which was the estimated yield of citrate from pyruvate in some large-scale experiments on bovine brain carried out by Simola & Alapeuso (1943).

As shown by the results in Table 5, a classification of tissue preparations into slices, breis and dispersions is somewhat arbitrary and in certain respects misleading. This should not, however, obscure the fact that the metabolism of the more organized preparations is better systematized to the extent that intermediate reactants accumulate to a lesser degree in them, although the appearance of such intermediates at all in recognizable form is in a sense a measure of the artificiality of the experimental conditions.

SUMMARY

1. The accumulation of citrate in association with the oxidation of pyruvate by breis and slices of pigeon brain is reported.

2. The extent of citrate formation in such preparations is compared with that previously found in dispersions, and the implications of this comparison are discussed.

3. Some reactions between *cis*-aconitate and *D*-isocitrate and citrate in dispersions of pigeon brain have been studied.

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A Third Unsaturated Amino Acid in Groundnut Plants: Evidence for the Occurrence of γ -Amino- α -methylenebutyric Acid

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In recent years several new amino acids have been isolated from plant materials, whilst the presence of many other compounds of similar type, as yet lacking definite characterization, has been indicated by the methods of paper chromatography. Two amino acids isolated from groundnut plants (*Arachis hypogaea*) and provisionally identified as γ -methyleneglutamine and γ -methyleneglutamic acid, have proved unusual in that they are unsaturated compounds (Done & Fowden, 1952).

These compounds are subsequently referred to as I and II respectively. No general survey of their distribution within the plant kingdom was then undertaken, but evidence for their presence in the leaves of tulips and certain other members of the Liliaceae has been obtained more recently. Prof. F. C. Steward of Cornell University has informed us that he and his co-workers are investigating two compounds in tulips which appear to be identical with those found in groundnut plants. It would

seem that the two compounds correspond to the unknown spots numbered 13 (the amide) and 4 (the acidic amino acid) on the diagram prepared by Steward & Thompson (1950).

The presence of much smaller amounts of a third new amino acid in groundnut plants was also indicated in the earlier work, and this substance was then described as compound IV. The original designation is retained in the present paper in which evidence is presented that IV is probably γ -amino- α -methylenebutyric acid (4-amino-2-methylenebutanoic acid), the product of α -decarboxylation of II. A brief report of this work has already been published (Fowden & Done, 1953).

By chromatographic techniques a small quantity of IV was isolated in pure form from some of the fractions obtained during the isolation of II (Done & Fowden, 1952). IV was a white powder, very soluble in cold water, and had elementary composition corresponding to the empirical formula $C_6H_9O_2N$. The material did not form a complex with copper, an indication that it was probably not an α -amino acid. Insufficient material was available for optical-rotation studies.

The behaviour of IV on paper chromatograms, especially the colour of the spots obtained with ninhydrin, indicated that it was probably an unsaturated amino acid. This was confirmed by hydrogenation using a palladium catalyst. Assuming that the empirical formula above also represents the molecular formula of IV, the amino acid absorbed 0.97 molecular proportions of hydrogen, indicating the presence of a single double bond within the molecule.

Ozonolysis of aqueous solutions of IV gave formic acid and β -alanine as the final oxidation products. These two substances are the end products that would be formed by ozonolysis of γ -amino- α -methylenebutyric acid as in the scheme below:

IV could therefore not be 2-aminopent-4-enoic acid, an isomer of γ -amino- α -methylenebutyric acid (4-amino-2-methylenebutanoic acid), which would be formed by γ -decarboxylation of II, for it would then have yielded aspartic acid on ozonolysis, a product readily distinguished from β -alanine on paper chromatograms.

The position occupied by IV on two-dimensional

paper chromatograms was extremely close to a position that could be predicted for γ -amino- α -methylenebutyric acid; the R_f values determined for IV and other amino acids in two different solvent systems indicated that IV differed structurally from II by the same features as γ -aminobutyric acid from glutamic acid and β -alanine from aspartic acid.

Further support for the proposed structure was obtained from experiments in which extracts of certain plant materials capable of α -decarboxylating glutamic acid were used to decarboxylate II *in vitro*. The course of the decarboxylations was followed manometrically; when the reactions were terminated the amounts of II that had disappeared and of a non- α -amino acid that was produced were estimated. The determinations proved that the non- α -amino acid was produced in the molecular proportions required by a reaction in which one molecule of carbon dioxide was lost by one molecule of II, and was therefore almost certainly γ -amino- α -methylenebutyric acid. This decarboxylation product of II could not be separated from IV when mixtures of the two substances were run on paper chromatograms developed in four different solvent systems. It was, however, easily separated from 2-aminopent-4-enoic acid when a butanol-acetic acid mixture was used as the developing solvent.

EXPERIMENTAL

Isolation of IV

Compound IV was isolated from certain fractions obtained during the isolation of compound II from groundnut plant extracts. These fractions contained most of the IV originally present in the vegetative parts of approximately 2000 3-week-old groundnut plants. The fractions were pooled, and after as much II as possible had been removed by crystallization (its solubility is very low in ice-cold water), the solution contained in addition to IV and II appreciable amounts of aspartic acid, glutamic acid, serine, tyrosine and phenylalanine. The last two amino acids were removed by adsorption on charcoal as described by Partridge (1949). The filtrate was evaporated to small bulk below 40° and finally to dryness over H_2SO_4 *in vacuo*. Of this residue (slightly more than 0.5 g.), 10–20% appeared to be IV as judged by qualitative paper chromatography.

IV was further purified using a cellulose-powder column as described by Synge (1951). The developing solvent used was the upper layer obtained by equilibrating 4 vol. of *n*-butanol, 1 vol. of glacial acetic acid and 5 vol. of water. The amino acid mixture was dissolved in the minimum volume of the developing solvent and applied to a column (2.5 cm. diam.) prepared from 100 g. of Whatman cellulose powder. As development proceeded the eluate was collected in 20 ml. fractions. Compound IV was the first amino acid to appear in the eluate, although traces of the other amino acids were also eluted in the early fractions. The fractions containing substantial amounts of IV were pooled and evaporated as

before to about 10 ml. The solution was then decolorized with 0.1 g. of charcoal, and the filtrate evaporated to dryness *in vacuo*. The residue, a hard, white, crystalline mass (about 100 mg.), consisted of about 80–90% of IV, with smaller amounts of aspartic acid, glutamic acid and serine. This material was used as a source of IV in many of the later comparison tests.

Several months after the separation of IV on cellulose columns had been attempted, we were informed (private communication from Dr J. F. Thompson, Ithaca) of a new method of separating non- α -amino acids in plant materials. We have used this method in the following manner to isolate a small quantity of IV uncontaminated by other amino acids.

5 g. of Merck alumina (standardized according to Brockmann) and 1.7 g. of CuCO_3 were ground in a mortar to a uniform powder, and then poured as a slurry in distilled water into a small column. The column was washed with further small amounts of water until the packing appeared firm. Suction was used to obtain a satisfactory percolation rate (3–4 ml./hr.). 50 mg. of the sample prepared above was dissolved in 12 ml. of water and the pH adjusted to between 6 and 7. This solution was applied to the column at the same rate, followed again by distilled water, and the eluate was collected (fifteen 2 ml. fractions). 10 μ l. of each fraction were examined chromatographically, and it was found that fractions 4–10 inclusive contained only IV. In the later fractions trace amounts of IV were present, but no aspartic acid, glutamic acid or serine, the original contaminating amino acids, were present in any of the fractions. These α -amino acids were absorbed quite strongly on to the packing mixture, their position being indicated by the presence of a narrow, faintly blue band due to copper-salt formation near the top of the column.

Fractions 4–10 inclusive were combined and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum amount of water and crystallized by the addition of ethanol. In this way about 30 mg. of IV were obtained. (Found: C, 52.6; H, 7.9; N, 11.9. $\text{C}_5\text{H}_9\text{O}_2\text{N}$ requires C, 52.2; H, 7.8; N, 12.2%.) IV did not melt sharply but began to darken in colour and partially liquefy at about 152°.

Chromatographic techniques

All paper chromatograms were prepared on Whatman no. 4 filter-paper sheets by methods essentially those of

Consden, Gordon & Martin (1944). The solvents used were mainly redistilled phenol saturated with 0.3% (w/v) aqueous NH_3 (referred to as phenol- NH_3 hereafter) and the *n*-butanol-acetic acid mixture of Partridge (1948). These two solvent systems were always used in the order stated for the development of two-dimensional chromatograms. The collidine-lutidine mixture of Dent (1947) and *tert*-amyl alcohol in the presence of diethylamine (Work, 1949) were also used occasionally for the preparation of one-dimensional chromatograms. 0.1% (w/v) ninhydrin in water-saturated *n*-butanol was used as the spraying agent.

The position of IV on chromatograms

IV moved as a well-defined spot in all solvents, and its position on two-dimensional chromatograms was such that it would not normally be completely resolved from valine. The R_F values determined when a mixture of IV and a number of other amino acids was run on a two-dimensional chromatogram are given in Table 1. On theoretical grounds (following Martin, 1949; Bate-Smith & Westall, 1950; Isherwood & Jermyn, 1951; Pardee, 1951) it can be predicted (and has been verified in practice with a variety of compounds) that for a particular solvent system, a similar structural change brought about in any of the three dicarboxylic amino acids listed in Table 1 would result in products such that the change in the function $\log_{10}(1/R_F - 1)$, calculated for the original and final molecules, would be identical. Thus when any of these amino acids are converted to their ω -amide forms, $\log_{10}(1/R_F - 1)$ is altered by about 0.60 for phenol- NH_3 and 0.26 for butanol-acetic acid. The differences in $\log_{10}(1/R_F - 1)$ found for II and IV for each of the solvent systems agreed with the differences found between the pairs glutamic acid- γ -aminobutyric acid and aspartic acid- β -alanine. This evidence then supports the idea that IV differs structurally from II by the loss of the α -carboxyl group.

Colour given by IV with ninhydrin

The colour of the spot produced from IV on chromatograms after ninhydrin treatment was at first yellowish brown, changing with further heating to a deep orange-brown. Compounds I and II react under these conditions in a similar manner, and the three compounds may easily be recognized on chromatograms by reason of this unusual

Table 1. R_F values of certain amino acids, and differences between the values of $\log_{10}(1/R_F - 1)$ for various pairs of amino acids

Amino acid	Phenol- NH_3				<i>n</i> -Butanol-acetic acid			
	R_F	$\log_{10}\left(\frac{1}{R_F} - 1\right)$	Differences in $\log_{10}\left(\frac{1}{R_F} - 1\right)$ (bracketed pairs)		R_F	$\log_{10}\left(\frac{1}{R_F} - 1\right)$	Differences in $\log_{10}\left(\frac{1}{R_F} - 1\right)$ (bracketed pairs)	
Asparagine	0.44	0.11	0.61	0.075	1.90	0.26	0.26	0.26
Aspartic acid	0.16	0.72			0.13			
β -Alanine	0.62	0.21	0.93	0.28	0.41	0.42	0.42	0.42
Glutamine	0.60	0.17	0.58	0.10	0.95	0.26	0.26	0.26
Glutamic acid	0.28	0.41		0.17	0.69			
γ -Aminobutyric acid	0.77	0.52	0.93	0.36	0.25	0.44	0.44	0.44
Compound I	0.67	0.32	0.60	0.115	0.86	0.26	0.26	0.26
Compound II	0.34	0.28		0.20	0.60			
Compound IV	0.83	0.68	0.96	0.42	0.14	0.46	0.46	0.46

(For details see text.)

colour. In the cases of I and II, the abnormal colour has been shown to be associated in some way with the unsaturated nature of the compounds, since they could be reduced to compounds giving normal blue-purple spots on ninhydrin-treated chromatograms (Done & Fowden, 1952).

Similar behaviour was observed for IV. Reduction of small amounts of IV by nascent hydrogen liberated at the mercury cathode of an electrolytic desalting apparatus (Consden, Gordon & Martin, 1947) gave rise to a new compound which moved to a very similar position on a two-dimensional chromatogram to that occupied originally by IV, but the colour of the spot was now blue-purple.

Four other unsaturated amino acids (synthesized by Goering, Cristol & Dittmer (1948); samples kindly supplied by Dr K. Dittmer, Boulder) showed similar behaviour with ninhydrin. 2-Aminopent-4-enoic acid, 2-aminohept-4-enoic acid and 2-aminopent-4-ynoic acid all gave a similar yellowish brown colour on chromatograms, whilst 2-amino-4-methylpent-4-enoic acid gave a yellowish green colour. Each of these compounds can be reduced by hydrogen to the corresponding saturated amino acids which react normally with ninhydrin on chromatograms.

Though unsaturation in these amino acids leads to abnormal ninhydrin colours on paper chromatograms, all the compounds, both natural and synthetic, react normally in solution with excess ninhydrin dissolved in water or *n*-butanol, producing blue-purple coloured solutions. The behaviour of these compounds may be compared with that of asparagine under similar conditions (Steward & Thompson, 1952).

Hydrogenation of IV

The unsaturated nature of IV was confirmed by catalytic hydrogenation. The uptake of H_2 by IV in the presence of a palladium-on-carbon catalyst was followed in the Warburg manometric apparatus. The catalyst was prepared by reducing a solution of 0.1 g. of $PdCl_2$ in 50 ml. 2*N*-HCl with H_2 in the presence of 0.5 g. of absorbent charcoal (British Drug Houses Ltd.) which had previously been heated to 120° for 1 hr. A suspension of the catalyst in distilled water (approx. 5 mg./ml.) was saturated with H_2 and 2 ml. portions were introduced into the main compartments of three Warburg flasks. 0.5 ml. of distilled water was placed in the side bulb of one flask to serve as a control and 0.5 ml. of a solution of pure IV into the side bulbs of the other two flasks. The flasks and manometers were then flushed with H_2 and, after equilibrating at 25° for 15 min., the flasks were tipped and the H_2 uptake measured manometrically, readings being continued until the amount of H_2 absorbed by IV had remained constant over a period of 1 hr.

1.59 mg. of pure IV absorbed 300 μ l. (mean value) of H_2 measured at s.t.p. The uptake calc. for $C_5H_9O_2N$ containing one $C=C$ bond is 310 μ l.

Attempts to form copper complex of IV

IV was run on chromatograms which had been dusted with copper carbonate (Crumpler & Dent, 1949) before solvent development. On later ninhydrin treatment IV reacted normally to give a coloured spot, whereas when II was treated similarly it could not be detected on the chromatograms. This indicated that IV did not form a copper complex and was probably a non- α -amino acid.

The inability of IV to form a copper complex was also demonstrated using copper acetylacetonate as suggested by

Jones (1949). A one-dimensional chromatogram containing spots of II and IV which had been dried after development in phenol: NH_3 was dipped in a 5% (w/v) solution of copper acetylacetonate in A.R. chloroform. The excess copper was washed from the paper by passing it through several changes of chloroform, and then it was lightly sprayed with a 0.1% (w/v) solution of sodium diethyldithiocarbamate in water. A yellow-brown spot was produced in the position normally occupied by II indicating that it had formed a copper complex, but there was no indication of a spot in the position expected for IV. These findings for paper chromatograms were in agreement with the earlier evidence obtained using alumina-copper carbonate columns (above).

Ozonolysis of IV

An aqueous solution of a few mg. of IV was treated with ozone for 5–10 min. The ozonized air used contained about 5% (v/v) ozone and was passed at the rate of 30–40 ml./min. The solution was neutralized with dilute NaOH solution and then N_2 gas was immediately bubbled rapidly through the solution for 15 min. before concentration *in vacuo* above H_2SO_4 . This was found to diminish the apparent charring that otherwise took place when the passage of ozone was stopped. Small portions of the concentrate were examined chromatographically for the presence of amino acids while other portions were tested for the presence of organic acids by the chromatographic procedure of Brown & Hall (1950).

The amino acid product of ozonolysis gave a blue ninhydrin-reacting spot on chromatograms, which was inseparable from the spot given by β -alanine when mixtures of the two compounds were run on one-dimensional chromatograms developed in each of the four solvent systems listed earlier.

The concentrate contained small amounts of an anionic material which was inseparable from formate when mixtures of the two compounds were run on chromatograms developed with *n*-butanol saturated with aqueous 1.5*N*- NH_3 . As with formate, the spot could be located as a dark-brown area using ammoniacal $AgNO_3$ solutions as spraying agent, as well as by the usual indicator-spray techniques.

Decarboxylation of II

Since IV may possibly be formed by decarboxylation of II within the groundnut tissues, attempts were made to prepare extracts of plant materials containing enzyme systems capable of removing CO_2 from II *in vitro* in order to compare the product of such a reaction with IV.

Groundnut-plant tissue extracts. Leaves and roots of 3-week-old groundnut plants were separately harvested and extracts made by one of the following techniques:

(a) The plant material was minced in a domestic meat chopper and then squeezed through muslin and the filtrate centrifuged at 1000 *g* for 5 min. The supernatant was buffered by adding one-fifth of its volume of 0.4*M*-potassium phosphate buffer (pH 5.6) before use.

(b) The plant material was roughly chopped and then ground to a smooth paste in a mortar with a minimum volume of ice-cold 0.2*M*-potassium phosphate buffer (pH 5.6) and a small amount of acid-washed sand. The paste was squeezed through muslin and the filtrate centrifuged as above, the supernatant being used in the later experiments.

(c) Extracts were as in (b) but using distilled water instead of buffer. The final supernatants were then freeze-dried, and

the residues taken up in a small amount of the 0.2M-phosphate buffer at pH 5.6 just before use.

The pH of 5.6 was chosen for the tests since this appeared from a survey of the literature to be about the mean pH determined for glutamic decarboxylase activity in many higher-plant tissues (Schales & Schaless, 1946*a, b*; Schaless, Mims & Schaless, 1946; Okunuki, 1937; Hasse & Schumacher, 1950; Beevers, 1951). Reaction mixtures were made up by taking 1 ml. of each extract and adding 0.5 ml. of a solution of II containing 4 mg./ml. titrated to pH 5.6 with dilute NaOH solution. The mixtures were incubated at 25° for various periods up to 24 hr. The reactions were stopped by adding 2 vol. of ethanol to each tube and heating. The precipitated proteins were removed by centrifugation, and 100 μ l. of each supernatant applied to a chromatogram later developed with butanol-acetic acid.

When the extracts of both the leaves and the roots of groundnut plants prepared by methods (a) and (b) were used, a small yellowish-brown spot not initially visible on chromatograms of the extracts appeared with the reaction mixtures after the longer incubation periods. In position and colour this new spot exactly reproduced the behaviour of IV, and was inseparable from IV in all four chromatographic solvents listed above. No new spot was seen when the reaction mixtures were immediately treated with ethanol and when the extracts alone were incubated since the amounts of II contained in them were small compared with the amount of II normally added. Similarly no new spot was produced when boiled extracts were employed. The new spot thus appeared to be produced from II and was probably identical with IV. Not more than 10% of the II added appeared to have been converted to the new compound, and manometric measurement of any CO₂ simultaneously produced would therefore have been impossible in such a slow reaction. The low decarboxylase activity of these extracts could explain the very low concentrations of IV compared with those of I and II normally found in the intact groundnut plants.

Other plant extracts. Extracts of other plant materials were prepared as in (b) above and the decarboxylase activities of the extracts were qualitatively assayed by chromatography as before, the size and intensity of the new amino acid spot produced being used as a guide to the amount produced during each incubation period. Such preliminary experiments indicated that extracts of barley roots and the fruits of a West African red pepper (*Capsicum* sp.) contained higher II decarboxylase activities than that found in any of the groundnut extracts. Using these extracts, II was decarboxylated at a rate sufficient to allow the CO₂ produced to be measured manometrically.

Manometric measurement of the carbon dioxide produced during decarboxylation of II. (1) Experiments with *Capsicum* extracts: three small fruits were chopped and extracted as in (b) above. Samples (2 ml.) of final extract were placed in the main compartments of four Warburg flasks. 0.5 ml. of a solution of II adjusted to pH 5.6 was placed in two of the side bulbs, whilst distilled water (0.5 ml.) was added to the side bulbs of the other two flasks to serve as controls. 0.2 ml. of 15% (w/v) KOH solution was added to the centre well of one control and one experimental flask. The flasks and manometers were flushed with O₂-free N₂ before transference to the thermostat bath (25°). After 15 min. the contents of the side bulbs were tipped, and manometric readings were taken every 15 min. until the reactions were stopped, immediately after the reading at 225 min. 1 ml. of

the reaction mixture was immediately withdrawn from each flask, pipetted into 2 ml. of ethanol and heated. The amounts of II remaining and of the new amino acid formed were determined in portions of the supernatants obtained after the removal of the precipitated proteins by the quantitative chromatographic procedure of Fowden (1951). In assaying the product amino acid, the assumption was made that this compound gave the same molar colour sensitivity with the ninhydrin reagent as that given by II. As stated earlier, the colour produced by these unsaturated compounds in the presence of excess ninhydrin was bluish purple and colorimetric readings were made at the usual wavelength of 570 m μ . The determinations indicated that the amount of the new compound produced was equivalent to 60.7% of the amount of II initially added to each flask, and that 36.2% of the II remained unchanged when the reaction was terminated. At this time 63.0% of the CO₂ that would have been produced by complete decarboxylation of II had been liberated.

(2) Experiments with barley-root extracts: roots taken from 15-day-old barley plants (Pioneer) grown in acid-washed sand were extracted by method (b). 2 ml. of the supernatant extract were introduced into the main compartments of the Warburg flasks, the other manometric arrangements being the same as those used in the experiments with the *Capsicum* extracts. The reactions were terminated immediately after the reading taken at 165 min., when the new amino acid produced accounted for 60.5% of the II initially present, whilst 40.8% of the II remained unchanged. The CO₂ evolved was equivalent to a decarboxylation of 65.0% of the II used.

Both IV and the new compound produced from II could be separated from 2-aminopent-4-enoic acid on one-dimensional chromatograms run in butanol-acetic acid mixtures. Since 2-aminopent-4-enoic acid would result from γ -decarboxylation of II, it was concluded that the plant extracts were catalysing the loss of CO₂ from the α -COOH group of II.

DISCUSSION

The evidence presented makes it highly probable that IV is γ -amino- α -methylenebutyric acid, COOH.C(:CH₂).CH₂.CH₂(NH₂), the product of α -decarboxylation of γ -methyleneglutamic acid. The course of enzymic decarboxylation of II is similar to that of glutamic acid (see Beevers, 1951). Decarboxylase activity for II appeared to be low in groundnut tissues and we also observed that glutamic decarboxylase activity was low. The products of decarboxylation, IV and γ -aminobutyric acid, were both present in groundnut plants but only in very low concentrations. The fact that over 50% of the II used could be enzymically decarboxylated by plant extracts probably indicated that the natural material isolated from groundnut plants and used here had the L configuration.

As noted earlier, IV, like I and II and certain other synthetic unsaturated amino acids tested, reacted with ninhydrin to give spots of an abnormal yellowish-brown colour on chromatograms. There have been numerous reports in the literature of the

presence in plant extracts of similarly coloured spots of an unknown nature. It is perhaps premature to assert that some of these may finally be shown to be unsaturated amino acids, but an indication of unsaturation may rapidly be obtained by the following simple method. The plant extract containing substances yielding an unknown spot or spots is desalted in the normal manner for 15–20 min.; a small amount of dilute hydrochloric acid may be added if the salt content is initially very low. A new chromatogram is then developed under similar conditions to the original one showing the one or more unknowns. If the unknown yellowish-brown spot or spots represented unsaturated amino acids, a new spot or spots, probably reacting normally with ninhydrin, will be seen on the chromatogram in positions very similar to those occupied by the original compounds. Since yellow spots on chromatograms may also be due simply to the presence of salts (Consden & Gordon, 1948), it is essential that a new spot or spots should result after electrolytic reduction as above before unsaturation can be considered as a probability.

In conclusion, we would state that although IV has been shown to be present in hot 70% (v/v) ethanol extracts of freshly harvested material, it is

possible that some of the IV present in the groundnut extracts from which it was isolated may have resulted by post-mortem change from the I and II also present, since enzyme activity was not completely destroyed for some hours after the groundnut plant tissue had been macerated.

SUMMARY

1. A third unsaturated amino acid has been isolated in small quantity from groundnut plants (*Arachis hypogaea*). Elementary analysis indicated the empirical formula $C_6H_9O_2N$.

2. Chromatographic and other evidence indicated that the new compound is probably identical with the product of enzymic decarboxylation of γ -methyleneglutamic acid, γ -amino- α -methylenebutyric acid (4-amino-2-methylenebutanoic acid).

3. Extracts of the fruits of a *Capsicum* species (red pepper) and of barley roots possess high decarboxylase activity for the α -carboxyl group of γ -methyleneglutamic acid.

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