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On the Biosynthesis of some Recently Discovered Derivatives of Glutamic Acid in Plants

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1- and 2-^{14}C-pyruvates, respectively, were fed to excised leaves of *Asplenium septentrionale* and *Phlox decussata* by different methods, and the incorporation of radioactivity into the amino compounds was followed as a function of time. No significant differences in the metabolic pattern could be found in these plants containing "new" amino acids as compared to the other plants examined. Transaminase activity is very high, alanine being most rapidly labelled. Already after a few minutes from the beginning of infiltration amino compounds contained up to 75 % of the total in 70 % ethanol soluble activity. After about 15 h glutamine was the major labelled product.

γ-Methyl-γ-hydroxyglutamic acid is shown to exist free in *Phlox decussata* by means of isotopic techniques. Hence this amino acid may be a normal metabolite in plants. The main biosynthetic route to γ-methyl-γ-hydroxyglutamic acid is most probably an aldol condensation of two pyruvate molecules, following transamination. γ-Hydroxyglutamic acid is not noticeably synthesized in the leaves of *Phlox decussata*.

The role of γ-aminobutyric acid in relation to glutamine is discussed. The most probable route from γ-AB to glutamic acid seems to be *via* glutamine.

The results indicate that glycine is not the main precursor of serine in these plants.

A great number of previously unknown amino compounds have been detected in living organisms during the last few years. Most of these compounds are found only in the 70 % ethanol soluble part, but some are thought to be protein components, too. Though it appears probable that these amino derivatives are not central metabolites in plants, it seems to be quite possible that many of them occur widely distributed in the plant kingdom.

Very little is still known about the metabolism of most of these "new" compounds. Not much could be done to solve this problem before the techniques using radioactive isotopes as tracers were available. It will be of great impor-

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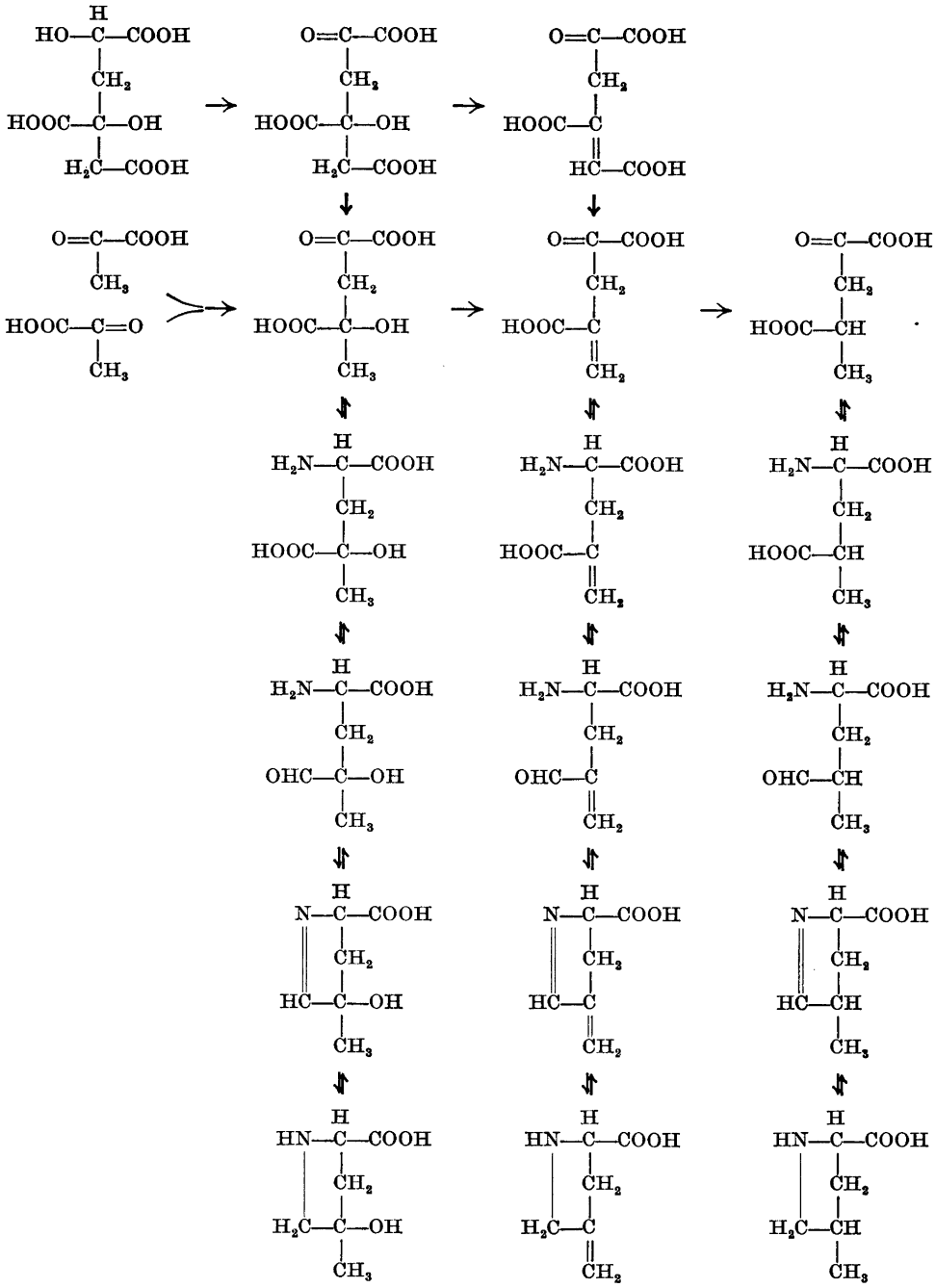

Fig. 1. Scheme of the possible biosynthetic reactions leading to γ-derivatives of glutamic acid.

tance to discover whether the metabolism of plants containing large amounts of these compounds is entirely different from that of others or if there is merely a simple enzyme block present. The latter hypothesis would explain the fact that some of these compounds, when present, are predominant among the free amino acids. In other cases they would be only very short lifetime intermediates and therefore not detectable without tracer techniques.

Already eleven monoaminodicarboxylic acids have been found to exist in plants. Four of them are derivatives of glutamic acid: γ-methyleneglutamic acid and the corresponding amide [1], γ-methylglutamic acid [2], γ-methyl-γ-hydroxyglutamic acid, γ-MHGA [2,3], and γ-hydroxyglutamic acid, γ-HGA [4]. All of these, as well as the known derivatives of γ-aminoadipic and pimelic acids [5], contain the substituents in γ-position. This has led to the assumption [6] that the primary reaction of the biosynthesis of these compounds would be an aldol condensation of either two molecules of pyruvate or one pyruvate molecule and one molecule of some other oxo compound as glyoxylic acid. Transamination would then result to the corresponding amino acid. Many of these keto acids are now known to exist free in plants [7–10].

A different possibility has been postulated by Bentley and Thiessen [11]. According to them, 2,4-dihydroxy-1,2,4-butanetricarboxylic acid, which is known to exist in dog's liver [12] and in bacteria [13], would be oxidized and dehydrated to 4-oxo-1-butene-1,2,4-tricarboxylic acid. The carboxyl group in position 1 would then be decarboxylated by means of a specific decarboxylase. Oxalocitramalic acid, which is a postulated intermediate in this scheme, is, however, not a normal metabolite [14].

A third possibility would be the biosynthesis from the corresponding proline derivatives. This hypothesis seems to be quite possible at least when γ-HGA is in question. A summary of all these reactions is shown in Fig. 1.

To test the aldol condensation hypothesis sodium 1- and 2-^{14}C-pyruvates were fed to *Asplenium septentrionale* [15] and *Phlox decussata*. In the former γ-MHGA [16] and in the latter γ-HGA [4] were known to occur.

MATERIALS AND METHODS

Plant material. *Asplenium septentrionale* was harvested in September and October. Most experiments were performed with freshly picked plants, but in some cases *Asplenium*, kept up to several months at +4°C in an open jar, was used. The results in different cases did not markedly differ from each other. *Phlox decussata* was grown in the greenhouse from a piece of root picked early in the spring under snow. Plants were used for experiments when about 10 cm high.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, England.

Experiments of two different types were performed. I. 25 *Asplenium* plants (*ca.* 700 mg fresh wt.) were put in each of a series of small test tubes containing 4 ml of water. Half a cm of the plants was cut off under water, and 3 ml of the liquid was carefully pipetted away. To each of the test tubes 100 μl of sodium pyruvate solution (0.067 mg = 2.7 μC ml of water was added mixing carefully. After 2, 6 and 13 h 0.5 ml of water was added to each tube. Samples were collected after ½, 1, 2, 4, 6, 12, 24 and 48 h. The uptake of radioactivity was much higher when plants stored (and dried) at +4°C were used. II. Most of the experiments were performed by the vacuum infiltration method. About 500 —700 mg fresh wt. of plants were cut to pieces, *ca.* 1 cm long, and put into small glass jars each containing 2 ml of radioactive pyruvate solution (2—4 μC in different experiments). Samples were then subjected to a small vacuum by means of a vacuum desicca-

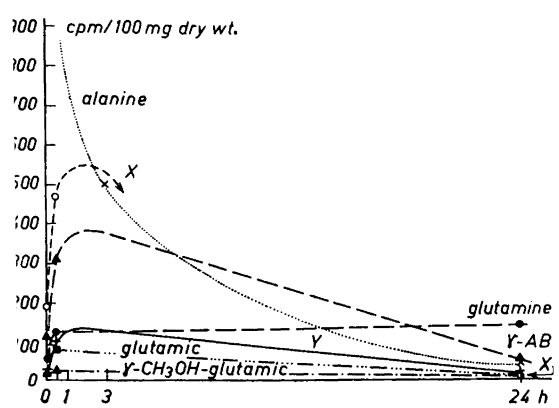

Fig. 2. Total radioactivity changes in the amino compounds of *Asplenium septentrionale* after 2-^{14}C-pyruvate infiltration.

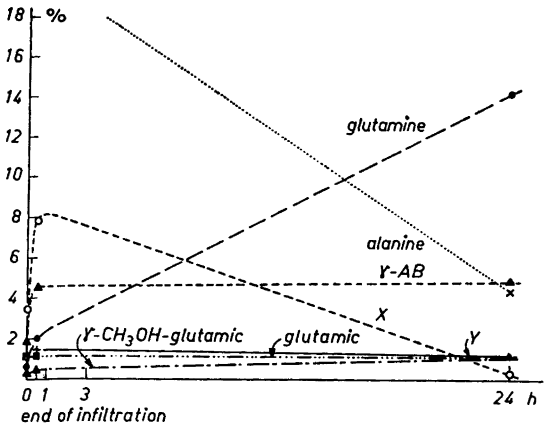

Fig. 3. Percentage of radioactivity in the individual amino compounds calculated from the total ethanol soluble activity in *Asplenium septentrionale* after 2-^{14}C-pyruvate infiltration.

tor several times after each other. The time of infiltration varied from 5 to 30 min. During this period practically all of the solution was taken up by the plants. Samples were dried lightly between filter papers and put on moist filter papers into light for different periods of time. Sometimes all of the plant material was subjected to infiltration simultaneously, and aliquots were taken after certain time intervals. All experiments with *Phlox decussata* were performed by this method.

The extraction was done with 70 % ethanol until the residue was practically colorless. Extracts were purified from salts and other materials, disturbing in paper chromatography, by passing through an Amerberlite IR-120 cation exchange resin, the extract thus being divided into the amino compound and "peptide" fractions. The amino compounds were eluted from the resin with 1 N NH_4OH. Both fractions were evaporated to a small volume *in vacuo* at 40°C. It was noticed using algae, from which good chromatograms can be made even without ion exchange treatment, that the "peptide" fraction does not continue to give a correct picture of the "anionic" compounds [17]. This is largely due to decomposition of phosphates and some other easily hydrolysable compounds including even some amino derivatives.

For chromatography Whatman No. 4 paper was used without prewashing. First solvent: phenol (Merck, "für Chromatographie", without distillation) saturated with water in ammonia atmosphere. Ammonia was omitted for chromatography of the "peptide" fraction. Second solvent: *n*-butanol: acetic acid: water (63:10:27). In both solvent systems HCN was used to prevent the "pink front" on ninhydrin sprayed amino acids.

Radioautograms were taken either on Ilford Ilfex X-Ray film or on Kodak's single coated Medical X-Ray tinted back film.

Radioactivity measurements of the spots on paper chromatograms were first done by extracting the spots with small amounts of water and counting the evaporated extracts on aluminium discs with Friesecke & Hoepfner, G.m.b.H., Erlangen-Bruck, scaler using Geiger tube FHZ 15/5716 (window -2.1 mg/cm^2). Later, spots were counted directly on chromatograms with a Tracerlab Autoscaler using a self made gasflow counter (DuPont mylar window). If found necessary, the results were corrected for the self absorption and coincidence.

Only semiquantitative paperchromatographical estimations were made in cases when the specific activity was determined.

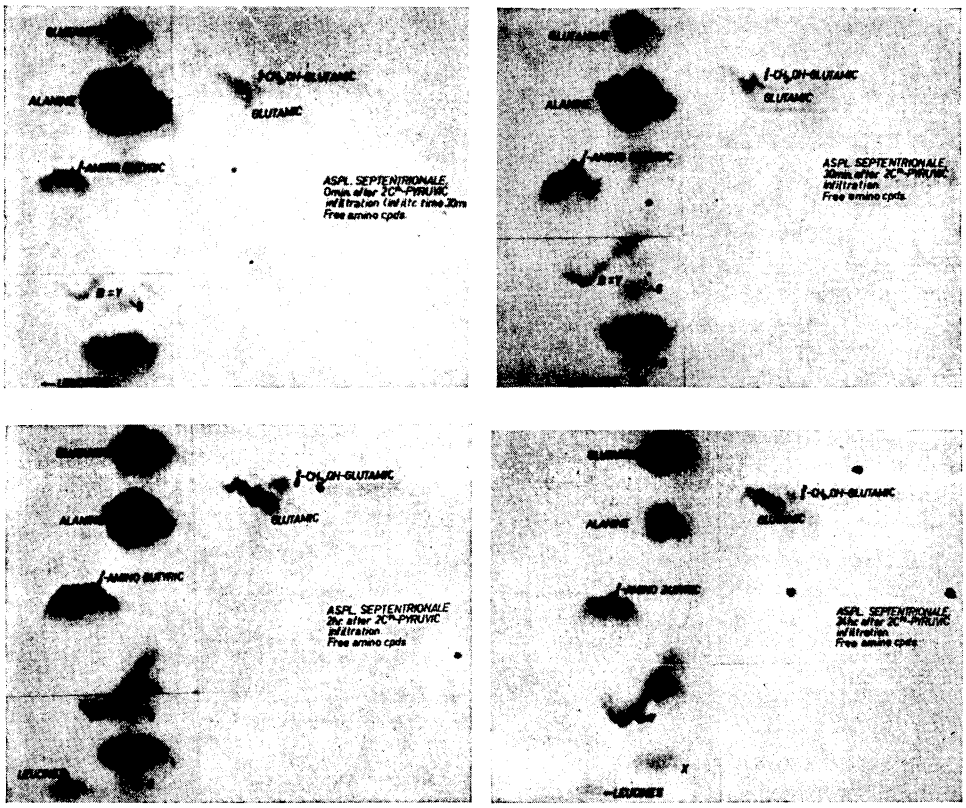

Fig. 4. Radioautograms of the amino compound fraction of *Asplenium septentrionale*. After 2-^{14}C-pyruvate infiltration.

RESULTS AND DISCUSSION

Radioactive pyruvate was readily metabolised in all experiments performed with *Asplenium septentrionale* (Figs. 2, 3 and 4). When excised shoots were allowed to take up active pyruvate solution the activity of the nutrient solution dropped to about 30 % in 2 h and to 4 % in 24 h (Table 1). A slightly higher percentage of the 70 % ethanol soluble radioactivity was incorporated into the amino compounds when pyruvate was infiltrated into the detached plants. The amino compounds contained relatively more activity after 2-^{14}C-pyruvate infiltration than when 1-^{14}C-pyruvate was used. Though pyruvate itself is found in the "peptide" fraction already after a few minutes amino compounds contained more than 50 % of the soluble activity. This amount dropped to about 10 % in 20 h when 1-^{14}C-pyruvate was given, but on 2-^{14}C-pyruvate infiltration after 24 h the amino compound fraction still contained 46 % of the activity (Table 2). The rapid labelling of this fraction was largely due to a very high transaminase activity, hence alanine being formed most

Table 1. Percentage of activity of 1-^{14}C-pyruvate in the nutrient solution when pyruvate was given to the excised shoots of *Asplenium septentrionale.*

Time, h	%
2	28.3
4	12.2
12	6.5
24	4.2

rapidly and with a rather high specific activity. When 1-^{14}C-pyruvate is used part of the active alanine may also be formed secondarily from the refixed carbon dioxide *via* 3-phosphoglyceric acid and the tricarboxylic acid cycle. The initially high activity of alanine then decreased rapidly.

Generally, 2-^{14}C-pyruvate was much more effectively utilized for the synthesis of labelled amino acids than was 1-^{14}C-pyruvate (Figs. 5 and 6). The latter is readily decarboxylated in the plant, the active CO_2 then being

Table 2. Percentage of activity in amino compounds from the total 70 % ethanol soluble in *Asplenium septentrionale.*

Time	1-^{14}C-pyruvate, no infiltration	1-^{14}C-pyruvate, infiltration	2-^{14}C-pyruvate, infiltration
h	%	%	%
½	42	55	75
1	36	45	67
2	26	—	65
3	—	37	—
20	—	11	—
24	—	—	46
70	—	6	—

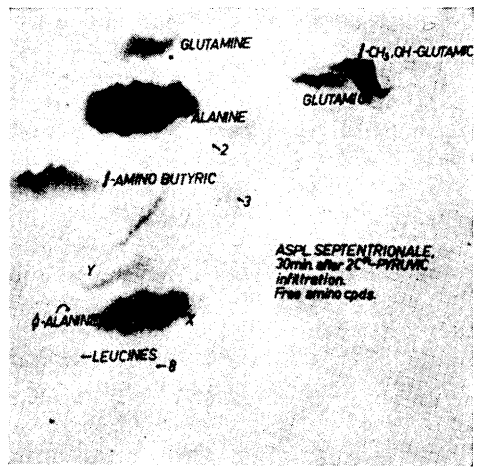

Fig. 5. Radioautogram of the amino compound fraction of *Asplenium septentrionale.* 30 min after 2-^{14}C-pyruvate infiltration.

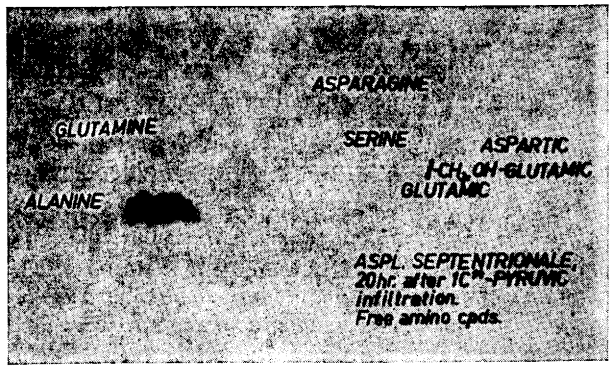

Fig. 6. Radioautogram of the amino compound fraction of *Asplenium septentrionale.* 20 h after 1-^{14}C-pyruvate infiltration.

reutilized to a great extent for carbohydrate synthesis etc. In this case most of the amino acids were rapidly found labelled with a low specific activity. This indicates that decarboxylation to acetyl-CoA is probably more important in the plant than is the carboxylation of pyruvate to oxalacetic acid. The relatively highest specific activity was found in the γ-MHGA. The results confirm those obtained by Miettinen [18] who showed that carboxyl labelled alanine was taken up with out breakdown by a shoot of the pea plant *(Pisum sativum)* detached from the root but was then immediately decarboxylated. On the contrary, no active CO_2 was primarily lost from 2-^{14}C-pyruvate.

An unknown amino compound X was also very rapidly labelled reaching its peak within one hour. Its activity then decreased very rapidly as did that of alanine. After 24 h almost no more activity could be found in this compound. The spot did not give a positive ninhydrin reaction, but this does not exclude the possibility that X is present in only such a low concentration that it cannot be detected by ninhydrin spray. In this case compound X would have had the highest specific activity.

It was of interest to notice that after 2-^{14}C-pyruvate infiltration no activity could be found in either aspartic acid or its amide. If aspartic acid is mainly formed by transamination from oxalacetic acid formed in turn either by the Wood-Werkman reaction or *via* the tricarboxylic acid cycle the resulting aspartate would be radioactive. Both aspartic acid and its amide were readily labelled when 1-^{14}C-pyruvate was given to the plant. Aspartic acid is known to be among the first radioactive compounds formed during photosynthetic $^{14}CO_2$ fixation by algae [17]. This agrees very well with the fact that carboxyl labelled pyruvate is rapidly decarboxylated in plants, carbon dioxide then being reutilized. Several observations have shown that the main biosynthetic route to aspartic acid in plants is not *via* transamination from oxalacetic acid [19,20]. Nelson and Krotkov [20] postulate a condensation for its formation. It now looks very probable that the two-carbon pieces possibly involved in this reaction are not directly derived from pyruvate.

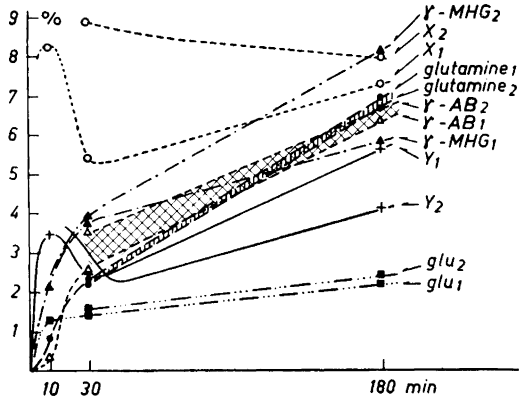

Fig. 7. The relative radioactivity changes of the individual amino compounds in *Asplenium septentrionale* after 2-^{14}C-pyruvate infiltration with (2) and without (1) inactive γ-AB addition.

In all experiments serine was labelled before glycine, which clearly indicates that it is not synthesized from the latter in *Asplenium septentrionale*. Similar results have been obtained by Tolbert and Gailey with wheat leaves [21]. In animal tissues [22,23] and in yeast [24] serine is found to be the precursor of glycine.

The radioactivity of glutamic acid rises rapidly to a certain level remaining then at a constant value. The same thing happens also with γ-aminobutyric acid (γ-AB). The specific activities of both of them are about the same. The activity of glutamine rises rapidly after a short lag period, being after about 15 h the major active compound. It is thought that the main route to γ-AB should be decarboxylation of glutamic acid [25,26]. Steward *et al.* [27,28] found only a slight activity of L-glutamic acid decarboxylase in potato tubers, which contain a high concentration of γ-AB. They suggested therefore that γ-AB was merely a precursor of glutamic acid and not its decarboxylation product. Miettinen and Virtanen [29] detected a considerable glutamic acid decarboxylase activity both in pea and alder but found no direct correlation between the concentration of γ-AB and the enzyme activity in plant tissues. According to Baptist [30] γ-AB may arise as a result of some reaction connected with protein synthesis. This is specially interesting because glutamine is thought to have some important role in protein synthesis [31,32]. Hence the role of γ-AB in relation to glutamine becomes an important question. Glutamate is known to be rapidly metabolised to glutamine and γ-ketoglutaric acid but not much to γ-AB in higher plants [33,34].

To try to trace the role of γ-AB, two experiments were performed with *Asplenium septentrionale*, in which 2-^{14}C-pyruvate only and 2-^{14}C-pyruvate with inactive γ-AB, respectively, were infiltrated to the plants (Fig. 7). The activity of the γ-AB itself was slightly increased in the latter case. This was probably due to the fact that while the rate of synthesis of active γ-AB remains the same, the γ-AB pool is diluted with the added inactive product, and hence less of the active molecules react further in a given time. This results in the enrichment of active γ-AB. The primary metabolic products from γ-AB should have slightly lowered the relative activity when inactive γ-AB is added. This

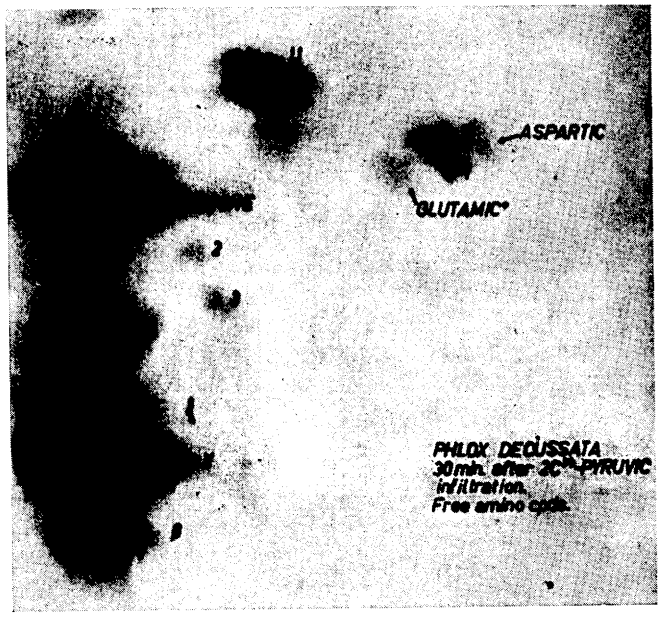

Fig. 8. Radioautogram of the amino compound fraction of *Phlox decussata*. 30 min after 2-^{14}C-pyruvate infiltration. The numbered spots are unknown. No. 1 is identified as γ-MHGA.

was found only in the case of glutamine and an unknown amino compound Y. The radioactivity of compound Y had a minimum after about 30 min, which could be explained under the assumption that two different metabolic pools of the precursor of Y exist, one of these two becoming much more slowly active. Because the radioactivity of glutamic acid was slightly increased in the latter experiment it may not be a primary carboxylation product of γ-AB. It seems much more probable that glutamic acid arises from γ-AB *via* glutamine. However, the increase of radioactivity of glutamic acid can at least be partly explained by the fact that the addition of inactive γ-AB increases the amount of nitrogen available for transamination reactions. The enzyme catalysing transamination between γ-AB and α-ketoglutaric acid is known to exist in pea [35].

γ-MHGA is rapidly labelled both with 1- and 2-^{14}C-pyruvates. In both cases the amount of radioactivity increases all the time resulting in a high specific activity as compared to other amino compounds. If the aldol condensation hypothesis is correct, pyruvic acid should be incorporated intact into the γ-derivative of glutamic acid. If this is the fact, 1-^{14}C-pyruvate would give a relatively high activity in γ-MHGA compared with the activities of glutamate and aspartate, since in this case the active carbon atom would enter directly into the molecule of γ-MHGA. Because the pyruvate molecules would undergo decarboxylation to give inactive acetyl-CoA, the only way labelled aspartic acid could be formed were the Wood-Werkman reaction of the liberated active

CO_2. From this labelled glutamic acid could then be formed. Therefore only a slight labelling of glutamic and aspartic acids would be expected. If the carbon chain of pyruvate would not enter intact into γ-MHGA, the radioactive compound could only arise from the reutilized carbon dioxide produced by decarboxylation. In such a case the Wood-Werkman reaction is probably the most important fixation mechanism, and the relative specific activities of aspartic and glutamic acids would be expected to be much higher than that of γ-MHGA. However, when 1-^{14}C-pyruvate was infiltrated to the plant γ-MHGA gained much greater specific activity than did the other amino acids. The observed difference was greater than what can be expected to arise from the possible dilution of the activities of the latter compounds during hydrolysis of inactive protein.

Active pyruvate, active pyruvate + inactive glycine, and active pyruvate + inactive glyoxylic acid, respectively, were infiltrated to the leaves of *Phlox decussata* to study whether γ-hydroxyglutamic acid would arise by a similar type of aldol condensation. In no experiments could any activity be incorporated into γ-HGA. Since the activity was rapidly incorporated into most of the other amino acids including a number of unknown amino compounds (Fig. 8) it seems very probable that γ-HGA is synthesized very slowly in the leaves, if at all. Even if this compound were to be synthesized *via* any intermediates of the tricarboxylic acid cycle in the leaves at least some radioactivity would be expected in it. However, synthesis from the protein amino acid hydroxyproline would result in an inactive product during short time experiments. It is also possible that the main site of the synthesis of γ-HGA is located elsewhere. This assumption is in good agreement with Fowden's [36] discovery that γ-methyleneglutamic acid and its amide arise during the early stages of seedling growth.

A spot with a very high specific activity was found on the place of γ-MHGA on paper chromatograms of the extracts of *Phlox decussata*. When isolated, it could indeed be shown to be identical with γ-MHGA isolated from *Asplenium*. This compound was not previously known to exist in *Phlox* and it could not even in this case be found by ninhydrin spray. The infiltration of inactive glycine did not have any significant effect on the labelling of this compound.

An interesting finding was that serine was rapidly labelled when inactive glycine was also infiltrated, but practically no formation of active serine was obtained with pyruvate infiltration alone.

All these experiments show that the most probable biosynthetic pathway of γ-MHGA in higher plants is *via* an aldol condensation from two pyruvate molecules. The final proof of this hypothesis would require a stepwise degradation of the γ-MHGA molecule and the determination of the radioactivities of each of the six carbon atoms, using pyruvate labelled in all three different positions, respectively. However, the total activity obtained in this compound was not great enough for such an experiment. The experiments with *Phlox decussata* showed in addition that γ-MHGA may be a normal metabolite in higher plants, but usually present in such a low concentration that the detection by the usual methods is not possible. This may be due to most of the newly discovered amino acids.

That no radioactivity could be incorporated into γ-HGA did not rule out the possibility of the existence of an aldol condensation with pyruvate and glyoxylate, but merely showed that this compound is not an active metabolite in the leaves of *Phlox decussata*. Generally, no significant differences in the metabolic pattern could be found in these plants containing "new" amino acids as compared to the other plants examined.

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