Metabolism of γ -Hydroxy- γ -Methylglutamic Acid in the Leaves of Groundnut

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Abstract

The metabolism of γ -hydroxy- γ -methylglutamic acid (γ -HMGA) in groundnut leaves was investigated by the tracer techniques. The radioactivity of uniformly labelled ^{14}C -pyruvate was rapidly incorporated into this amino acid, suggesting that the six-carbon skeleton of γ -HMGA might be, at least in part, derived from the carbons of pyruvic acid.

Uniformly labelled ^{14}C - γ -HMGA was, upon feeding, relatively rapidly metabolized in groundnut leaves. A quantity of radioactivity therefrom was incorporated into an amino acid which seemed to be γ -hydroxyglutamic acid (γ -HGA) at an early stage of incubation, and the radioactivity of this compound was gradually decreased with incubation time due to incorporation into most other amino acids. On the other hand, an enzyme preparation, by the action of which alanine was produced from γ -HMGA, was obtained from the groundnut leaves. On the basis of these findings it was assumed that γ -HGA might be formed from γ -HMGA via some intermediates such as pyruvate rather than by demethylation at the γ -carbon atom.

The metabolism of γ -hydroxy- γ -methylglutamic acid (γ -HMGA) has received little investigations, before the tracer techniques have been introduced to the studies of amino acid metabolism in plants. By the observations of Linko and Virtanen¹⁾ and Fowden and Webb²⁾ on the biosynthesis of γ -HMGA in some ferns and on that of γ -methyleneglutamic acid (γ -MGA) in groundnut, it was suggested that these γ -substituted glutamic acid derivatives with six carbon atoms might be synthesized *in vivo* from pyruvate or its related compounds. However, no conclusive evidences for supporting this assumption have so far been obtained.

The present authors³⁾ recently found that γ -HMGA occurred widely in fern plants and changed in its content during their growth period in the roughly reciprocal proportion with that of glutamic acid and glutamine. From the result it was suggested that γ -HMGA might play some physiological role in fern plants. The present authors, therefore, investigated precisely the metabolism of γ -HMGA in plants using uniformly labelled ^{14}C -pyruvate and ^{14}C - γ -HMGA. The results obtained will be reported in this paper. As the experimental material, the groundnut at various stages of growth were employed.

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Material and Methods

I. *Plant material.*

Groundnut (*Arachis hypogaea*) used in this study was cultivated outdoors in a period from May to August. Young leaves at the flowering stage of the plant were picked at an appropriate period between noon and two o'clock p.m. on a sunny day. After being washed with water, the leaves were cut into small discs of 7 mm diameter with a cork borer in a small amount of ice-cooled distilled water. The leaf discs were carefully pressed between two sheets of filter paper to make free from sticking water, and quickly used for experiments.

II. *Preparation of radioactive compounds.*

Uniformly labelled sodium ^{14}C -pyruvate was purchased from the Radiochemical Centre (Amersham, England).

Uniformly labelled ^{14}C - γ -HMGA was prepared from the ^{14}C -pyruvate, according to the method of Steward *et al.*⁴⁾ The product was isolated as a salt of hydrochloric acid. The substance behaved as a single component in paper chromatogram and its Rf-value was in agreement with that of a racemic compound reported by Steward⁴⁾.

III. *Feeding experiments with ^{14}C -labelled compounds.*

In these experiments, all works were carried out in a cold room at 0°. Two hundred eighty pieces of leaf discs of 2100 mg on a fresh weight basis were prepared. They were placed in a small glass jar and immersed in 20 ml of 0.01 M ^{14}C -pyruvate or ^{14}C - γ -HMGA solution. In some experiments, 0.02 M ^{14}C - γ -HMGA was used. The jar was evacuated for 30 min. in a small vacuum desiccator to infiltrate the ^{14}C -pyruvate into the discs. After the pressure had been equilibrated, the leaf discs were still allowed to stand in the glass jar for additional 30 min., and then the sticking liquid was removed by careful blotting between two sheets of filter paper. They were divided into seven groups, each of which consisted of 40 pieces, and each group was then placed in a Thunberg tube. The side arm of the tube contained 2 ml of a 0.1 N potassium hydroxide solution to trap carbon dioxide produced by the respiration of leaf discs. Each 1 mg of the ^{14}C -pyruvate and of the ^{14}C - γ -HMGA contained 1.25 μCi and 0.5 μCi , respectively.

These Thunberg tubes were closed and then incubated at 30°. After being incubated for an appropriate period, the leaf discs were removed from the tubes, soaked at first into about 5 ml of hot 70 per cent (v/v) aqueous ethanol to kill them, and then heated in a boiling water bath for 15 min to extract soluble materials. Similar extraction was repeated several times. The combined aqueous extract was adjusted at pH 7.0 and concentrated to a syrup under reduced pressure at 40°. After being dissolved in small quantities of water, the syrup was applied to a column of Amberlite IR-120 (H^+ -type) to absorb amino and keto acids. The column was eluted at first with water and then with 1.85 N ammonium hydroxide. The alkaline eluate was neutralized and dried up under reduced pressure at 25°. Finally the dried material was dissolved in 1 ml of water to be analyzed for amino and keto acids.

IV. *Separation and determination of individual amino acids by paper chromatography.*

Individual amino acids including γ -derivatives of glutamic acid in the extracts from leaf discs were identified by two dimensional paper chromatography using Toyo filter paper No. 51 (40 $\times$ 45 cm). Phenol-saturated water was used for the first

direction by ascending method, and the upper layer of a mixture of n-butanol:gracial acetic acid: water (4:1:5) was employed for the second direction by descending method. After being developed, the filter paper was dried, and sprayed with a ninhydrin reagent (prepared by dissolving 0.5 g ninhydrin in a mixture of 100 ml of 70 per cent aqueous ethanol and 0.5 ml of 0.1 N sodium hydroxide) and heated at 65° for 20 min. Each area stained by the reagent on a paper chromatogram was cut out, extracted with 5 ml of 70 per cent aqueous ethanol under centrifuging at 2,000 rpm for 5 min. The solution was colorimetrically measured at 570 m μ with a Shimadzu AKA Type 5-D photoelectric photometer.

V. *Determination of the radioactivity of individual amino acids.*

An aliquot of the above extract was evaporated on an aluminum planchet at 80°. The radioactivity of the residue was measured by an Aloka Type LBC-6 windowless gas flow counter.

VI. *Determination of the radioactivity of α -keto acid fractions.*

Part of the concentrated initial extract containing amino and keto acids obtained in the experiment III was deproteinized with the trichloroacetic acid solution of a 5 per cent final concentration. After removing the protein precipitate by filtration, one tenth volume of 1 per cent (w/v) 2, 4-dinitrophenylhydrazine in 5 N sulphuric acid was added to the filtrate. The mixture was allowed to stand for 45 min at room temperature. A derivative of phenylhydrazone obtained was extracted four times with a half volume of ethylacetate⁶⁾. The combined extracts were evaporated on an aluminum planchet, and the radioactivity of the residue was counted in the same way as above.

VII. *Preparation of the dinitrophenylamino acids.*

Dinitrophenylamino acids were prepared by the method of Ley *et al.*⁶⁾ These amino acid derivatives were then subjected to one-dimensional paper chromatography with the solvent system of isoamylalcohol saturated with 1 per cent acetic acid by ascending method⁷⁾.

VIII. *Crude extracts for enzyme assays.*

The following plant materials were used for the preparation of enzyme solution for the possible degradation and synthesis of γ -HMGA; seedlings of *Glycine soja*, seedlings, young plants and ripening seeds of *Arachis hypogaea*, growing and matured leaves of *Adiantum pedatum*, matured leaves of *Dryopteris crassirhizoma*, growing leaves of *Osmunda japonica* and growing leaves of *Pteridium aquilinum*. The fresh samples of these materials were homogenized with an equal amount of distilled water, and squeezed through two layers of cheese cloth. The filtrate was centrifuged at 4,000 $\times$ g for 10 min at 2°. The supernatant was dialyzed overnight against four volumes of distilled water, and then centrifuged at 7,000 $\times$ g for 20 min. at 2°. The supernatant was used as a crude enzyme preparation.

IX. *Enzyme assay.*

Possible enzymic synthesis of γ -HMGA was tested with a reaction mixture composed of 0.1 ml of 0.05 M sodium pyruvate or alanine as substrate and 0.1 ml of 0.05 M glutamate as the donor of amino group and 0.2 ml of buffer. For testing the enzymic degradation of γ -HMGA, a reaction mixture composed of 0.1 ml of 0.05 M

γ -HMGA hydrochloride of pH 7.0 as substrate, and 0.2 ml of buffer was used. In some experiments, disodium salt of adenosine triphosphate (ATP) and magnesium chloride was added to the reaction mixture to 1.25 μ moles and 1.0 μ mole final concentration, respectively. As the buffer solutions, 0.1 M acetate buffers of pH's 3.8, 5.2 and 6.0 and 0.1 M Veronal buffers of pH's 7.0 and 8.0 were used.

After a given period of incubation at 30°, 0.1 ml aliquots of the reaction mixtures were applied to Toyo filter paper No. 50 and chromatographed by ascending technique using phenol-saturated water as solvent system and 0.2 per cent ninhydrin in *n*-butanol saturated with water as staining reagent.

Results

(1) *Incorporation of the radioactivity of ^{14}C -pyruvate into amino acids in the groundnut leaves.*

Leaf discs of groundnut were incubated with ^{14}C -pyruvate. During the incubation, some of the leaf discs were removed at an appropriate intervals, extracted with 70 per cent aqueous ethanol, and the radioactivity of amino acid fractions obtained from the extracts were determined. The results are shown in Table 1.

^{14}C -Pyruvate appeared to be readily metabolized in the groundnut leaves, since the radioactivity was rapidly incorporated into amino acid fractions as much as 40 per cent of the total activity in the extract within a short period such as 15 min, although the radioactivity decreased thereafter to some extent. As the next experiment, the distribution of radioactivities from ^{14}C -pyruvate among several individual amino acids was investigated and the results are shown in Table 2 in terms of specific activity (cpm/mg of amino acid).

At an early stage of incubation, a considerably high radioactivity was

Table 1. Radioactivities incorporated into amino acid fractions from groundnut leaves incubated with ^{14}C -pyruvate.

Incubation time	Radioactivity in amino acid fraction
min.	%*
15	40.3
30	17.5
60	—
120	15.6
180	20.6

* Rate per the total activity of the extract.

Table 2. Radioactivities (cpm/mg) incorporated into various amino acids in groundnut leaves incubated with ^{14}C -pyruvate.

Amino acid	Incubation time (min.)		
	15 min.	30 min.	120 min.
Asparagine	5654	80	0
Alanine	4333	2087	2444
γ -ABA ^a	500	375	318
γ -MG ^b	0	414	0
γ -HMGA	2720	444	900
Glutamic acid	261	400	157

a= γ -aminobutyric acid, b= γ -methyleneglutamine

found in γ -HMGA, although the incorporation was far less than that in asparagine and alanine. However, the radioactivities of γ -HMGA and asparagine decreased rapidly, in particular with the latter in prolonged incubation, whereas that of alanine very slowly. These results suggest that pyruvate molecule may be either directly condensed to form the carbon skeleton of γ -HMGA or previously converted to alanine by transamination followed by condensation with pyruvate to form γ -HMGA.

A high incorporation of radioactivity in asparagine, on the other hand, suggests that this amino acid also may be derived from ^{14}C -pyruvate, possibly through several steps of pathway. The radioactivity of asparagine, however, was decreased very rapidly during the incubation, so that this acid appears to be metabolized easily in groundnut.

The radioactivity of γ -methyleneglutamine (γ -MG) was detected at 5 minute incubation, although it was not shown in the Table. Presumably, this substance might be secondarily formed from γ -HMGA, since it is assumed that γ -methyleneglutamic acid (γ -MGA) may be derived from γ -HMGA by dehydration and the γ -MG from the γ -MGA in the same way as glutamine from glutamic acid⁹⁾. This assumption can be supported by the fact that Fowden has found that the radioactivity of ^{14}C - γ -MGA was incorporated into γ -MG in groundnut⁹⁾.

The radioactivity of γ -aminobutyric acid (γ -ABA) was not high, but showed only a slight decrease in prolonged incubation. This indicated that this compound may be considerably inert in its metabolism.

(2) Degradation of γ -HMGA in vivo.

^{14}C - γ -HMGA was infiltrated *in vacuo* into the leaf discs of groundnut, and some of them were removed at intervals from the incubation medium to treat with 70 per cent aqueous ethanol. Radioactivities of γ -HMGA, total amino acids and total α -keto acids in each extract were determined. The results are shown in Table 3.

As will be seen from Table 3, γ -HMGA was very slowly metabolized. However, considerable amounts of the radioactivity were incorporated into amino acids other than γ -HMGA, and in addition they decreased gradually as the incubation period was prolonged. Relatively little radioactivity was found in keto acid fractions, but it also decreased with the incubation period.

The distribution of radioactivity from γ -HMGA among individual amino acids are shown in Table 4.

It is to be noticed in the Table that an amino compound named "unknown A"

Table 3. Degradation of ^{14}C - γ -HMGA and the radioactivities incorporated into amino acid and keto acid fractions from groundnut leaves during the incubation.

Incubation time	γ -HMGA degraded	Amino acid fraction except γ -HMGA	α -keto acid fraction
min.	%*	%**	%**
30	12	40	15
60	—	45	18
90	—	34	—
120	28	32	—
1440	48	24	6

* Per cent ratio between the radioactivity of (initial - residual) and that of (initial).

** Rate per the total activity of the extract.

was predominantly labelled at early stages of incubation and that its radioactivity increased for the first 30 minute incubation, but rapidly decreased with incubation time. In contrast, the radioactivities of most other amino acids including γ -ABA continued to increase with prolonged incubation, although the amounts of radioactivities were much lower than that of the "unknown A" in early stages of incubation.

(3) *Identification of "unknown A" substance.*

"Unknown A" showed a characteristic reddish violet color with ninhydrin reagent and located on the chromatogram very close to the spots of aspartic acid and authentic γ -hydroxyglutamic acid (γ -HGA) as listed in Table 5.

Upon fractionation of di- and monocarboxylic acids by column chromatography with acid-activated aluminum oxide¹¹⁾, the "unknown A" was not eluted in monocarboxylic acid fraction. A dinitrophenyl-derivative of this substance was prepared, and the Rf value was compared with that of authentic dinitrophenyl- γ -HGA, as shown in Fig. 1.

The Rf values of these two substances were in agreement with each other. The "unknown A", therefore, may be tentatively identified as γ -hydroxyglutamic acid (γ -HGA).

Table 4. Radioactivities (cpm/mg) incorporated into various amino acids in groundnut leaves incubated with ^{14}C - γ -HMGA.

Amino acid	Incubation time (min.)				
	5	10	30	60	120
Unknown A	2360	2190	4744	3155	2041
Glutamic acid	48	24	275	385	647
Serine	55	127	488	670	2625
Asparagine	56	33	0	0	0
Glutamine	88	80	0	323	1468
Alanine	245	309	800	571	1678
γ -ABA	310	237	1744	2100	4116
Valine	0	0	0	0	1694
Leucines	165	87	0	0	0
γ -MG	104	289	279	357	0

Table 5. Rf-values of unknown A together with those of aspartic acid and γ -HGA and their colors with ninhydrin reagent.

Compound	Rf-values with solvent systems		Color
	Phenol-water	Butanol-acetic acid	
Unknown A	0.18	0.06	Reddish violet
Aspartic acid	0.18	0.07	Blueish violet
γ -HGA	0.17	0.06	Reddish violet

Fig. 1. Diagram of the paper chromatogram showing the dinitrophenyl-derivatives of amino acids. DNP- γ -HGA: Dinitrophenyl γ -hydroxyglutamic acid. DNP-U.A.: Dinitrophenyl derivative of "unknown A". DNP-Asp.A.: Dinitrophenyl aspartic acid.

(4) *Enzymic degradation of γ -HMGA in vitro.*

An enzyme extract from the seedling of *Arachis* was incubated with γ -HMGA at pH 5.6 for 13 hrs., and it was found by paper chromatography a small quantity of alanine was formed in the incubation mixture. The production of alanine was also observed under the same conditions when an enzyme extract from maturing seeds was used as enzyme solution. Furthermore, this enzyme activity was found at pH's 3.8 and 6.0 in place of pH 5.6 (Fig. 2).

(5) *Attempt for the enzymic synthesis of γ -HMGA in vitro.*

Sodium pyruvate or alanine was incubated as substrate with each of several crude enzyme solutions obtained from various plant sources, as described in the part of material and methods, in the presence of sodium glutamate as amino donor. No enzymic formation of γ -HMGA from pyruvate or alanine was observed under present condition, and ATP added as a possible energy source was not effective for this formation.

Fig. 2. Diagram of the paper chromatogram showing the degradation products from γ -HMGA by an enzyme preparation from the maturing seeds of groundnut.

Incubation time: 60 min. Solvent system: phenol-saturated water. Spray reagent: ninhydrin in water-saturated butanol.

1, pH 3.8; 2, pH 3.8, enzyme omitted; 3, pH 5.2; 4, pH 6.0; 5, pH 7.0; 6, pH 8.0.

Discussion

γ -Derivatives of glutamic acid have often been considered to be rather secondary inert products in the metabolism of amino acids⁹⁾. In the present work, however, it was shown that ^{14}C -labelled- γ -HMGA (γ -hydroxy- γ -methylglutamic acid) supplied

to groundnut leaves was rapidly metabolized and its radioactivity was quickly distributed into a variety of other amino acids. This fact suggests that γ -HMGA plays some important metabolic roles in this plant.

The concept that the six-carbon skeleton of γ -HMGA may be directly derived from pyruvate molecules has been supported by some tracer experiments of Fowden and Webb²⁾ and of Linko and Virtanen¹⁾. On the contrary, Marcus and Shannon^{12,13)} could not succeed to synthesize γ -hydroxy- γ -methyl- α -ketoglutaric acid, an α -keto acid closely related to γ -HMGA, from pyruvate by a purified enzyme preparation from groundnut seedlings, whereas the enzymic degradation of this amino acid was succeeded by these workers. Under the conditions employed in the present work, the formation of γ -HMGA from pyruvate or alanine by crude enzymes from groundnut seedlings and other plant materials was not confirmed. However, the present finding that the radioactivity of ^{14}C -pyruvate was incorporated into γ -HMGA at a high rate in the groundnut leaves, suggests that γ -HMGA may be synthesized *in vivo* from pyruvate by the reaction of a type of aldol condensation.

The radioactivity of ^{14}C - γ -HMGA supplied to groundnut leaves was very rapidly incorporated into an amino compound which seemed to be γ -HGA (γ -hydroxyglutamic acid), suggesting that γ -HMGA would be directly converted to γ -HGA.

The radioactivity once incorporated into γ -HGA was decreased gradually with incubation period, whereas the radioactivities of most other amino acids including alanine were remarkably increased in prolonged incubation. In addition, it was found in the present work that alanine was produced from γ -HMGA by the action of the extract from the ripening seeds of groundnut. Shannon and Marcus¹²⁾ also demonstrated that pyruvate, the precursor of alanine, was enzymically produced from the α -keto acid corresponding to γ -HMGA by the cleavage of the middle bond of this keto acid. Therefore, it is also tempted to assume that γ -HGA would be reversibly formed via some intermediate products of γ -HMGA, probably via pyruvate. This assumption might be also supported by the finding that γ -HGA was reversibly decomposed into alanine (or pyruvate) and glyoxylate^{14,15,16,17)} by animal tissues, although Linko and Virtanen¹⁾ failed to demonstrate that ^{14}C -pyruvate was utilized as a precursor for the synthesis of γ -HGA in a plant, *Phlox*, where γ -HGA occurs under the natural conditions¹⁸⁾. Therefore, the possible existence of a pathway through which γ -HGA would be decomposed into alanine and glyoxylic acid should not still be excluded, in particular in the groundnut leaves. However, the specific activity of alanine was relatively small as compared with that of γ -HGA when ^{14}C - γ -HMGA was supplied to groundnut leaves. In this respect, there may be strong possibility that the γ -HGA would be derived directly from ^{14}C - γ -HMGA by demethylation, although this reaction seems hardly to occur in view of enzymic reaction.

The compound which seemed to be γ -HGA obtained from groundnut leaves in the present work was not isolated as a single entity. The identification of this compound was based only on paper chromatography, so that further confirmation is necessary for this discussion. However, a good agreement between Rf value of a DNP-derivative of this compound and that of authentic one makes the present tentative identification more reliable. Thus, the possible metabolic pathway of γ -HMGA in groundnut leaves may be as follows, where the reactions with more positive evidences are indicated by solid arrows and those with the less by dotted ones.

摘 要

田中 実*・西沢一俊**・三輪知雄**： ナンキンマメ葉における
 γ -ヒドロキシ- γ -メチルグルタミン酸の代謝

グルタミン酸の γ -位置換体は植物界に広くその分布が確認されているが、その生理的意義ならびに代謝系路についてはほとんど未詳である。著者等はナンキンマメ葉に *vacuum infiltration* 法によって与えた ^{14}C -標識化合物の放射能を追跡することによって、それら置換体の一つ γ -ヒドロキシ- γ -メチルグルタミン酸 (γ -HMGA) の代謝を調べた。

$\text{U-}^{14}\text{C}$ -ビルビン酸を与えると γ -HMGA は他のアミノ酸と同様にすみやかに標識化される。したがって γ -HMGA の炭素骨格は、少くともその一部は、ビルビン酸に由来すると考えられる。

与えられた $\text{U-}^{14}\text{C}$ - γ -HMGA は比較的すみやかに代謝される。その放射能は5分後にすでに多くのアミノ酸にとりこまれるが、その際もっとも高い比放射能を示す物質は、二、三のクロマトグラフ的性質の一致から γ -ヒドロキシグルタミン酸 (γ -HGA) と推定された。しかしながら、ナンキンマメ葉から抽出された粗酵素標品によると、 γ -HMGA から γ -HGA はできず、アラニンの生成を認める。

これらの知見に基づいて γ -HMGA の代謝系路に考察を加え、 γ -HGA が生ずる2,3の可能性について議論を試みた。(*,**東京教育大学理学部植物学教室・*現在 三共株式会社中央研究所)