3. The method of Adcock (1957) for estimating sorbitol in biological material was adapted for use with leaf extracts.

4. The sorbitol content of plum leaves under natural conditions was followed from 1 p.m. on one day to 9 a.m. the next day. The amount present remained constant at 110 mg./g. of alcohol-insoluble leaf residue (45 mg./g. dry wt. of leaf).

The investigation formed part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research. The authors wish to thank Mr E. W. Hobbs and the Director of the Long Ashton Agricultural and Research Station for facilities placed at their disposal, and Dr D. I. Rees for helpful advice.

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A New Asparagine Derivative, N⁴-(2-Hydroxyethyl)-L-asparagine, from Bryony (*Bryonia dioica*)

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(Received 15 March 1961)

Many amino acids additional to those normally present in protein have been identified as components of higher plants (see reviews, Hunt, 1959; Fowden, 1958, 1960). Several of the newly characterized acids are derivatives of either glutamic acid or glutamine; others based on aspartic acid or asparagine are now known. N⁴-Ethyl-L-asparagine (I) was isolated recently from squirting cucumber (*Ecballium elaterium*) by Gray & Fowden (1961). The present paper reports the isolation of larger quantities from another member of the Curcubitaceae, *Bryonia dioica* (white bryony), together with another substituted amide identified as N⁴-(2-hydroxyethyl)-L-asparagine (II).

ISOLATION AND IDENTIFICATION

The substances were encountered as ninhydrin-reacting spots (brown) on paper chromatograms of 75% (v/v) ethanol extracts of macerated bryony

shoots. About 7 g. of ethylasparagine and 1.2 g. of hydroxyethylasparagine were isolated from an aqueous extract of 20 kg. of shoots. Hydroxyethylasparagine, having empirical formula C₈H₁₂O₄N₂, gave equimolar amounts of aspartic acid and ethanolamine after acid hydrolysis. Extracts of rat liver also converted hydroxyethylasparagine into aspartic acid and ethanolamine; ethylasparagine was converted similarly into aspartic acid and ethylamine. The pK_a values of hydroxyethylasparagine were 2.32 and 8.92 [cf. values for asparagine, 2.02 and 8.80 (Meister, 1957)]. Ammonia displaced hydroxyethylasparagine from cation-exchange resin columns together with glutamic acid and before displacement of ethylasparagine and asparagine. This result was in agreement with structure for hydroxyethylasparagine which contains a free hydroxyl group; a possible alternative structure, 4-(2-aminoethyl)-hydrogen aspartate, an ester of ethanolamine and aspartic acid, presumably would behave as a basic amino acid on ion-exchange resins.

Confirmation of the hydroxyethylasparagine structure was obtained by chemical synthesis. The

synthetic and natural compounds were shown to be identical by comparison of their m.p., $[\alpha]_D^{20}$, R_F values and infrared spectra. The spectra confirmed the absence of an ester linkage.

EXPERIMENTAL

Chromatographic techniques. Paper chromatograms were run on Whatman no. 3MM chromatographic-grade filter paper. Sheets were washed thoroughly with 5% (v/v) acetic acid solution before being used for preparative work. Solvents used included: *A*, 75% (w/v) phenol solution in the presence of NH₃; *B*, a one-phase mixture of butan-1-ol-acetic acid-water (90:10:29, by vol.); *C*, 70% (v/v) propan-1-ol; *D*, the upper phase of ethyl acetate-pyridine-water (2:1:2, by vol.). A 0.1% (w/v) solution of ninhydrin in 95% (v/v) ethanol was normally used as a spraying agent.

Detection of ethyl- and hydroxyethyl-asparagine in bryony shoots. Two-dimensional paper chromatograms (solvent *A* followed by solvent *B*) of 75% (v/v) ethanol extracts of macerated bryony leaves and stems showed three strong brown spots after treatment with ninhydrin. Two of these were identified as asparagine and *N*-ethylasparagine (I); the latter partially overlapped the yellow spot of proline. The third brown spot (hydroxyethylasparagine; R_F in solvent *A*, 0.65) partially overlapped glutamine (R_F 0.61); the two substances had identical R_F values in solvent *B*. Ethyl- and hydroxyethyl-asparagine were not detected on chromatograms prepared from seed extracts.

Preliminary fractionation on cation-exchange resins. Bryony shoots (20 kg.) were collected in August and kept at -40°. The frozen material was crushed and batches (250 g.) were macerated with chloroform-saturated water (500 ml.) at 2°. The macerate was pressed (6000 lb./in.²); the expressed juice was adjusted immediately to pH 4.6-4.8 and warmed (60°) for 10 min. to flocculate the protein. The combined filtrate (about 50 l.) was applied at a rate of 2 l./hr. to a Zeo-Karb 215 (resin mesh 80-100) column (120 cm. × 10 cm. diam.).

All the amino acids were adsorbed. The column was washed with 200 l. of water and eluted with 0.5N-NH₃ solution. Amino acids were present in 12 l. of eluate collected in 500 ml. fractions. Resolution was not good, but the first five fractions contained only aspartic acid, glutamic acid, threonine and serine, and were discarded. All later fractions were combined and evaporated *in vacuo* at 50° to remove NH₃. The sticky residue was redissolved in water (4 l.), and the solution (pH 6.8) was decolorized by treating it three times with charcoal (50 g., prepared as by Partridge, 1949).

This solution was applied at the rate of 50 ml./hr. to Dowex 50 X 8 (mesh 100-200) columns (upper: 60 cm. × 3.5 cm. diam.; lower: 30 cm. × 2 cm. diam.). After adsorption the amino acids formed a band of depth 27 cm. upon the upper column. The columns were washed with water (6 l.) and then were eluted with 0.25N-NH₃ solution (flow rate 50 ml./hr.). After the break-through of aspartic acid 85 fractions (15 ml.) were collected. Hydroxyethyl-asparagine was present in fraction nos. 7-16 together with aspartic acid, glutamic acid, an unidentified amino acid (purple colour with ninhydrin, designated P1) and small amounts of threonine and serine. Ethylasparagine was present in fraction nos. 15-38 and asparagine (in small amount) in nos. 23-36.

Isolation of N⁴-ethyl-L-asparagine (I). Fraction nos. 18-33 contained large amounts of ethylasparagine (about 10 g.) together with traces of several other amino acids. The combined fractions were evaporated *in vacuo* and the residue was recrystallized twice from 75% (v/v) ethanol to yield pure ethylasparagine (7.15 g.). The properties and synthesis of ethylasparagine have been reported by Gray & Fowden (1961).

Isolation of N⁴-(2-hydroxyethyl)-L-asparagine (II). On storage at 2° aspartic acid (1.8 g.) separated from fraction nos. 5-9 and glutamic acid (1.1 g.) from nos. 12-16. After removal of the crystals, fraction nos. 7-14 were pooled and evaporated *in vacuo* (yield 3.8 g.). This residue was redissolved in water (250 ml.) and the solution was applied to a Dowex 1 (acetate form; resin mesh 100-200) column (30 cm. × 2 cm. diam.) to absorb residual aspartic acid and glutamic acid. The column was washed with water (500 ml.) and, after evaporation of the combined eluates, the residue (1.9 g.) contained hydroxyethylasparagine and P1 together with traces of threonine and serine. The residue, dissolved in a minimum volume of warm water, was streaked across eight sheets of washed filter paper (27 in. × 23 in.), which were developed with solvent *A*. The four amino acids were resolved completely and the bands of hydroxyethylasparagine and P1 were cut out and eluted with water. After clarification with charcoal and evaporation, hydroxyethyl-asparagine (1.22 g.) and P1 (0.3 g.) were obtained. Recrystallization from 80% (v/v) ethanol gave pure hydroxyethylasparagine as lustrous plates (760 mg.).

Properties of hydroxyethylasparagine. This had m.p. 199-200° and $[\alpha]_D^{20} = -2.9 \pm 0.5^\circ$ in water (*c*, 5.0) (Found: C, 40.6, 40.4; H, 7.1, 6.9; N, 15.8, 15.9. C₆H₁₂O₄N₂ requires C, 40.9; H, 6.9; N, 15.9%). When a 0.1M-solution of hydroxyethylasparagine was titrated against 0.1N-HCl, a pK_A of 2.32 was determined; titration against 0.1N-NaOH gave a pK_A of 8.92.

Hydroxyethylasparagine was destroyed completely when heated with 6N-HCl at 100° for 24 hr. Paper chromatography showed two products, which were inseparable from aspartic acid and ethanolamine in solvents *A-D*. Quantitative paper chromatography (Fowden & Webb, 1958; Bryant & Fowden, 1959) with solvent *B* established that 17.6 mg. of hydroxyethylasparagine (100 μmoles) gave 13.0 mg. of aspartic acid (98 μmoles) and 6.3 mg. of ethanolamine (103 μmoles).

Under the conditions used for quantitative conversion of asparagine into NH₃ and aspartic acid, namely 2N-HCl at 100° for 3 hr., less than 50% of hydroxyethylasparagine was hydrolysed. The hydrolysate contained an intermediate compound, giving a yellow ninhydrin-reacting spot of $R_F \sim 0.95$ in solvent *A*. This intermediate, possibly a cyclization product, disappeared during prolonged hydrolysis with 2N-HCl.

Enzymic degradation. Fresh rat liver was homogenized with 2 vol. of phosphate buffer (pH 7.8) containing 100 ml. of 0.2M-KH₂PO₄ and 90.4 ml. of 0.2N-KOH/l. After centrifuging at 10 000*g* for 15 min., the supernatant was concentrated to one-third of the original volume by dialysis against moistened Carbowax 20M polyethylene glycol (supplied by G. T. Gurr Ltd., London, S.W. 6). The concentrate was dialysed against several changes of 0.1M-phosphate (pH 7.8) for 24 hr. at 2° and then used as a source of crude asparaginase (Greenstein & Carter, 1946).

Fig. 1. Infrared spectra of N^4 -(2-hydroxyethyl)-L-asparagine. A, Synthetic material; B, natural material. Both spectra were determined with liquid-paraffin mulls of the amide.

The hydrolysis of asparagine and ethyl- and hydroxyethyl-asparagine by the liver preparation was studied at 37°. After 3 hr., ethylamine and aspartic acid were identified chromatographically as degradation products of ethylasparagine, whereas hydroxyethylasparagine gave ethanolamine and aspartic acid. Asparagine was hydrolysed faster than either ethyl- or hydroxyethyl-asparagine.

The rate of breakdown of asparagine by rat-liver extracts was increased markedly when a keto acid, particularly pyruvic acid, was added to the incubation mixtures (Greenstein & Carter, 1946; Meister, Sober, Tice & Fraser, 1952). Under these conditions the α -amino group of asparagine is donated to the added keto acid; the amide group is also hydrolysed and oxaloacetate and NH_3 form the main reaction products. The rate of disappearance of either ethyl- or hydroxyethyl-asparagine from reaction mixtures was not influenced appreciably by the addition of pyruvate and so transamination of the substituted amides must have been negligible. This behaviour was similar to that of L-isoasparagine (Meister *et al.* 1952).

Synthesis of N^4 -(2-hydroxyethyl)-L-asparagine (II). 4-Ethyl hydrogen L-aspartate was prepared by the method of Curtis & Koch (1888). Dry HCl gas was bubbled through a suspension of L-aspartic acid (20 g.) in dry ethanol (200 ml.) until no solid remained. The solution was evaporated to dryness *in vacuo* at 40°. The residue was dissolved in ethanol and re-evaporated to remove traces of HCl when 4-ethyl hydrogen L-aspartate hydrochloride (25 g.) remained. An equivalent amount of methanolic KOH was added to 4-ethyl hydrogen L-aspartate hydrochloride (20 g.) dissolved in ethanol. After removal of precipitated KCl, the filtrate was concentrated and 4-ethyl hydrogen L-aspartate (12.5 g.) crystallized.

N^4 -(2-Hydroxyethyl)-L-asparagine was prepared by refluxing 4-ethyl hydrogen L-aspartate (8 g.) for 3 hr. with 10 ml. of ethanolamine dissolved in 10 ml. of ethanol. Ethanol was removed by evaporation *in vacuo*, and the sticky residue was dissolved in water (300 ml.). This solution was fractionated on a Dowex 50X 8 (mesh 100–200) column (30 cm. $\times$ 2 cm. diam.). During elution with 0.2N- NH_3 solution, hydroxyethylasparagine and some aspartic acid appeared together in the early fractions; unchanged ethanolamine and small quantities of ninhydrin-reacting by-products appeared in later fractions. Hydroxy-

ethylasparagine was freed from aspartic acid by absorbing the latter on a Dowex 1 (mesh 100–200) column (20 cm. long, 1.5 cm. diam.). Evaporation of the eluate left a residue of hydroxyethylasparagine (5.1 g.). Recrystallization from 80% (v/v) ethanol (50 ml.) gave pure hydroxyethylasparagine (3.2 g.), m.p. 202–203°, $[\alpha]_D^{20} - 2.5 \pm 0.4^\circ$ in water (*c.* 5.0) (Found: C, 40.3; H, 6.8; N, 15.9. $\text{C}_6\text{H}_{12}\text{O}_4\text{N}_2$ requires C, 40.9; H, 6.9; N, 15.9%).

The synthetic material was hydrolysed completely by treatment with 6N-HCl at 100° for 24 hr. to yield aspartic acid and ethanolamine.

Identity of natural and synthetic amides. The two substances showed identical chromatographic behaviour in all four chromatographic solvents. The agreement between their m.p. and $[\alpha]_D^{20}$ values and their behaviour upon treatment with HCl solutions at 100° also served to confirm their identity. Final proof of identity was obtained by comparison of their i.r. spectra, which showed that all absorption peaks were common to both substances (Fig. 1). No absorption peaks were observed at wavelengths (5.6–5.7 μ) characteristically associated with ester groups.

SUMMARY

1. N^4 -(2-Hydroxyethyl)-L-asparagine has been newly characterized as a component of higher plants, being isolated from shoots of *Bryonia dioica* (white bryony) together with larger amounts of N^4 -ethyl-L-asparagine.

2. N^4 -(2-Hydroxyethyl)-L-asparagine was converted into an equimolar mixture of aspartic acid and ethanolamine on hydrolysis with hydrochloric acid. Hydrolysis proceeded more slowly than that of the parent amide, asparagine.

3. N^4 -(2-Hydroxyethyl)-L-asparagine was prepared by refluxing an ethanolic solution of 4-ethyl hydrogen L-aspartate with ethanolamine.

The author wishes to acknowledge grants from the Central Research Fund's Committee of the University of London. Miss J. Chessun gave technical assistance during the investigation. Bryony shoots were supplied by the University of London Botanical Supply Unit, Egham.

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