 column. Natural γ -methylglutamic acid was shown to be identical with the isomer eluted second from the resin by applying paper-chromatographic, infrared-spectroscopic and optical-rotation methods.

The configuration of the γ -carbon atom was established by degrading natural γ -methylglutamic acid to yield D-(+)- α -methylsuccinic acid. The two asymmetric carbon atoms therefore exhibit a *cis* relationship and the natural product becomes *erythro*- γ -methyl-L-glutamic acid. Nuclear-magnetic-resonance studies on cyclized forms (see below), although in themselves inconclusive, also supported the assignment of an *erythro* structure.

γ -Hydroxy- γ -methylglutamic acid behaved identically on paper chromatograms with synthetic material supplied by Dr J. K. Pollard. Consistent with the γ -hydroxy acid structure, considerable lactonization occurred during its elution from Dowex 1 columns with dilute acetic acid. The acid

was very insoluble in water and crystallized readily as long needles of the monohydrate; it had $[\alpha]_D^{18} - 18.5^\circ$ in 6N-HCl (calculated for the anhydrous compound). It was converted completely into γ -methylglutamic acid by treatment with hydroiodic acid and red phosphorus, but reduction was accompanied by racemization at both asymmetric carbon atoms, to yield an optically inactive mixture of *erythro* and *threo* forms. However, partial reduction to γ -methylglutamic acid was effected under milder conditions by catalytic hydrogenation to yield the *erythro* form accompanied by only about 10% of the *threo* isomer. The orientation of the methyl group at the γ -carbon atom is therefore presumed to be the same as that in natural *erythro*- γ -methylglutamate.

The configuration at the α -carbon atom of γ -hydroxy- γ -methylglutamic acid was established by conversion into aspartic acid by oxidation with acidic permanganate. As expected, the yield was low, but sufficient aspartic acid was obtained for measurement of its specific rotation, which indicated the L-form. The Fischer projections of the structures assigned to the natural isomers of γ -methylglutamic acid (I) and γ -hydroxy- γ -methylglutamic acid (II) are shown.

The unidentified acid (see above, p. 136) gave a brown spot with ninhydrin. It was stable when heated with 6N-HCl, but was converted by catalytic hydrogenation into a substance giving a purple colour. The reduction product gave a single spot on paper chromatograms developed in several solvents but the 2-methylbutan-2-ol-acetic acid-water mixture resolved it into two compounds. These properties of the unknown resembled those established for γ -methyleneglutamic acid and may indicate a related structure in which an unsaturated carbon atom occurs at the branch point in the carbon skeleton. Oxidative degradation with permanganate gave aspartic acid as the major product and so indicated that the unsaturated linkage could not be present in the chain before the γ -carbon atom is reached.

Cyclization reactions

Cyclization of amides. Glutamine is converted readily into pyrrolid-2-one-5-carboxylic acid with loss of a molecule of ammonia. The rate of cycliza-

tion is dependent on pH, but is affected in a more pronounced way by phosphate ion (see Greenstein & Winitz, 1961, vol. 3, p. 1933). γ -Methyleneglutamine was shown to cyclize at least as rapidly as glutamine at pH 7.0 in the presence of 0.1M-phosphate, yielding an acid of low solubility, $[\alpha]_D^{25} + 6^\circ$ in N-NaOH, having an analysis consistent with 3-methylenepyrrolid-2-one-5-carboxylic acid. Gentle catalytic hydrogenation of the latter compound gave another acid presumed to be 3-methylpyrrolid-2-one-5-carboxylic acid.

Cyclization of dicarboxylic amino acids. Glutamic acid cyclizes more slowly than glutamine, yielding pyrrolid-2-one-5-carboxylic acid with loss of water. Maximum rates are observed at about pH 3 (Greenstein & Winitz, 1961, vol. 3, p. 1933). When heated in acetic acid solution at pH 3, γ -methyleneglutamic acid, γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid were cyclized to acidic compounds that failed to react with ninhydrin but gave positive reactions in the starch-iodide chloroimide test for peptides (Rydon & Smith, 1952), which also detects lactams sensitively.

The product formed from γ -methyleneglutamic acid was identical with 3-methylenepyrrolid-2-one-5-carboxylic acid obtained from γ -methyleneglutamine; that from γ -methylglutamic acid was identified provisionally as 3-methylpyrrolid-2-one-5-carboxylic acid (see above).

erythro- γ -Methylglutamic acid cyclized more rapidly than the *threo* form and both cyclized faster than glutamic acid treated under the same conditions. γ -Methyleneglutamic acid was converted into a pyrrolidone at approximately the same rate as glutamic acid. Some transient lactone formation was noticed during cyclization of γ -hydroxy- γ -methylglutamic acid at pH 3, but the final product was almost entirely lactam.

Enzymic studies

During the first few days of growth of peanut seedlings γ -methyleneglutamine becomes the predominant free amino acid, although it cannot be detected in dormant seeds (Fowden, 1954). The young seedlings must contain an active biosynthetic enzyme system, and an attempt was made to show whether the enzyme acted similarly to glutamine synthetase from animal and plant tissues. Previous work has indicated that a purified glutamine synthetase from pea seeds can utilize γ -methyleneglutamic acid very slowly as substrate in comparison with glutamic acid (Levintow, Meister, Hogeboom & Kuff, 1955). However, the conversion reported was no greater than that we have observed to occur as a non-enzymic reaction between substrates (see below), and, even if truly enzymic, this unspecific action of glutamine synthetase seems insufficient to explain the massive accumulation of

γ -methyleneglutamine observed in peanut seedlings. More recently peanut-seedling extracts have been used as an enzyme source when glutamine-synthetase activity was demonstrated by glutamate-dependent hydrolysis of ATP; however, γ -methyleneglutamic acid was completely inactive in similar assay systems (Marcus, Feeley & Shannon, 1963).

In searching for an enzyme synthesizing γ -methyleneglutamine, crude non-particulate preparations from peanut cotyledons were observed to possess such high adenosine-triphosphatase activity that it was impossible to obtain reproducible results with a glutamine-synthetase-type assay. However, the large amounts of γ -methyleneglutamine made available by isolation enabled us to use a glutamine-transferase-type assay, which requires not ATP but catalytic quantities of ADP, although it is known that the two reactions are catalysed by the same enzyme (Elliot, 1953). Transferase activity with glutamine as substrate was readily demonstrated in the seedling extracts, but all attempts to show hydroxamate formation from γ -methyleneglutamine proved inconclusive, despite variations in the conditions of the reaction, e.g. in substrate concentration, pH, Mg^{2+} , Mn^{2+} and other metal ions. Table 1 shows the results of an experiment in which a non-particulate enzyme fraction converted over half the glutamine into its hydroxamate in 20 min. at 37° , although no γ -methyleneglutamohydroxamate was detected. Although peanut seedlings contain an active γ -methyleneglutamine deamidase, the failure to obtain transferase activity in this experiment could not be attributed to the rapid hydrolysis of substrate, because other experiments had shown that the deamidase was located entirely in the mitochondrial fraction.

Hydroxamate formation from γ -methyleneglutamic acid could not normally be demonstrated (Table 1), although it was observed in two experiments with shoot extracts of 10-day-old plants.

These results, together with those of previous workers, suggest that γ -methyleneglutamine is synthesized by a mechanism somewhat different from that associated with glutamine synthesis.

DISCUSSION

The coexistence of these γ -substituted acids in a few distantly related members of the plant kingdom suggests that they form a closely-associated metabolic group. However, no enzymes are yet known to catalyse interconversion reactions, although experiments in which labelled pyruvic acid or alanine was supplied to peanuts (Fowden & Webb, 1958) and *Phyllitis* (Linko & Virtanen, 1958) indicated a common metabolic precursor. The similar configuration established for the methyl group at the γ -carbon atom in γ -methyl- and

Table 1. *Hydroxamic acid formation from glutamic acid and γ -methyleneglutamic acid and from glutamine and γ -methyleneglutamine catalysed by peanut-seedling extracts*

The reaction mixtures used in the synthetase-type assay contained the following substances (μ moles): tris (pH 7.8), 400; MgSO_4 , 50; ATP, 75; sodium thioglycollate, 20; hydroxylamine hydrochloride, 200; L-glutamic acid, 250, or DL- γ -methyleneglutamic acid, 500, and 0.5 ml. of enzyme preparation (in sucrose-tris grinding solution) in a total volume of 2 ml. The transferase-type system contained: potassium maleate (pH 6.4), 100; MnSO_4 , 1; ATP, 0.2; sodium thioglycollate, 20; sodium arsenate, 20; hydroxylamine hydrochloride, 20; L-glutamine or γ -methylene-L-glutamine, 40, and 0.5 ml. of enzyme preparation (in sucrose-tris grinding solution) in a total volume of 2 ml. Results are expressed as μ moles of hydroxamate formed in 20 min. at 37°.

Type of assay system	Amino acid substrate	Hydroxamate with boiled enzyme	Hydroxamate with active enzyme	Corrected enzymic hydroxamate formation
Synthetase	Glutamic acid	1.0	11.5	10.5
	γ -Methyleneglutamic acid	1.5	1.5	0
Transferase	Glutamine	0.25	26.25	26.0
	γ -Methyleneglutamine	0.50	0.50	0

γ -hydroxy- γ -methyl-glutamic acid may be viewed as favouring a direct oxidation of the former compound into the hydroxy acid. However, an inter-conversion of these two compounds through γ -methyleneglutamic acid is perhaps more likely, because this pathway would be analogous with other established examples of interconversion by hydration-dehydration and hydrogenation-dehydrogenation reactions, e.g. in the conversion of succinic acid into malic acid and in the metabolism of the CoA thiol esters of fatty acids. The asymmetric character of the γ -carbon atom would be lost at the stage of the γ -methylene intermediate, and so configurational comparisons cannot be used to support or refute this latter pathway.

EXPERIMENTAL

Chromatographic and ionophoretic techniques. Solvents used for paper chromatography were: A, 75% (w/w) phenol solution in the presence of NH_3 ; B, a one-phase mixture of butan-1-ol-acetic acid-water (90:10:29, by vol.); C, the upper phase of 2-methylbutan-2-ol (British Drug Houses Ltd., technical)-acetic acid-water (10:1:10, by vol.). A 0.1% solution of ninhydrin in 95% (v/v) ethanol was normally used to detect the amino acids, and an aniline-xylose reagent (Smith, 1960) or the starch-iodide-chloroimide technique of Rydon & Smith (1952) was used to locate the pyrrolidonecarboxylic acids produced after cyclization of the γ -substituted amino acids.

Paper-ionophoretic separations were performed in pyridinium acetate buffer, pH 3.55 (pyridine 5 ml., acetic acid 30 ml. and water 950 ml.) by using a horizontal method essentially that of Gross (1961).

Isolation of compounds from tulips. Leaves (55 kg.) were collected, deep-frozen and minced. Immediately on thawing, the mush was pressed (6000 lb./in.²) to yield juice whose pH was adjusted to 4.5. Protein was coagulated by warming to 60° and removed by filtration. The clarified extract was divided into three portions and each was applied to a squat column containing Zeo-Karb 225 (H^+ form, 10 lb., mesh 52-100). After thorough washing, the amino acid fraction was displaced from the resin by 2N- NH_3 . The

eluates were evaporated to small volume to remove NH_3 , and combined.

The combined amino acid fraction was applied to a column (20 cm. $\times$ 10 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100-200). Acidic amino acids were retained, but neutral and basic acids were present in the eluate and wash water was collected at this stage. γ -Methyleneglutamine formed the predominant component of the eluate, and was readily separated from most other amino acids by fractional elution from a Dowex 50 (X8; mesh 100-200) column with 0.25N- NH_3 ; yield 16.7 g. A further 10-20 g. remained in the mother liquors.

The acidic amino acids retained on the Dowex 1 column were displaced with 0.25N-acetic acid. Fractions (17 ml.) of the eluate were collected after the breakthrough of the first acid, glutamic acid. Fractions 15-63 (fraction A) when pooled contained large quantities of glutamic acid and small amounts of α -aminoadipic acid and γ -methylglutamic acid, whereas fractions 103-175 (fraction B) contained mainly aspartic acid and γ -methyleneglutamic acid.

The components of fractions A and B were further resolved in a countercurrent apparatus having 20 ml. upper and lower phases. The phases were prepared by refluxing a butan-1-ol-water-acetic acid mixture (5:7:1, by vol.) at 100° for 6 hr. followed by cooling and separation. Fraction A solids were dissolved in the lower phase of the first tube and then 720 transfers of upper phase were effected. Tubes 78-96 now contained the peak concentrations of γ -methylglutamic acid. Their contents were pooled and evaporated, and the residue was subjected to a further absorption-elution cycle on a small Dowex-1 column, to yield 210 mg. of a preparation thought to be γ -methylglutamic acid.

Paper ionophoresis of a portion of this preparation revealed for the first time the presence of the unknown acidic amino acid; in routine examination of fractions by paper chromatography with solvent B, its presence had been obscured by γ -methylglutamic acid, which had an identical R_F . The two substances were separated when the residue was streaked across sheets of Whatman no. 3MM paper soaked in pH 3.55 buffer and subjected to ionophoresis at 30 v/cm. for 2.5 hr. γ -Methylglutamic acid had moved about 1.5 cm. towards the anode while the unknown remained almost on the starting line. The unknown was eluted from the dried sheets and obtained as a crude, yellowish solid.

Fraction B (see above) solids were placed in the first two tubes of the countercurrent apparatus, which then effected 840 transfers. The contents of tubes 82–102 after evaporation and crystallization yielded the purest sample (0.5 g.) of γ -methylene-glutamic acid. Tubes 70–81 gave a slightly impure sample (0.6 g.).

Isolation of compounds from Phyllitis. Fronds (18 kg.) were processed in a similar way to the tulip leaves to obtain the free amino acid fraction after treatment with Zeo-Karb 225. The eluate was concentrated to 400 ml., and applied to a column (50 cm. $\times$ 3 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100–200). After washing, acidic amino acids were displaced with 0.1 N-acetic acid until fraction 340 had been collected, and then with 0.5 N-acetic acid. Fractions 96–300, containing high concentrations of γ -methylglutamic acid, were pooled and evaporated to small volume, when the acid (9.3 g.) crystallized out. γ -Hydroxy- γ -methylglutamic acid (7 g.) crystallized from fractions 391–462 without prior concentration. γ -Methylene-glutamic acid was present in fractions 311–370 but was not isolated.

Studies with γ -methylglutamic acid. The isolated material had $[\alpha]_D^{18} -1.9^\circ$ (c 5 in water) and $+21^\circ$ in 6N-HCl (c 2.5) (Found: C, 44.9; H, 7.0; N, 8.3. Calc. for $C_6H_{11}NO_4$: C, 44.7; H, 6.9; N, 8.7%). It had R_F 0.37 in solvent A and R_{Leu} 0.46 in solvent B. Synthetic γ -methylglutamic acid behaved identically. The diastereoisomeric forms present in the synthetic material separated on chromatograms (Whatman no. 4 paper) run for 3 days in solvent C, and had R_{Leu} values of 0.40 and 0.46. Natural γ -methylglutamic acid (identified below as the *erythro*-L-isomer) was inseparable from the slower-moving compound.

A mixture of *erythro*-L- and *threo*-L- γ -methylglutamic acid was prepared by catalytic hydrogenation of γ -methylene-L-glutamic acid (0.6 g.) in water (10 ml.) in the presence of PtO_2 (120 mg.). Complete reduction was effected by bubbling H_2 through the mixture kept at 40° . The isomers were separated on a column (65 cm. $\times$ 13 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100–200), being eluted with 0.2 N-acetic acid. Fractions (4.5 ml.) were collected when ninhydrin-positive material emerged. The *threo*-L-form, $[\alpha]_D^{18} -0.8^\circ$ (c 5 in water) and $+22.2^\circ$ in 6N-HCl (c 2.5), was isolated from fractions 1–20, and the *erythro*-L-form, $[\alpha]_D^{18} -5.1^\circ$ (c 5 in water) and $+19.2^\circ$ in 6N-HCl (c 2.5), from fractions 27–42. *Erythro*-DL- and *threo*-DL-preparations were obtained when synthetic material was separated in a similar way. The natural product had an infrared-absorption spectrum identical with that of the isomer eluted second from the Dowex 1 resin. The spectrum of the isomer first eluted, although basically similar, showed important differences in the 7–12 μ range.

Conversion into α -methylsuccinic acid was used to establish the configuration at the γ -carbon atom. Natural γ -methylglutamic acid (1 g.) was refluxed with ninhydrin (2 g.) in N-acetic acid (50 ml.), to form β -methylsuccinic semialdehyde. Insoluble material was removed and the filtrate evaporated to small volume at 40° . Water was added to 50 ml. and the solution adjusted to pH 7 with NaOH and clarified with charcoal. After filtration it was applied to a small column of Dowex 1 resin; unchanged ninhydrin was washed through and then the semialdehyde and any unchanged γ -methylglutamic acid were displaced by N-HCl. The eluate was evaporated at 40° and the residue dissolved in 15% (w/v) H_2O_2 (5 ml.). After being kept

at 50° for 15 min. to effect oxidation to α -methylsuccinic acid, the solution was taken to dryness. The residue was dissolved in water and any unchanged γ -methylglutamic acid still present was removed by shaking with Dowex 50 (H^+ form) resin. After re-evaporation, the residue was dissolved in ethanol (5 ml.) and aq. NH_3 (sp.gr. 0.88) added to precipitate diammonium α -methylsuccinate (85 mg.) (Found: C, 35.8; H, 8.6; N, 16.5. Calc. for $C_6H_{14}N_2O_4$: C, 36.1; H, 8.5; N, 16.9%). After dissolving in an equivalent amount of HCl to yield the free acid, it had $[\alpha]_D^{18} +6.3^\circ$ (c 4.5); authentic D-(+)- α -methylsuccinic acid has $[\alpha]_D +9.2^\circ$ in water (Fredga, 1942).

Studies with γ -hydroxy- γ -methylglutamic acid. The isolated material was extremely insoluble and determination of an $[\alpha]_D$ value in water was impossible. However, the compound had $[\alpha]_D^{18} -16.1^\circ$ in 6N-HCl (c 2.9) and -10.3° in N-NaOH (c 4.6) (Found: C, 37.3; H, 6.8; N, 7.4; loss at 103° , 9.0. $C_6H_{11}NO_5 \cdot H_2O$ requires C, 36.9; H, 6.7; N, 7.2; H_2O , 9.2%). The isolate was inseparable from synthetic γ -hydroxy- γ -methylglutamic acid on paper chromatograms run in all three solvent systems. Solvent C did not effect any separation of diastereoisomers expected in the synthetic sample.

The natural isomer (100 mg.) was converted completely into γ -methylglutamic acid by heating with 55% (w/v) HI (4 ml.) and red phosphorus (20 mg.) at 130° for 18 hr. Reduction under these conditions was accompanied by racemization at both asymmetric carbon atoms; the isolated γ -methylglutamic acid showed no optical rotation in 6N-HCl, and chromatography in solvent C indicated an approximately equal mixture of *erythro*- and *threo*-forms. In contrast, when H_2 was bubbled for 2 hr. through a suspension of γ -hydroxy- γ -methylglutamic acid (10 mg.) and PtO_2 (5 mg.) in water (1 ml.) at 70° , producing about 30% reduction, *erythro*- γ -methylglutamic acid was the major product, and only a trace of the *threo*-isomer was detected.

The α -carbon was assigned an L-configuration after conversion of the isolate into L-aspartic acid. γ -Hydroxy- γ -methylglutamic acid (0.25 g.) was dissolved in 10% (w/v) H_2SO_4 at 50° . $KMnO_4$ (1 g.) in 1% (w/v) H_2SO_4 (50 ml.) at 50° was added gradually over 6 hr. and the mixture kept overnight at this temperature. Sulphate was precipitated with barium and removed together with some MnO_2 by filtration. The filtrate contained aspartic acid, glycine and β -alanine; no γ -hydroxy- γ -methylglutamic acid remained. Aspartic acid was separated from the other amino acids by adsorption on a small Dowex 1 column and eluted with 0.5 N-acetic acid. The eluate after clarifying with charcoal was evaporated to yield aspartic acid (14 mg.). $[\alpha]_D^{18} +21^\circ$ (c 2 in 6N-HCl); authentic L-aspartic acid has $[\alpha]_D +24.6^\circ$ in 6N-HCl.

Studies with the unidentified acid. This compound gave a brown spot with ninhydrin that moved close to natural γ -methylglutamic acid in all solvents, having R_F 0.34 in A and R_{Leu} 0.46 and 0.36 in B and C respectively. After catalytic hydrogenation, solvent C resolved the product into two purple-reacting spots, R_{Leu} 0.62 and 0.72. When it was oxidized at 50° by dropwise addition of 1% $KMnO_4$ in 10% (w/v) H_2SO_4 , aspartic acid was identified chromatographically as the major amino acid product.

Cyclization reactions. When an aqueous solution of γ -methylene-glutamine (5 mg./ml.) was refluxed for 2 hr., some loss of amide was noticed, coupled with the formation of an acidic substance, R_F 0.61 in solvent B, giving positive

reactions with the aniline-xylose and the Rydon & Smith (1952) reagents. If a solution of the same concentration was refluxed in the presence of 0.1 M-phosphate at pH 7, the loss of amide was almost complete in 30 min. Glutamine treated under these conditions cyclized slightly more slowly to yield pyrrolid-2-one-5-carboxylic acid.

The reaction product from γ -methyleneglutamine was isolated by refluxing the amide (0.5 g.) in 50 ml. of 0.1 M-potassium phosphate, pH 7, for 1 hr. After concentration to 25 ml., the solution was adjusted to pH 3 with HCl, saturated with ether and cooled to 0°. The precipitate was collected and recrystallized from ether-saturated water to yield 3-methylenepyrrolid-2-one-5-carboxylic acid (105 mg.) (Found: C, 50.7; H, 4.9; N, 9.6. $C_6H_7NO_3$ requires: C, 51.1; H, 5.0; N, 9.9%). The substance had $[\alpha]_D^{25} + 6.2^\circ$ (c 1.5 in N-NaOH) and when heated it decomposed slowly above 200° without melting.

When an aqueous solution of 3-methylenepyrrolid-2-one-5-carboxylic acid (5 mg./ml.) containing PtO_2 was hydrogenated, another acid, R_F 0.67 in solvent B, was obtained. This gave positive aniline-xylose and Rydon & Smith tests and was presumed to be 3-methylpyrrolid-2-one-5-carboxylic acid.

The rates of cyclization of the γ -substituted dicarboxylic acids were compared with that observed for glutamic acid in acetic acid solution at pH 3. The natural isomers of γ -methyl- and γ -hydroxy- γ -methyl-glutamic acid lactamized faster than glutamic acid or γ -methyleneglutamic acid. At 105°, approximately half of the two former acids (initial concentration 5 mg./ml.) had undergone cyclization after 2 hr. and formation of pyrrolidones was complete after 24 hr.; even after 24 hr. under these conditions some 10–20% of glutamic acid and γ -methyleneglutamic acid remained unchanged. *erythro*- γ -Methylglutamic acid cyclized appreciably faster than the *threo*-form. When γ -hydroxy- γ -methylglutamic acid was heated, its γ -lactone formed rapidly but, after more prolonged heating, no lactone could be detected and only lactam was present. The products formed from γ -methylene- and γ -methyl-glutamic acids were chromatographically identical with 3-methylene- and 3-methyl-pyrrolid-2-one-5-carboxylic acids respectively (see above). The lactam formed from γ -hydroxy- γ -methylglutamic acid, presumably 3-hydroxy-3-methylpyrrolid-2-one-5-carboxylic acid, had a lower R_F (solvent B, 0.47) than the other cyclization products.

Nuclear-magnetic-resonance spectra were determined on D_2O solutions of the two 3-methylpyrrolid-2-one-5-carboxylic acids obtained from *erythro*- and *threo*- γ -methyl-L-glutamic acid. The spectra, measured on a 60 Mcyc. Varian instrument, could be integrated and indicated the correct proton number required for the cyclic compounds. However, the interaction of the protons on adjacent carbon atoms is complex and may be expected to yield a multi-split pattern for the stereochemically significant γ -proton peak. In practice insufficient resolution was achieved to enable authoritative stereochemical predictions to be made; but the fact that the peak associated with the γ -proton of the cyclized *threo*- (*trans*-) form was remarkably high upfield, whereas the corresponding peak for the *erythro*-form had a normal τ value, is in agreement with the configurations assigned after conversion of the parent amino acid into α -methylsuccinic acid (see above).

Enzyme preparations. Cotyledons of three-day-old peanut seedlings grown at 30–33° were homogenized with an equal

weight of 0.1 M-tris-HCl or 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.4 M-sucrose; the macerate was strained through muslin and centrifuged to remove coarse debris. The supernatant was then centrifuged at 10 000g for 45 min. to separate microsomal and non-particulate supernatant fractions. The mitochondrial and microsomal preparations, after washing by recentrifuging from new grinding medium, were resuspended in small volumes of 0.1 M-buffer appropriate to the assay to be undertaken.

Each fraction was tested for synthetase and transferase activity in reaction mixtures similar to those described in Table 1, but only the non-particulate preparation catalysed hydroxamate formation. Reaction mixtures were normally incubated at 37° for 20 min., when the amount of hydroxamate formed was determined by the procedure of Lipmann & Tuttle (1945), extinctions being measured at 480 μ . Glutamohydroxamic acid purchased from Sigma Chemical Co. was used for colour calibrations.

SUMMARY

1. γ -Methyleneglutamine, γ -methyleneglutamic acid and an unidentified acidic amino acid were isolated from tulip leaves. Pure γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid, as its monohydrate, were obtained from fronds of the fern, *Phyllitis scolopendrium*.

2. Both γ -methylene compounds were identified as L-isomers. By comparison with *erythro*- and *threo*-isomers and by conversion into D(+)- α -methylsuccinic acid, natural γ -methylglutamic acid was shown to have an *erythro*-L-configuration.

3. Natural γ -hydroxy- γ -methylglutamic acid was shown to have an α -L-configuration by oxidizing it to L-aspartic acid. The arrangement of the methyl group about the γ -carbon atom was the same as that in natural γ -methylglutamate.

4. All the compounds cyclized to the corresponding lactams (3-substituted pyrrolid-2-one-5-carboxylic acids).

5. A search for a glutamine-synthetase-type enzyme able to convert γ -methyleneglutamic acid into γ -methyleneglutamine in peanut seedlings was unsuccessful.

We thank the Central Research Fund of the University of London and the Agricultural Research Council for grants in support of this work, Dr E. A. Bell and Dr W. Hafferl for help with electrophoretic and nuclear-magnetic-resonance studies respectively, and Miss Jean Chesson for skilled technical assistance.

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Biochem. J. (1964), 92, 142

Trans- β -glucosylation by Alfalfa-Seed Enzymes

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(Received 11 December 1963)

An early comparison between the glycosidase system of alfalfa (lucerne, *Medicago sativa*) seeds and sweet almonds (Hill, 1934) revealed a similar distribution of activities except that alfalfa 'emulsin' contained only traces of β -glucosidase. The various activities were assayed with synthetic phenyl glycosides and naturally occurring phenolic glycosides such as salicin and amygdalin, i.e. glycosides with non-carbohydrate aglycones. A survey of the disaccharidases of alfalfa, however, has now shown (D. H. Hutson & D. J. Manners, unpublished work) that β -glucosidases are present in substantial amounts, although only weak activity was displayed towards phenyl β -D-glucopyranoside, salicin and amygdalin. Moreover, of the common glycosidases found in the seed extracts, this activity was the only one that showed trans-glucosylation properties when incubated with a 1% solution of substrate for 24 hr.

Plant and fungal β -glucosidases, in general, appear to possess a group rather than an absolute specificity (Peat, Whelan & Hinson, 1952; Manners, 1960), although only a few fractionation studies have been reported (e.g. Krishna-Murti & Stone, 1961; Anderson, Cunningham & Manners, 1963). Investigations of the trans- β -glucosylation action of various plant and fungal preparations on cellobiose have been carried out by several workers (e.g. Barker, Bourne, Hewitt & Stacey, 1955; Buston & Khan, 1956; Crook & Stone, 1957; Anderson & Manners, 1959) and the acceptor specificities of the enzyme preparations determined. In these experiments, transglucosylation occurred predominantly

on the C₆-hydroxyl group, but was also observed on the C₃-, C₃'- and C₄-hydroxyl groups of D-glucopyranose and D-glucopyranosides. There appear to be, however, certain minor differences in acceptor specificity in these systems.

Trans- β -glucosylation by alfalfa-seed extracts has now been examined with a view to determination of the acceptor specificity pattern with cellobiose as substrate. Since β -(1 $\rightarrow$ 6)- and β -(1 $\rightarrow$ 3)-linkage synthesis is usually encountered, it was pertinent to study the behaviour of gentiobiose and laminariobiose under similar conditions.

MATERIALS AND METHODS

Enzyme preparation. Alfalfa seeds (var. de Puits, 300 g.) were milled and sieved to remove husk material to yield a fine flour (170 g.). This was extracted at room temperature with 0.4M-sodium citrate buffer, pH 6.8 (final volume 800 ml.), and stored at -20° overnight. The thawed extract, after filtration through muslin, was centrifuged and the supernatant solution dialysed against 2 l. of 0.5M-sodium citrate buffer, pH 5.8. Protein was precipitated from the centrifuged dialysis residue by the addition of ammonium sulphate to 70% saturation (at 0°). The precipitated protein was redissolved in 0.01M-sodium citrate buffer, pH 5.8 (140 ml.), and dialysed against the same buffer at 0°. The resultant preparation was stored at 0° and used within 1 week.

Incubation conditions. All digests were incubated at 37° under toluene. One-quarter of the digest volume consisted of 0.2M-disodium hydrogen phosphate-0.1M-citric acid buffer (Mollvaine, 1921).

Chromatography and paper electrophoresis. Paper chromatograms were developed with the following solvents: