Phytochemistry, 1972, Vol. 11, pp. 663 to 673. Pergamon Press. Printed in England

# THE BIOSYNTHESIS OF γ-SUBSTITUTED GLUTAMIC ACIDS IN *GLEDITSIA TRIACANTHOS*

P. J. PETERSON* and L. FOWDEN

Department of Botany and Microbiology, University College London, Gower Street, London

(*Received* 29 *July* 1971)

**Abstract**—The concentrations of several glutamic acid derivatives, each having a γ-methylene or γ-methyl substituent, increased rapidly during the early stages of germination and growth of *Gleditsia triacanthos* seeds. *Gleditsia* seedlings therefore formed a suitable system for biogenetic studies with this group of amino acids. Various $^{14}$C-labelled substances were supplied to seedlings and, after 2 or 4 days metabolism, the incorporation of radioactivity into individual substituted glutamic acids was determined. These experiments showed that the branched $C_6$ skeleton was unlikely to arise directly by the addition of a $C_1$ unit (from methionine) at the γ-carbon atom of glutamic acid. In contrast, the seedlings converted [1-$^{14}$C]leucine into *erythro*-γ-methylglutamic acid, in which the $^{14}$C label was located essentially in the α-carboxy C atom, i.e. in the expected position for a direct pathway converting a terminal methyl group of leucine into a γ-carboxyl group by oxidative processes. 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid was probably formed by direct hydroxylation of the parent acid, *erythro*-γ-methylglutamic acid. When [1-$^{14}$C]pyruvate was supplied to seedlings, considerable amounts of radioactivity were incorporated into the γ-substituted glutamic acids. However, only very small percentages of the $^{14}$C were present in the α-carboxyl group of the $C_6$ amino acids, so the idea that the branched carbon skeleton arises by condensation of two molecules of pyruvate (or some activated derivative) is untenable.

## INTRODUCTION

A SERIES of γ- and β-substituted glutamic acids have been characterized during the past 20 years from species in unrelated groups of the plant kingdom, e.g. Liliaceae, Leguminosae, Filicales.[1] In some plants both diastereoisomers of a particular amino acid can be found, but more usually only one is present in any single species. However, little definitive information is available concerning the biosynthesis of these amino acids, although several groups of workers have investigated this problem employing species selected from the orders in which they occur, e.g. *Lilium regale*[2] and *Tulipa gesneriana*[3] (Liliaceae), *Arachis hypogaea*[4] (Leguminosae), *Adiantum pedatum*[5] (Filicales).

The general co-existence of some of the compounds, e.g. γ-methyl-, γ-hydroxy-γ-methyl- and γ-methylene-glutamic acids, might suggest that the carbon skeletons of the amino acids have a common origin, and that interconversion of these three substances would take place via reversible oxidation-reduction or hydration-dehydration processes about the γ-carbon atom. Previous studies have provided some inconclusive evidence for the concept that the initial biosynthetic step involves an aldol condensation between two molecules of pyruvic acid to give a keto acid which is then transaminated.[5–7] This idea

---

* Present address: Dept. of Botany, Westfield College, University of London, London NW3.

[1] L. FOWDEN, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, pp. 203–266, Wiley, New York (1970).

[2] M. WICKSON and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **34**, 502 (1956).

[3] L. FOWDEN, *J. Exptl Bot.* **5**, 28 (1954).

[4] L. FOWDEN and J. A. WEBB, *Ann. Bot. Lond.* **22**, 73 (1958).

[5] P. LINKO and A. I. VIRTANEN, *Acta Chem. Scand.* **12**, 68 (1958).

[6] N. GROBBELAAR, J. K. POLLARD and F. C. STEWARD, *Nature, Lond.* **175**, 703 (1955).

[7] L. FOWDEN, *Ann. Bot. Lond.* **18**, 417 (1954).

has a basis, by analogy, in the chemical synthesis of $\gamma$-hydroxy-$\gamma$-methyl-$\alpha$-ketoglutaric acid from pyruvic acid.[8] Keto acids corresponding to the $\gamma$-substituted glutamic acids also have been identified in a number of the above plants.[2] However, the validity of this biosynthetic concept has been questioned by Marcus and Shannon[9] following their demonstration that seedlings of *Arachis hypogaea* contain an aldolase that can catalyse a facile exchange reaction between its substrate, $\gamma$-hydroxy-$\gamma$-methyl-$\alpha$-ketoglutaric acid and $^{14}C$-pyruvic acid; as a result the $C_6$ keto acid becomes labelled.

The possibility that the $C_6$ branched carbon skeleton of these $\gamma$-substituted glutamic acids might arise from glutamic acid by a $C_1$ transfer mechanism, or by alternative routes from other amino acids, has received little attention.

Perhaps one of the main difficulties encountered in earlier investigations was the slow growth rate of the plants employed; these circumstances would not favour biosynthesis and consequently the incorporation of $^{14}C$ into $\gamma$-substituted glutamic acids was low. Recently, this group of compounds has been identified in seedlings of the legume genus, *Gleditsia* in particular, *G. triacanthos* seems ideally suitable for biosynthetic studies because its seeds are relatively small, they germinate and grow rapidly, when they produce considerable quantities of $\gamma$-substituted glutamic acids.[10] We have used this species in the experiments now reported, which provide new data concerning the pathways leading to $\gamma$-methyl-, $\gamma$-hydroxy-$\gamma$-methyl-, $\beta$-hydroxy-$\gamma$-methyl-, and $\gamma$-methylene-glutamic acids, and to the amide, $\gamma$-methyleneglutamine.

## RESULTS

### *Amino Acid Levels in Seeds and Seedlings*

*G. triacanthos* seeds were germinated and grown for varying periods of time, either in the light or in the dark, to select the stage of growth at which the greatest increase in the concentration of substituted glutamic acids occurs. Samples were assayed for their content of each of the substituted glutamic acids using either high voltage paper electrophoresis or two-dimensional paper chromatography. Figures 1a and 1b show that $\gamma$-methyleneglutamic acid and $\gamma$-methyleneglutamine could not be detected in the dry seed, but on germination in the dark the concentration of both substances increased dramatically. When seedlings were grown in the light, this effect was less pronounced. There is a predominance of the 2(S),4(S)-(*erythro*-)isomer of $\gamma$-hydroxy-$\gamma$-methylglutamic acid in the seed and this increases on germination; only trace amounts of the 2(S),4(R)-(*threo*-)isomer are present and its concentration is not shown in Fig. 1. *Erythro*-$\gamma$-methylglutamic acid was present at all stages but the *threo*-isomer could not be detected. However, it may occur in trace amounts, since it would be a logical precursor of 2(S),4(R)-$\gamma$-hydroxy-$\gamma$-methylglutamic acid. *G. triacanthos* also contains 2(S),3(S),4(R)-$\beta$-hydroxy-$\gamma$-methylglutamic acid,[11] but the 2(S),3(R),4(R)-diastereoisomeric form, which is present in *Gymnocladus dioica* (a closely related legume),[11, 12] is absent although it occurs in some other *Gleditsia* species.[10] The concentrations of the 2(S),3(S),4(R)-isomer overlap those of $\gamma$-methylglutamic acid and so are not plotted on Fig. 1.

No qualitative differences in the distribution of $\gamma$-substituted glutamic acids were noted

---

[8] A. W. K. DE JONG, *Rec. Trav. Chim. Pays-Bas* **20,** 81 (1901).

[9] A. MARCUS and L. M. SHANNON, *J. Biol. Chem.* **237,** 3348 (1962).

[10] M.-L. SUNG and L. FOWDEN, unpublished results.

[11] G. A. DARDENNE, *Phytochem.* **9,** 924 (1970).

[12] E. A. BELL, personal communication.

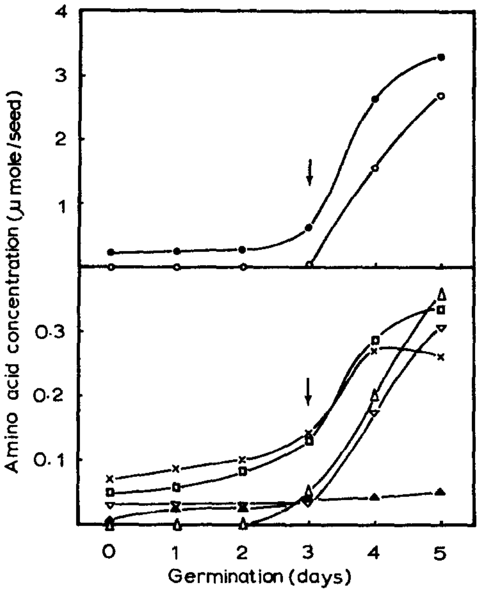


FIG. 1. PRODUCTION OF AMINO ACIDS DURING SEED GERMINATION IN THE DARK. THE ARROW INDICATES THE STAGE AT WHICH SEEDLINGS WERE TRANSFERRED TO RADIOACTIVE SOLUTIONS.

●—● Asparagine; ○—○ γ-methyleneglutamine; ×—× glutamic acid; □—□ aspartic acid; ▽—▽ 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid; △—△ γ-methyleneglutamic acid; ▲—▲ *erythro*-γ-methylglutamic acid.

between different parts of the plant, but small quantitative differences were apparent, e.g. the concentration of 2(S),3(S),4(R)-β-hydroxy-γ-methylglutamic acid in cotyledons was higher than in the radicle.

Seeds used in metabolic experiments were germinated in the dark for 3 days at 30° until the radicle was about 0·5 cm long. The seedlings were then transferred to tapered tubes containing the labelled amino acid solutions and allowed to grow for a further 2 or 4 days at 30°.

### *Biosynthesis of* erythro-*γ-Methylglutamic Acid*

[1-$^{14}$C]DL-Glutamic acid and L-[*methyl*-$^{14}$C]methionine were administered separately to 3-day-old seedlings to test the possibility that *erythro*-γ-methylglutamic acid was formed from glutamic acid by a $C_1$ transfer mechanism requiring the methyl group of methionine. After further growth, the seedlings were extracted and the amino acids were separated by high voltage paper electrophoresis, prior to determining their $^{14}$C-content using a radiochromatogram scanner. The distribution of radioactivity in the acidic amino acids is shown in Table 1. No $^{14}$C-label was detected in γ-methylglutamic acid, or in any other substituted glutamic acid, after methionine was administered. When [$^{14}$C]glutamic acid was administered, some label was found in *erythro*-γ-methylglutamic acid, and trace labelling of 2(S), 4(S)-γ-hydroxy-γ-methylglutamic acid was detected after 4 days' metabolism.

[1-$^{14}$C]L-Leucine was also supplied to seedlings because its structure resembles that of γ-methylglutamic acid (a methyl group of leucine might become the γ-carboxyl group of the latter amino acid). After growth for two days, $^{14}$C-label incorporated into γ-methylglutamic acid was about six times higher than that determined after administering [$^{14}$C]glutamic acid.

TABLE 1. DISTRIBUTION OF $^{14}$C-LABEL AMONG VARIOUS AMINO ACIDS AFTER SUPPLYING LABELLED PRECURSORS TO DEVELOPING SEEDLINGS OF *G. triacanthos*

| | | | | $^{14}$C-incorporated (nc) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Compound administered | Time (days) | Radioactivity supplied/seedling (μc) | Specific activity of compound fed (mc/mM) | γ-Hydroxy-γ-methylglutamic acid | γ-Methylglutamic acid | γ-Methyleneglutamic acid | Aspartic acid | Glutamic acid | γ-Methyleneglutamine plus neutral amino acids |
| [U-$^{14}$C]D-Glucose | 4 | 200 | 3 | 41·7 | 11·1 | 7·54 | 43·0 | 21·3 | 319·0 |
| [1-$^{14}$C]DL-Glutamic acid | 2 | 10 | 50 | 0 | 0·35 | 0 | 0 | 19·2 | 30·0 |
| [1-$^{14}$C]DL-Glutamic acid | 4 | 10 | 50 | tr* | 1·01 | 0 | 0 | 17·4 | 19·0 |
| [*Me*-$^{14}$C]L-Methionine | 4 | 3 | 46 | 0 | 0 | 0 | 0 | 0 | 9·01 |
| [1-$^{14}$C]L-Leucine | 2 | 10 | 62 | tr* | 2·14 | 0 | 0 | 0 | 27·1 |
| [1-$^{14}$C]L-Leucine | 4 | 10 | 62 | 1·72 | 2·09 | 0 | 0 | 0 | 14·7 |
| [1-$^{14}$C]Pyruvic acid | 2 | 25 | 24 | 8·51 | 1·19 | 1·53 | 2·09 | 0·44 | 10·6 |
| [1-$^{14}$C]Pyruvic acid | 4 | 25 | 24 | 3·25 | 0·98 | 0·86 | 2·18 | 0·64 | 34·9 |

* tr—Indicates that a trace of radioactivity was present in the compound.

In addition, [$^{14}$C]leucine gave rise to a trace of labelled γ-hydroxy-γ-methylglutamic acid after day 2, and a slightly heavier labelling after day 4. No other substituted glutamic acid became labelled, and so it seemed likely that γ-hydroxy-γ-methylglutamic acid was formed secondarily from γ-methylglutamic acid.

Additional evidence relating to the question of whether glutamic acid or leucine was the more direct precursor of γ-methylglutamic acid was obtained by decarboxylating the latter amino acid after elution from electrophoretograms using standard ninhydrin procedures. For comparison, γ-methylglutamic acid also was eluted from seedlings supplied [U-$^{14}$C]D-glucose. Van Slyke determination showed that ninhydrin released $CO_2$ from only one of the two carboxyl groups of the substituted glutamic acids; in contrast, both carboxyls of β-substituted aspartic acids are lost. Labelled γ-methylglutamic acid, synthesized from [$^{14}$C]glucose or from [$^{14}$C]glutamic acid lost 15·2% and 10·5%, respectively, of its total activity on decarboxylation with ninhydrin. If all six carbons had become equally labelled as expected following metabolism of [U-$^{14}$C]D-glucose, then 16·7% of the total activity would have been lost as $^{14}CO_2$ after ninhydrin treatment (Table 2). If [1-$^{14}$C]glutamic acid was converted into γ-methylglutamic acid by a direct route, the C-1 atom would have been exclusively labelled and all $^{14}$C activity should have been lost on decarboxylation. The loss of radioactivity measured after decarboxylation represented only a small fraction of the

TABLE 2. LOSS OF $^{14}$C-LABEL AFTER NINHYDRIN DECARBOXYLATION OF [$^{14}$C]γ-METHYLGLUTAMIC ACID SYNTHESIZED BY *G. triacanthos* SEEDLINGS AFTER SUPPLYING VARIOUS LABELLED PRECURSORS

| Compound administered | $^{14}$C lost (%) by ninhydrin treatment |
|---|---|
| [U-$^{14}$C]D-Glucose | 15·2 |
| [1-$^{14}$C]-Glutamic acid | 10·5 |
| [1-$^{14}$C]-Leucine | 85·3 |
| Calculated for uniformly labelled product | 16·7 |

total, and this evidence, in association with the absence of label in γ-methylglutamic acid after administration of [$^{14}$C]methionine, supported the conclusion that γ-methylglutamic acid did not arise directly from glutamic acid. In contrast, 85·3% of the total activity incorporated into γ-methylglutamic acid after supplying [1-$^{14}$C]leucine was released as $^{14}CO_2$ by ninhydrin treatment, indicating that leucine was converted into γ-methylglutamate with little randomization of $^{14}$C-label, i.e. by a direct pathway.

### *Involvement of Pyruvic Acid in Substituted Glutamic Acid Biosynthesis*

When [1-$^{14}$C]pyruvic acid was administered to seedlings, 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid was strongly labelled, whilst smaller amounts of radioactivity were incorporated into the other substituted glutamic acids (Table 1). This γ-hydroxy-γ-methylglutamic acid was isolated and decarboxylated with ninhydrin to check whether two molecules of pyruvic acid were undergoing an aldol-type condensation. If this mechanism was involved in biosynthesis, then ninhydrin treatment should release exactly 50% of the radioactivity present in the amino acid, whereas if some pyruvic acid molecules were first degraded and then the resulting $^{14}CO_2$ was reincorporated, label would tend to be more randomly distributed between the six carbon atoms of the amino acid. The results given in Table 3 show that ninhydrin treatment released very little activity (2·1%) from the γ-substituted glutamic acid isolated after 2 days' metabolism, but a little more (5·7%) was lost from the amino acid isolated after 4 days. Therefore, γ-hydroxy-γ-methylglutamic acid does not result from the condensation of two molecules of pyruvate. These $^{14}$C-distribution measurements indicate indirectly that much of the label incorporated into γ-hydroxy-γ-methylglutamic acid resided in the γ-carboxyl C atom, being introduced into that position by the aldolase-catalyzed exchange reaction mentioned above (see also Discussion). The small

TABLE 3. LOSS OF $^{14}$C-LABEL AFTER NINHYDRIN DECARBOXYLATION OF $^{14}$C-AMINO ACIDS SYNTHESIZED BY *G. triacanthos* SEEDLINGS AFTER SUPPLYING VARIOUS LABELLED PRECURSORS

| Compound administered | Day | $^{14}$C loss (%) by ninhydrin treatment<br>γ-Hydroxy-γ-methylglutamic acid | γ-Methyleneglutamic acid |
|---|---|---|---|
| [1-$^{14}$C]Pyruvic acid | 2 | 2.1 | −0·9 |
| [1-$^{14}$C]Pyruvic acid | 4 | 5·7 | 0 |
| [U-$^{14}$C]D-Glucose | 4 | 15·1 | 15·8 |
| Calculated for uniformly labelled product | — | 16·7 | 16·7 |

amount of label in the α-carboxyl group, which increased slightly with time, probably originated from metabolic $^{14}CO_2$.

### *Biosynthesis of* 2(*S*), 4(*S*)-*γ-hydroxy-γ-methylglutamic Acid*

When [1-$^{14}$C]leucine was administered to seedlings, γ-hydroxy-γ-methylglutamic acid, in addition to *erythro*-γ-methylglutamic acid, became labelled after 4 days. Therefore experiments were performed in which [2-$^{14}$C]*threo*- and [2-$^{14}$C]*erythro*-γ-methylglutamic acids were administered separately to seedlings to check whether γ-hydroxy-γ-methylglutamic acid was formed by direct hydroxylation of the γ-methyl derivative, rather than via γ-methyleneglutamic acid. The results of these experiments are shown in Table 4. A very small amount of radioactivity was present in 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid 2 days after feeding *erythro*-γ-methylglutamic acid, and this had increased by the fourth day. In addition, some of the γ-methylglutamic acid had been degraded by the seedlings.

TABLE 4. DISTRIBUTION OF $^{14}$C LABEL AMONG VARIOUS AMINO ACIDS AFTER SUPPLYING LABELLED PRECURSORS TO DEVELOPING SEEDLINGS OF *G. triacanthos*

| | Time (days) | Supplied radioactivity/ seedling (uc) | Specific activity of compound fed (mc/mM) | $^{14}$C-incorporated (nc) | |
|---|---|---|---|---|---|
| | | | | γ-Hydroxy-γ-methylglutamic acid | γ-Methylglutamic acid |
| [2-$^{14}$C]*Erythro*-γ-methyl-DL-glutamic acid | 2 | 4 | 2 | tr† | 58·2 |
| [2-$^{14}$C]*Erythro*-γ-methyl-DL-glutamic acid | 4 | 4 | 2 | 0·58 | 27·6 |
| [2-$^{14}$C]*Erythro*-γ-methyl-DL-glutamic acid* | 4 | 4 | 2 | 9·87 | 31·5 |
| [$^{14}$C]2(S),4(S)-γ-Hydroxy-γ-methyl-L-glutamic acid‡ | 2 | 0·5 | 0·1 | 3·18 | 0 |
| | 4 | 0·5 | 0.1 | 0 | 0 |
| [$^{14}$C]γ-Methylene-L-glutamic acid‡ | 2 | 1 | 0·1 | 0 | 0 |
| | 4 | 1 | 0·1 | 0 | 0 |

| | $^{14}$C-incorporated (nc) | | | | |
|---|---|---|---|---|---|
| | γ-Methyleneglutamic acid | γ-Methylene-glutamine | γ-Hydroxy-γ-methyl-glutamic acid | Unkown acidic amino acid | Neutral amino acids |
| [2-$^{14}$C]*Erythro*-γ-methyl-DL-glutamic acid | 0 | 0 | 0·25 | 0 | 6·68 |
| [2-$^{14}$C]*Erythro*-γ-methyl-DL-glutamic acid | 0 | 0 | 0·25 | 0 | 10·54 |
| [2-$^{14}$C]*Erythro*-γ-methyl-DL-glutamic acid* | 0 | 0 | 0·61 | 0 | 20·30 |
| [$^{14}$C]2(S),4(S)-γ-Hydroxy-γ-methyl-L-glutamic acid‡ | 0 | 0 | 0 | 0 | 2·22 |
| | 0 | 0 | 0 | 0 | 4·50 |
| [$^{14}$C]γ-Methylene-L-glutamic acid‡ | 4·53 | 5·65 | 0 | 2·43 | tr† |
| | 2·25 | 5·91 | 0 | 1·75 | tr† |

* Seed used in this experiment was obtained from Palermo; commercial seed from U.S.A. was used in other experiments.

† tr—indicates that a trace of radioactivity was present in the compound.

‡ These two compounds were biosynthesized: the distribution of $^{14}$C was unknown.

These observations were extended and confirmed by feeding [2-$^{14}$C]*erythro*-γ-methyl-DL-glutamic acid to *G. triacanthos* seedlings grown from seed of different origin (Giardino Botanico e Coloniale di Palermo). These seedlings converted a higher percentage of the compound supplied to 2(S), 4(S)-γ-hydroxy-γ-methylglutamic acid: about 16% of the radioactivity remaining in the seedling after 4 days' metabolism was present in the latter amino acid, whilst 50% was still associated with γ-methylglutamic acid. In a previous experiment, [2-$^{14}$C]γ-methylglutamic acid (mixed diastereoisomers) was supplied to these seeds together with the initial water of imbibition: under these conditions, approximately 40% of the radioactivity remaining in the seedlings after 4 days' growth was present in γ-hydroxy-γ-methylglutamic acid.[10] In both experiments, most of the radioactivity remaining in γ-methylglutamic acid presumably was in the form of D-isomers.

Although *threo*-γ-methylglutamic acid could not be detected in the seedlings, a small amount of labelled 2(S),4(R)-γ-hydroxy-γ-methylglutamic acid was formed when the labelled *threo*-isomer was fed to seedlings. However, no further study was made of this 2(S),4(R)-isomer of the γ-hydroxy derivative, since the 2(S),4(S)-diastereoisomer was clearly predominant.

In all these experiments, no radioactivity could be detected in either γ-methyleneglutamic acid or γ-methyleneglutamine, so indicating that the conversion of γ-methylglutamic acid into γ-hydroxy-γ-methylglutamic acid was probably a direct one, and did not involve successive dehydrogenation and hydration steps utilizing γ-methyleneglutamic acid as an intermediate.

### *Hydroxylation of γ-Methylglutamic Acid*

A mixture of [2-$^{14}$C]*erythro*-γ-methylglutamic acid (4 μc) and [$^{3}$H]*erythro*-γ-methylglutamic acid (2 mc) ($^{3}$H label was on the γ-C atom, and in one of the 3 possible positions in the methyl group) was supplied to a seedling (Palermo Botanic Garden seed) which then was grown for 4 further days. The seedling was extracted and the amino acid fraction resolved by electrophoresis on paper. The area corresponding to labelled 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid was eluted and the $^{14}$C-$^{3}$H ratio measured and compared with that of the amino acid originally supplied to the seedlings. From these ratios (Table 5), 42·3% of the initial $^{3}$H in γ-methylglutamic acid was lost during conversion into the γ-hydroxy derivative, indicating that one of the two $^{3}$H atoms was eliminated in the reaction. Direct hydroxylation of γ-methylglutamic acid would involve the replacement of the γ-proton by a hydroxyl group.

TABLE 5. LOSS OF $^{3}$H-LABEL AFTER METABOLISM OF $^{14}$C-$^{3}$H DOUBLY-LABELLED γ-METHYLGLUTAMIC ACID BY *G. triacanthos* SEEDLINGS

| Radioactivity measurements | $^{14}$C (counts/min) | $^{3}$H (counts/min) |
|---|---|---|
| Supplied γ-methylglutamic acid | 2945 | 472,866 |
| | 2936 | 467,191 |
| Isolated γ-hydroxy-γ-methylglutamic acid | 2940* | 271,905 |
| | | 270,465 |
| Tritium loss | | 200,961 |
| | | 196,726 |
| Mean tritium loss (%) | | 42.3 |

* The amount of labelled γ-hydroxy-γ-methylglutamic acid added to the counting vial was computed to give the same $^{14}$C count as the administered γ-methylglutamic acid.

### *The Biosynthesis of 2(S),3(S),4(R)-β-hydroxy-γ-methylglutamic Acid*

When [2-$^{14}$C]*erythro*-γ-methylglutamic acid was fed to *Gleditsia* seedlings, no label was detected on electrophoretograms in the position of the 2(S),3(R),(4R)-β-hydroxy-γ-methylglutamic acid. As the 2(S),3(S),4(R)-isomer does not separate clearly from γ-methylglutamic acid, the area corresponding to the position of the two compounds was eluted:

the two amino acids were subsequently separated on chromatograms developed in butan-1-ol–acetic acid–water. A scan for radioactivity indicated a small quantity present in a ninhydrin-positive spot exactly coinciding with the known position of 2(S),3(S),4(R)-β-hydroxy-γ-methylglutamic acids. Therefore both γ-hydroxy- and β-hydroxy- derivatives of γ-methylglutamic acid can arise from the parent amino acid. No radioactivity was detected in either isomer of β-hydroxy-γ-methylglutamic acid when other labelled γ-substituted amino acids were administered to seedlings.

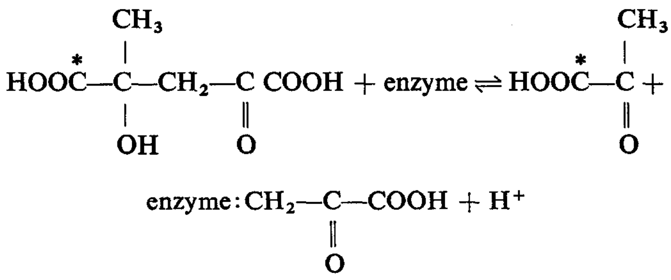
### *Metabolism of γ-Hydroxy-γ-methylglutamic Acid and γ-Methyleneglutamic Acid*

When $^{14}C$-labelled 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid (isolated from seedlings supplied [U-$^{14}C$]glucose) was supplied to seedlings, it was extensively metabolized and after 4 days no label remained in the administered compound, nor was label present in any of the other substituted glutamic acids.

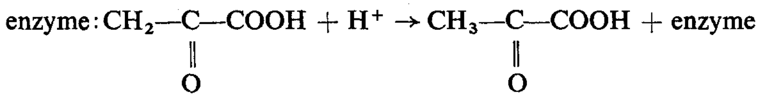
Labelled γ-methyleneglutamine was produced from [$^{14}C$]γ-methyleneglutamic acid (again obtained from seedlings supplied [U-$^{14}C$]glucose), but no radioactivity could be detected in other known γ-substituted glutamic acids. About 20% of the label supplied in this experiment was incorporated into an unidentified acidic amino acid that migrated slightly slower than aspartic acid during high voltage electrophoresis at pH 3·4. The possibility that this compound was a γ-glutamyl derivative of γ-methyleneglutamic acid was considered, but a study of the products of acid hydrolysis did not support this idea. The unknown compound occurred in a position close to that of the γ-hydroxy-γ-methylglutamic acids after two-dimensional chromatography in the system phenol–water followed by butan-1-ol–acetic acid–water.

## DISCUSSION

Previous investigations concerning the metabolism of γ-substituted glutamic acids have all provided evidence indirectly supporting the idea that the branched $C_6$-skeleton may arise by condensation of two molecules of pyruvic acid or some biogenetically-related $C_3$ compound.[5–7] In the work now reported, [1-$^{14}C$]pyruvic acid was actively incorporated into these compounds, but decarboxylation studies showed that only very small percentages of the $^{14}C$ incorporated resided in the α-carboxyl carbon. If two molecules of pyruvic acid (or a related activated form) had undergone direct condensation, then $^{14}C$ label should have been distributed equally between the two carboxyl groups: this type of biosynthetic mechanism is then no longer tenable. It seems likely that an exchange reaction, catalysed by an aldolase enzyme such as has been isolated from peanuts,[9] is responsible for the introduction of label into the substituted glutamic acids. This enzyme cleaves γ-hydroxy-γ-methyl-α-ketoglutaric acid (a compound linked with γ-hydroxy-γ-methylglutamic acid by a transamination reaction) in two stages. The first stage (I) is reversible, and it is this reaction in reverse that can lead to the incorporation of a labelled pyruvate moiety into a previously inactive product by a process that does not effect net synthesis. The second stage (II) brings about an irreversible breakdown of the enzyme–pyruvate complex. The small amount of label, shown by decarboxylation to be present in the α-carboxyl group, increased between day 2 and day 4; this label almost certainly arose by the re-utilization of $^{14}CO_2$ produced from [1-$^{14}C$]pyruvic acid during conversion into acetyl-CoA. Likewise, the small amount of label detected in aspartic and glutamic acids is attributable to re-fixation of this metabolic $^{14}CO_2$.



$$\mathrm{HOOC{-}\underset{\displaystyle OH}{\overset{\displaystyle CH_3}{\overset{*}{C}}}{-}CH_2{-}\underset{\displaystyle O}{\overset{}{\underset{\|}{C}}}\,COOH + enzyme \rightleftharpoons HOOC{-}\underset{\displaystyle O}{\underset{\|}{\overset{\displaystyle CH_3}{\overset{*}{C}}}} + enzyme{:}CH_2{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}COOH + H^+} \qquad \text{(I)}$$



$$\mathrm{enzyme{:}CH_2{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}COOH + H^+ \rightarrow CH_3{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}COOH + enzyme} \qquad \text{(II)}$$

The alternative biosynthetic hypothesis invoking the substitution of a $C_1$ unit on the carbon chain of glutamic acid was excluded by experiments in which [1-$^{14}$C]glutamic acid and [*methyl*-$^{14}$C]methionine were supplied separately to growing seedlings. Certainly, glutamic acid was not converted directly into γ-methylglutamic acid; only 10·5% of the total label incorporated into the latter amino acid was located in the α-carboxyl carbon, a figure totally inconsistent with such a conversion. Likewise, the absence of label in any of the γ-substituted glutamic acids after administration of [$^{14}$C]methionine gave no support to the idea of a $C_1$ group transfer reaction.

A biosynthetic pathway leading from leucine to *erythro*-γ-methylglutamic acid is proposed in this communication. Strong support for this idea came from an experiment in which [1-$^{14}$C]leucine, supplied to seedlings, gave rise to γ-methylglutamic acid labelled almost exclusively on the α-carboxyl group. Presumably the conversion proceeds via a stepwise oxidation of one of the methyl groups of leucine; δ-hydroxyleucine may act as an intermediate but, by analogy with a number of other systems converting hydroxymethyl to carboxyl, the transient aldehyde form may remain firmly enzyme-bound. 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid also became labelled in this experiment, suggesting that it arose by further conversion of γ-methylglutamic acid. More direct conversions of [2-$^{14}$C]-*erythro*-γ-methylglutamic acid into labelled 2(S),4(S)-γ-hydroxy-γ-methylglutamic acid and of [2-$^{14}$C]*threo*-γ-methylglutamic acid into labelled 2(S),4(R)-γ-hydroxy-γ-methylglutamic acid were demonstrated after administration of the two isomeric γ-methyl derivatives. A double-labelling experiment utilizing [$^{14}$C,$^{3}$H]γ-methylglutamic acid provided data consistent with a direct hydroxylation process. In these experiments, some radioactivity was also detected in 2(S),3(S),4(R)-β-hydroxy-γ-methylglutamic acid (compare also[12]). At present it is uncertain whether a single non-specific hydroxylase catalyses these three oxidations or whether three highly-specific enzymes are involved. However, certain evidence suggests the existence of more than one enzyme; for instance the relative degree of labelling of β-hydroxy-γ-methylglutamic acid and γ-hydroxy-γ-methylglutamic acid differed for seedlings grown from the two batches of seed available, while other analyses showed that only one of these isomeric acids was present in particular species. Some plants contain γ-hydroxyglutamic acid,[1] but radioactivity was never found in a position associated with this compound after electrophoresis.

The quantitative difference in the biosynthetic activities of the two batches of seed used is not surprising, for their different origins are almost certainly associated with different gene contents. In a similar manner, Bell and Tirimanna[13] have clearly shown that the concentration of three different $\gamma$-hydroxyamino acids in *Vicia unijuga* varied markedly with the seed source.

The biosynthetic origin of $\gamma$-methyleneglutamic acid is still obscure, despite the fact that it is both formed rapidly in germinating seeds and converted in large amounts to its amide, $\gamma$-methyleneglutamine (compare earlier work with peanut seedlings and tulip leaf discs[14]). The lack of $^{14}C$-incorporation into this amino acid from labelled $\gamma$-methyl- or $\gamma$-hydroxy-$\gamma$-methylglutamic acids fed to seedlings, or imbibing seeds,[10] does not prove conclusively that it cannot arise from leucine; nevertheless, an alternative route is strongly indicated by such negative results. In contrast to $\gamma$-methylglutamic acid and $\gamma$-hydroxy-$\gamma$-methylglutamic acid, which both can be present in the seed, $\gamma$-methyleneglutamic acid is formed only on seed germination in *G. triacanthos* (compare similar situations in *Lilium regale*,[2] *Tamarindus indica*,[15] *Delonix regia* and many *Caesalpinia* species[10]). Perhaps it originates from an unidentified product formed in the cotyledons during seed germination; this substance may be confined in a sub-cellular metabolic pool that remains distinct from the labelled exogenous precursors supplied in our experiments.

## EXPERIMENTAL

*Plant material.* Seeds of *G. triacanthos* L. (common honey locust, obtained from F. W. Schumacher, Sandwich, Mass., U.S.A.) were sacrified, dusted with Thiram (Murphy Chemical Co., Wheathampstead, Herts) and allowed to germinate in the dark for three days in moist vermiculite at 30°. Seeds obtained from the Giardino Botanico e Coloniale di Palermo were treated similarly, but were only used in a few experiments. Seedlings with radicles about 0·5 cm long were selected and placed individually in tapered tubes containing the appropriate radioactive amino acid solutions (0·2–0·5 ml). During growth, water was added to keep the elongating radicles covered and, after 2 or 4 days, seedlings were taken for analysis.

*Amino acid extraction and separation.* At the end of the assimilation period, the radicles were washed thoroughly to remove residual radioactivity and then the seedlings were ground with 75% (v/v) EtOH and extracted by continuous shaking during 24 hr. Amino acids present in the extracts were freed from non-cationic materials by using small Zeokarb 225 ($H^+$) resin columns.[16]

Acidic amino acids were separated by a high voltage paper electrophoresis procedure (Whatman 3MM paper, pH 3·4, 100 v. $cm^{-1}$, 2 hr) using a Locarte Co. (London) apparatus having 1 m plates.[17,18] The following compounds were separated readily from each other and are listed in increasing order of migration towards the anode: glutamic acid, *erythro*-$\gamma$-methylglutamic acid, 2(S),3(R),4(R)-$\beta$-hydroxy-$\gamma$-methylglutamic acid, $\gamma$-methyleneglutamic acid, aspartic acid, 2(S),4(S)-$\gamma$-hydroxy-$\gamma$-methylglutamic acid, and 2(S),4(R)-$\gamma$-hydroxy-$\gamma$-methylglutamic acid. *Threo*-$\gamma$-methyglutamic acid migrated more slowly than glutamic acid, while 2(S),3(S),4(R)-$\beta$-hydroxy-$\gamma$-methylglutamic acid migrated faster, but these three substances were inadequately separated under the electrophoretic conditions. When determinations were required on these compounds, the combined area from the electrophoretogram was eluted and the extracted amino acids were resolved by chromatography on Whatman No. 3MM paper using $n$-BuOH–HOAc–$H_2O$ (12:3:5, by vol.) as solvent.

The identity of each acidic amino acid was confirmed by two-dimensional chromatography employing 75% (w/v) phenol, in presence of ammonia vapour, and $n$-BuOH–HOAc–$H_2O$ as solvents.

Quantitative amino acid determinations were made after separation by high voltage paper electrophoresis or chromatography using the cadmium–ninhydrin reagent of Blackburn.[19]

*Radioactive assay.* High voltage paper electrophoretograms or one-dimensional chromatograms were

---

[13] E. A. BELL and A. S. L. TIRIMANNA, *Biochem. J.* **97**, 104 (1965).

[14] L. FOWDEN, *Nature, Lond.* **186**, 897 (1960).

[15] R. N. PALL and M. M. LALORAYA, *Physiol. Plant.* **20**, 789, (1967).

[16] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **4**, 933 (1965).

[17] P. J. PETERSON, *J. Chromatog.* **38**, 301 (1968).

[18] P. J. PETERSON and L. FOWDEN, *J. Chromatog.* **48**, 575 (1970).

[19] S. BLACKBURN, in *Methods of Biochemical Analysis* (edited by D. GLICK), Vol. 13, pp. 1–45, Interscience, New York (1965).

scanned for radioactivity with a Packard Radiochromatogram scanner. Radioautography was used for finger-printing, and for locating labelled substances separated by two-dimensional chromatography. $^{14}C$ present in labelled compounds eluted from electrophoretograms or chromatograms was determined by planchet counting (efficiency 5%), while $^{14}C$ and $^{3}H$ present in doubly-labelled compounds were assayed using a differential channel counting procedure and a Packard Tricarb liquid scintillation spectrometer [butyl-PBD (Koch-Light Laboratories Ltd., Colnbrook) was employed as scintillant]. A counting efficiency of 10% for $^{14}C$ and 2% for $^{3}H$ was obtained.

*Radioactive syntheses and biosyntheses.* [2-$^{14}C$]$\gamma$-Methylglutamic acid was synthesized from ethyl [2-$^{14}C$]-acetamidocyanoacetate by the method of Sung.[20] The labelled mixture of diastereoisomers resulting was clearly resolved into *threo-* and *erythro*-forms by high voltage paper electrophoresis (pH 3·4, 100 v. cm$^{-1}$ for 4 hr). After eluting each isomer from the electrophoresis strips, any contaminating materials were removed by applying to a Zeokarb 225 (H$^{+}$) column.

$^{14}C$-Labelled L-$\gamma$-methyleneglutamic acid and 2(S),4(S)-L-$\gamma$-hydroxy-$\gamma$-methylglutamic acid were isolated from *G. triacanthos* seedlings that had grown in the presence of [U-$^{14}C$]D-glucose for 5 days (each seedling received 200 $\mu$C labelled glucose). The amino acids present in the seedling extracts again were separated by high voltage paper electrophoresis. These seedlings also produced very large amounts of labelled $\gamma$-methyleneglutamine, and so the amide was eluted, together with other neutral amino acids, from the electrophoretogram: this extract was hydrolysed (2 N HCl, 18 hr, 100°) and again subjected to electrophoresis to separate the resulting [$^{14}C$]$\gamma$-methyleneglutamic acid from other components of the hydrolysate. Again, these biosynthetically-labelled products were finally purified using Zeokarb 225 (H$^{+}$) columns.

The [$^{3}H$]$\gamma$-methylglutamic acid used in the double-label experiment was prepared by shaking the diethyl ester of L-$\gamma$-methyleneglutamic acid (20 mg), dissolved in tetrahydrofuran (5 ml), with $^{3}H_2$ gas (10 c, 98%) and platinum catalyst (20 mg) in a sealed ampoule previously flushed with argon. Labile $^{3}H$ was removed by equilibration with $H_2O$, and then the ester was hydrolysed (2 N HCl, 18 hr, 100°). The *threo-* and *erythro-$\gamma$*-methylglutamic acids were separated by paper electrophoresis (pH 3·4, 100 v. cm$^{-1}$, 4 hr), and located by radioautography. The high specific activity of the preparations led to some radiochemical breakdown during storage, and so samples administered to seedlings were used quickly after their separation by electrophoresis.

*Acknowledgements*—The authors wish to thank the Science Research Council for their award of a Senior Visiting Fellowship that enabled P.J.P. to participate in this work, and also the New Zealand Department of Scientific and Industrial Research for financial support (P.J.P.).

---

[20] M. L. SUNG, Ph.D. thesis, University of London (1970).

*Key Word Index*—*Gleditsia triacathos*; Leguminosae; biosynthesis; amino acids; $\gamma$-methyleneglutamic acid.