Dihydroxyglutamic Acid in Plants

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In an earlier paper [1] we reported the discovery of dihydroxyglutamic acid in the seeds of *Lepidium sativum* and in the green parts of *Rheum rhaponticum*.

On the two-dimensional paper chromatogram (solvents: butanol-acetic acid-water and phenol-water in NH_3-atmosphere) of 70 % ethanol extracts of the seeds of *Lepidium*, and of many plants later on, a spot could be detected after ninhydrin spraying which moved much slower than aspartic, γ- and β-hydroxyglutamic acids [2] in both solvents (Fig. 1). In butanol-acetic acid it moved somewhat slower than hydroxyaspartic acid. The colour of the spot was at first weak but turned stronger and violet later on. The substance was unaffected by mild (1 N HCl, 20 h, 108° C) as well as strong (6 N HCl, 20 h, 108° C) acid hydrolysis. The colour became even more intense when the ethanol extract was hydrolyzed before paper chromatography. When separating basic and neutral amino acids in an Amberlite IR-4B column the new amino acid remained in the fraction with acidic amino acids. In paper electrophoresis 2 000 V, 18 mA Na-acetate buffer, pH 3.2) it proved to be more strongly acid than any other known natural aminodicarboxylic acid.

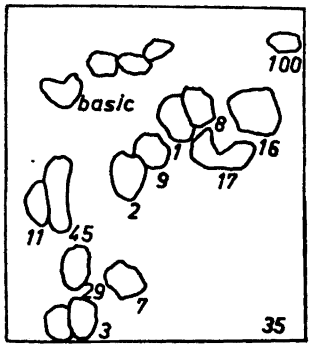


Fig. 1. Two-dimensional paper chromatogram of a 70 % ethanol extract (hydrolyzed with 6 N HCl) of the seeds of *Lepidium sativum*. Solvents: phenol-NH_3 and butanol-acetic acid. 100 = the new acidic amino acid (dihydroxyglutamic acid), 1 = gly, 2 = ala, 3 = val, 7 = tyr, 8 = ser, 9 = threo, 11 = pro, 16 = asp, 17 = glu, 29 = γ-aminobutyric acid, 45 = ethanolamine.

Isolation of the new amino acid from Rheum and the seeds of Lepidium. The amino acids in a 70 % ethanol extract were separated in an Amberlite IR-120 column. The eluate (1 N ammonia) was evaporated *in vacuo* and the extract hydrolyzed with 6 N HCl in order to split eventual peptides. Since the new amino acid moved slower in phenol than all other known natural amino acids, it was separated in a cellulose powder column using phenol as eluant. Before the run the cellulose powder column was washed with 1 % acetic acid and afterwards thoroughly with hot water. The fractions containing only the new amino acid were evaporated to dryness.

The structure of the new amino acid. The light coloured, slightly yellowish preparation obtained was on the basis of a two-dimensional paper chromatogram free from other amino acids. It did not contain sulphur or phosphorus. An aminodicarboxylic acid was thus obviously in question. An OH group in α-position to the NH_2 group was established with periodic acid and Nessler's reagent. On reduction with 66 % HI (d 1.96) and red phosphorus (4 mg substance + 18 μl HI + 3 mg P) at 140° C for 4 h mostly glutamic acid was found to be formed (Fig. 2). Small amounts of serine and glycine, and traces of threonine and alanine could also be found on the paper chromatogram. In some experiments a very faint spot was observed also in the position of β-hydroxyglutamic acid, but the substance could not be identified.

Using both phenol and butanol-acetic acid as solvents, the position of the new amino acid on the paper chromatogram (R_F values 0.05 and 0.04, respectively) showed that it was not β- or γ-hydroxyglutamic acid. Dihydroxyglutamic acid seemed thus probable. The amino acid contained 7.88 % N, calc. for dihydroxyglutamic acid $C_5H_9O_6N$, 7.85 % N.

Upon heating the amino acid at pH 3.3 at 125° C for 4 h the amino acid was transformed into the corresponding pyrroli-donecarboxylic acid to about 90 %. This compound was established by paper chromatography, treating the paper first with chlorine and then with a solution of 1 %

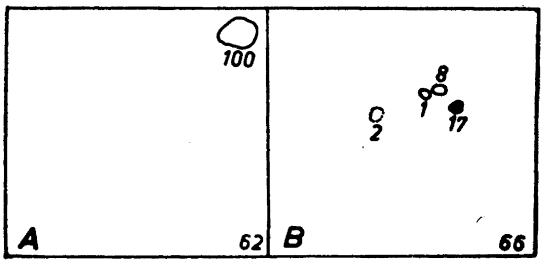


Fig. 2 A. Two-dimensional paper chromatogram of pure dihydroxyglutamic acid isolated from the seeds of *Lepidium sativum*. B. After reduction with HI and red P.

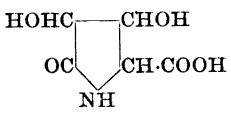
starch-KI, upon which the spot changed into bluish black [3]. The substance was isolated in a cellulose powder column using phenol-water as eluant. The fractions giving this colour reaction were evaporated to dryness *in vacuo* and extracted with ethyl-acetate, in which the pyrrolidonecarboxylic acid was difficult to dissolve. The substance was white, became darker upon heating at 235° C and decomposed at about 250° C. It contained 8.61 % N, calc. for $C_5H_7O_5N$, 8.70 % N.

The splitting of the carboxyl group as CO_2 by the effect of ninhydrin was determined according to Linko [4]. 0.269 mg of CO_2, or 26.4 %, were formed from 1.018 mg of the substance. Calc. for dihydroxypyrrolidonecarboxylic acid 27.3 %. Only little ammonia was formed, 27 % NH_3-N of total N. The determination, made for comparison, with pyrrolidonecarboxylic acid prepared from glutamic acid gave similar results: CO_2 formation 30.4 %, calc. 34.1 %, NH_3-N formation 22.1 % of total N.

The R_F value of the isolated dihydroxypyrrolidonecarboxylic acid was 0.33 in phenol-water. On hydrolysis with 6 N HCl the original aminodicarboxylic acid was re-formed.

The new amino acid had thus been established as dihydroxyglutamic acid,

The chromatographical and chemical properties of the new amino acid are compatible with the formula HOOC · CHOH · CHOH · $CHNH_2$ · COOH. The corresponding dihydroxypyrrolidonecarboxylic acid



seems not to be an artificial product only but also a very probable compound in fresh plants, because in the plant extract the amount of dihydroxyglutamic acid rises after strong acid hydrolysis. The increase is strongest in *Rheum* (pH of the sap 3.5—3.8) but can be noticed also in other plants (pH of the sap about 6.5).

The N-content of dihydroxyglutamic acid in the seeds of *Lepidium* is about 0.2 % of soluble N. On the whole the content of this amino acid is small in all the plants investigated. There is no experimental information yet about its biosynthesis. Hypothetically it can be assumed to be formed *via* transamination from the corresponding keto acid. The keto acid may be formed as an oxidation product of a ketopentose or also of γ-hydroxyglutamic acid.

Dihydroxyglutamic acid is comparatively common as a free amino acid in plants, and is to be found in all parts of the plants. By paper chromatography we have found it in the following plants: (The part of the plant in which the amino acid has been found is mentioned in parenthesis. Other parts have not been investigated. When both fruits and green parts have been investigated the amino acid has been found in both.)

Flowering plants. *Aesculus hippocastanum* (leaves and fruits), *Alchemilla vulgaris* (leaves and stem), *Angelica archangelica* (leaves), *Arctostaphylos uva-ursi* (leaves, stem and berries), *Brassica napa* (seeds), *Cornus alba* (berries), *Daucus carota* (stem and root), *Digitalis lanata* (leaves), *Lactuca sativa* (seeds), *Lepidium sativum* (seeds and sprouts), *Lilium bulbiferum* (leaves and stem), *Parthenocissus vitacea* (leaves), *Polygonum amphibium* (leaves and stem), *Polygonum aviculare* (leaves and stem), *Rheum rhaponticum* (leaves and stem), *Ribes alpinum*

(leaves), *Ribes grossularia* (leaves), *Ribes nigrum* (leaves and berries), *Ribes rubrum* (berries), *Spinacia oleracea* (leaves).

Ferns. *Struthiopteris filicastrum* (leaves).

Mosses. *Polytrichum commune.*

Mushrooms. *Amanita muscaria, Amanita virosa, Boletus luteus, Boletus versipellis.*

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*iso*Thiocyanates XXIV*. (+)-, (—) and (±)-α-Methylbenzyl *iso*Thiocyanate

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In connexion with other work in this laboratory pure specimens of the enantiomeric α-methylbenzyl *iso*thiocyanates became desirable. The racemic mustard oil has been described by Dyson and George [1] as a slightly yellow liquid, b.p. 240—244°, without presentation, however, of additional physical constants or analytical data. Recently, Luskin *et al.*[2] reported the preparation of (±)-α-methylbenzyl *iso*thiocyanate from α-methylstyrene and *iso*thiocyanic acid, as well as from α-methylbenzylamine by the customary decomposition of its dithiocarbamate.

In the present work the method of Schmidt *et al.*[3], involving hypochlorite oxidation of dithiocarbamates, was utilized for the synthesis of the racemic mustard oil, further characterized as its benzylthiourea-derivative. The pure, optically active α-methylbenzyl *iso*thiocyanates, (I) and (II), were produced on reaction of the corresponding amines with thiocarbonyl chloride by a slightly modified variation of the described procedure [1]. Again, the benzylthiourea-derivatives were synthesized for the purpose of characterization. Attempts to prepare the simple thioureas by reaction of the mustard oils with ammonia did not result in crystalline products.

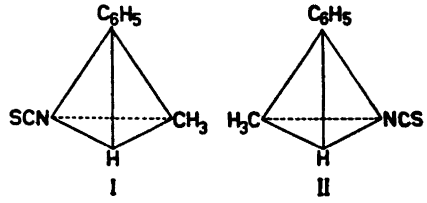

Fig. 1.

The symbols (I) and (II) are considered to represent the absolute configurations of (+)- and (—)-α-methylbenzyl *iso*thiocyanate, respectively. The former derives from (—)-α-methylbenzylamine, the transformation of which into (+)-alanine has been effected previously [4]. The configurational relationship of this amino acid with (—)-glyceraldehyde has been established by Wolfrom *et al.*[5] A unique three-dimensional representation of such molecules is required in consequence of the experimentally established absolute configuration of appropriate optically active molecules [6]. Adopting the recently proposed system for specification of absolute configuration [7] the sequence of transformations mentioned above is: *S*(—)-glyceraldehyde → *S*(+)-alanine → *S*(—)-α-methylbenzylamine → *S*(+)-α-methylbenzyl *iso*thiocyanate (I).

Experimental. Melting points are uncorrected and determined in capillary tubes in a slowly heated bath. Rotations are measured in a 1 dm tube.

S(+)-α-Methylbenzyl isothiocyanate (I). To (—)-α-methylbenzylamine (2.02 g, $[\alpha]_D^{24}$ —39.2° (neat)), dissolved in water, was added a chloroform solution of thiocarbonyl chloride (1.91 g). A total of 33.4 ml of 1 N NaOH was added in small portions to the vigorously stirred mixture. The organic layer was separated, the aqueous phase extracted twice with fresh portions of chloroform and the combined organic extracts dried over sodium sulphate. After removal of the solvent, the *iso*thiocyanate (1.12 g) distilled as a colourless oil, b.p. 126° at 16 mm, n_D^{25} 1.5802; $[\alpha]_D^{24}$ +17.5° (*c* 9.8, $CHCl_3$). (Found: C 66.50; H 5.52; N 8.91. Calc. for C_9H_9NS: C 66.24; H 5.56; N 8.59).

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