γ-Hydroxyglutamic Acid in Green Plants

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When it was found that the amino acid preparation which Dakin [1] more than 30 years ago isolated from proteins, and which he believed to be β-hydroxyglutamic acid, was a mixture of commonly occurring amino acids [2], hydroxyglutamic acid definitely disappeared from the list of amino acids occurring in nature. We have now, however, found γ-hydroxyglutamic acid in the green parts of *Phlox decussata*.

A 70 % alcohol extract (13 l) of *Phlox* (3.5 kg of fresh plants = 1.18 kg dry substance, = 24.8 g N, = 6.57 g soluble N) was prepared. A two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3) of the extract gave a spot immediately above that of aspartic acid (Fig. 1). In paper electrophoresis the amino acid in question (X) showed to be acidic. The isolation of X was performed as follows: the alcohol extract was passed through an Amberlite IR 120 column. The amino acids remained in the column and were eluted with 1 *N* ammonia. The ammonia was evaporated *in vacuo* and acidic amino acids were separated from the neutral and basic ones in an Amberlite IR-4B column. The acidic amino acids which remained in the column were eluted with 0.4 *N* hydrochloric acid. The solution was evaporated *in vacuo* to a syrup, which was dissolved in 200 ml of water. As the solution still contained great amounts of dark colcuring matter it was neutralized with NaOH. At a pH about 3.5 a dark precipitate was formed. When this precipitate had been separated by filtration the clear but still brown solution was neutralized to pH 7 and once more passed through an Amberlite IR 120 column. This procedure was repeated, ammonia being evaporated in the

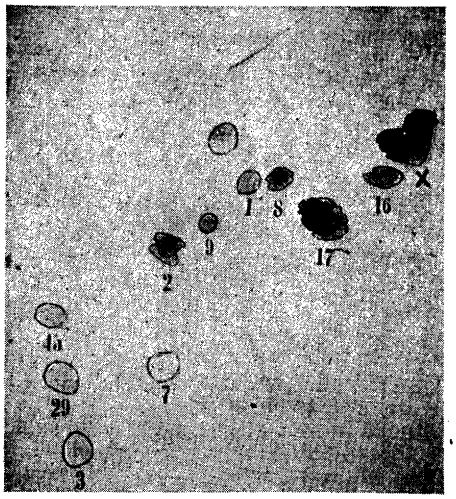

Fig. 1. Two-dimensional paper chromatogram of a 70 % alcohol extract of Phlox decussata. 1 = gly, 2 = ala, 7 = tyr, 8 = ser, 9 = threo, 16 = asp, 17 = glu, X = unknown acidic amino acid.

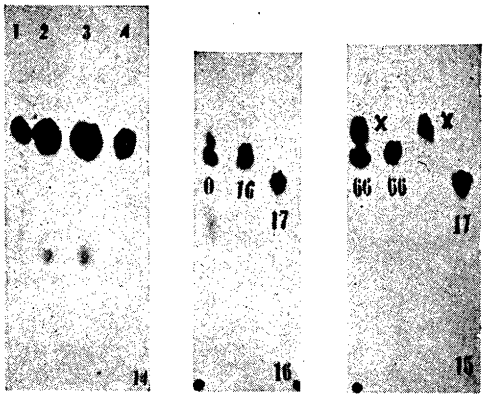

Fig. 2. Paper chromatogram of the pure preparation of X after reduction with HI and red P. 1, 2, and 3 = the reduction products of the amino acid X (increasing concentration of X from 1 to 3), 4 = glutamic acid. Solvent butanol-acetic acid.

Fig. 3. Paper chromatogram of the pure preparation of X after oxidation with $KMnO_4$ in sulphuric acid solution. O = the oxidation products of the amino acid X, 16 = aspartic acid, 17 = glutamic acid. Solvent butanol-acetic acid.

Fig. 4. Paper chromatogram of X and β-hydroxyglutamic acid (synth.). From left: a mixture of X and β-hydroxyglutamic acid (66), pure β-hydroxyglutamic acid (66), pure X and pure glutamic acid.

meantime. The solution was finally evaporated to dryness *in vacuo*, and the light solid substance remaining in the flask was dissolved in a small amount of water. 5 g of cellulose powder was poured into the flask after which the mass was evaporated to dryness, and placed on the top of a cellulose powder column (5.5 × 110 cm) containing as indicator 20 mg of methylorange. The elution was performed with a solution of butanol-acetic acid. After the indicator had come out 125 fractions of 36 ml were taken. Fractions 45—62 contained glutamic acid, 55—99 aspartic acid, and 71—125 the amino acid X. Fractions 100—125 contained only the unknown amino acid. They were evaporated to dryness *in vacuo*, the substance was dissolved in water and the solution poured into a glass jar. The solution was evaporated on a waterbath until crystallization began. After cooling the crystalline substance was separated by centrifugation and dried in a vacuum desiccator. The yield was 124 mg. M.p. 187° C (decomp.). Fractions 71—99 contained, besides a small amount of aspartic acid, about 620 mg of the amino acid X. 3.5 kg of fresh *Phlox* (= 6.57 g sol. N) thus contained about 750 mg (65 mg N) of the unknown amino acid (1.0 % of soluble N). Through crystallization a part of the amino acid X in fractions 71—99 could be separated from aspartic acid.

By the following experiments we established the structure of the amino acid X:

1. By reduction with 66 % HI (*d* 1.96) and red phosphorus at 140° C for 4 h glutamic acid was found to be formed (Fig. 2) from which we conclude that the amino acid X is a hydroxyglutamic acid.

2. Analysis of the amino acid X: C 36.82; H 5.35; N 8.60. Calc. for $C_5H_9O_5N$: C 36.81; H 5.52; N 8.59. Titration with phenolphtalein as indicator: 2.281 mg used 1.36 ml 0.00977 *N* NaOH. Calc. for monohydroxyglutamic acid 1.43 ml. The amino acid thus has the same empirical formula as monohydroxyglutamic acid.

3. Upon heating the amino acid X at pH 3.3 and 125° C for 4 h the amino N (van Slyke) diminished by 95 %; accordingly hydroxypyrrolidonecarboxylic acid was formed. From glutamic acid pyrrolidonecarboxylic acid is formed correspondingly (decrease of amino N 90 %).

4. By oxidation with potassium permanganate in sulphuric acid solution (10 mg of X + 7.5 mg of $KMnO_4$ in 189 µl of water + 90 µl of 20 % H_2SO_4) at + 10° C aspartic acid was formed (Fig. 3).

5. Potentiometric titration in parallel experiments gave the following pK-values (values approximate, taken from titration curves).

	pK_2	pK_3
Glutamic acid	4.2	9.7
Aspartic acid	3.7	9.6
β-Hydroxyglutamic acid	4.2	9.3
γ-Hydroxyglutamic acid (X)	3.6	9.7

6. Synthetic β-hydroxyglutamic acid can be distinguished from our γ-hydroxyglutamic acid on the paper chromatogram (butanol + acetic acid) (Fig. 4).

	butanol + acetic-acid	phenol + NH_3
R_F-value for γ-hydroxyglutamic acid (X)	0.06	0.17
» » β-hydroxyglutamic acid (synth.)	0.09	0.17
» » γ-hydroxy-α-amino pimelic acid [3]	0.15	0.34
» » glutamic acid	0.14	0.31

The new amino acid found in *Phlox* has thus been established as γ-hydroxyglutamic acid, HOOC · CHOH · CH_2 · $CHNH_2$ · COOH. On the basis of the paper chromatogram *Linaria vulgaris* also contains γ-hydroxyglutamic acid. The acid has not been isolated from this plant.

γ-Hydroxyglutamic acig is found also in the protein fraction of *Phlox*.

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