negative except for traces of methylamine in liver, and more in urine, some of which was derived from dimethylnitrosamine.

5. The results indicate that the dimethylamino groups are oxidized to one carbon-atom intermediates and ammonia, and that the nitroso group is partly reduced to ammonia.

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REFERENCES

Barnes, J. M. & Magee, P. N. (1954). *Brit. J. industr. Med.* **11**, 167.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. E. (1949). *Isotopic Carbon*, p. 93. New York: John Wiley and Sons, Inc.
Caster, W. O., Poncelet, J., Simon, A. B. & Armstrong, W. D. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 122.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Csaky, T. Z. (1948). *Acta chem. scand.* **2**, 450.
Dutton, A. H. & Heath, D. F. (1956*a*). *J. chem. Soc.*, p. 1892.
Dutton, A. H. & Heath, D. F. (1956*b*). *Nature, Lond.*, **178**, 644.
Elliot, W. H. (1948). *Biochem. J.* **42**, v.
Evans, E. A. & Huston, J. L. (1952). *Analyt. Chem.* **24**, 1482.
Friedrich, A. (1933). *Hoppe-Seyl. Z.* **216**, 68.
Heath, D. F. (1957). *J. chem. Soc.* p. 4152.
Heath, D. F. & Jarvis, J. A. E. (1955). *Analyst*, **80**, 613.
Hultin, T. (1956). *Exp. Cell Res.* **10**, 697.
Klevens, H. B. & Ellenbogen, E. (1955). *Symp. Faraday Soc.* **18**, 217.
Lindenbaum, A., Schubert, J. & Armstrong, W. D. (1948). *Analyt. Chem.* **20**, 1120.
McKennis, H. jun., Weatherby, J. H. & Witkin, L. B. (1955). *J. Pharmacol.* **114**, 385.
Magee, P. N. (1956). *Biochem. J.* **64**, 676.
Magee, P. N. & Barnes, J. M. (1956). *Brit. J. Cancer*, **10**, 114.
Miller, E. C. & Miller, J. A. (1947). *Cancer Res.* **7**, 468.
Miller, J. A. & Miller, E. C. (1953). *Advanc. Cancer Res.* **1**, 339.
O'Leary, J. F., Wills, J. H., Harrison, B. & Oikemus, A. (1957). *Proc. Soc. exp. Biol., N.Y.*, **94**, 775.
Ormsby, A. A. & Johnson, S. (1950). *J. biol. Chem.* **187**, 711.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, vol. 2, p. 547. London: Baillière, Tindall and Cox.
Rapi, G. (1953). *Sper. Sez. Chim. Biol.* **4**, 11.
Raschig, F. (1924). *Schwefel und Stickstoffstudien*, p. 183. Leipzig: Verlag Chemie.
Schneider, W. C. & Hogeboom, G. R. (1950). *J. biol. Chem.* **183**, 123.
Shinn, M. B. (1941). *Industr. Engng Chem.* **13**, 33.
Speck, J. F. (1947). *J. biol. Chem.* **168**, 403.
Speck, J. F. (1949). *J. biol. Chem.* **179**, 1387.
Watt, G. W. & Chrisp, J. D. (1952). *Analyt. Chem.* **24**, 2006.
Webster, G. C. & Varner, J. E. (1955). *J. biol. Chem.* **215**, 91.

Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

4. ISOLATION OF AZETIDINE-2-CARBOXYLIC ACID AND EVIDENCE FOR THE OCCURRENCE OF $\alpha\gamma$-DIAMINOBUTYRIC ACID IN *POLYGONATUM**

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Azetidine-2-carboxylic acid, the lower imino acid analogue of proline, has been isolated from two members of the Liliaceae family. Fowden (1955, 1956) isolated it from *Convallaria majalis* (lily of the valley) and characterized it chemically by comparison with synthetic material. An identical substance was isolated from *Polygonatum officinalis* (a Solomon's seal) by Virtanen and co-workers (Virtanen, 1955*a*). A survey of liliaceous and related plants by Fowden & Steward (1957) demonstrated that the imino acid was present in about one-quarter of the ninety species examined. Its distribution was practically confined to the members of the Liliaceae, although a few species of the Agavaceae contained small amounts of the acid. The observation that it is present in certain members of the latter family, a group of plants split off rather recently from the Liliaceae, is interesting since there is no report of its occurrence in other families. The imino acid often accumulates in very large amounts and may represent the major proportion of the non-protein-nitrogen of particular plants. This is the case for the two species from which it was isolated. Azetidine-2-carboxylic acid is not known to occur in plant proteins.

The present paper describes the isolation of azetidine-2-carboxylic acid and $\alpha\gamma$-diaminobutyric acid from a liliaceous plant. Diaminobutyric acid,

* Part 3: Zacharius, Cathey & Steward (1957).

aspartic acid and homoserine are possible precursors for the biosynthesis of azetidine-2-carboxylic acid. While aspartic acid is accepted as a normal constituent of the soluble nitrogeneous fraction of plants, and homoserine has been shown to be present in a number of plants (Virtanen, 1955*b*), $\alpha\gamma$-diaminobutyric acid has never been conclusively shown to occur in a higher plant. Evidence is now presented that the acid does occur in small amounts in the non-protein-nitrogen fraction of *Polygonatum*, together with the other two amino acids.

A secondary feature of this investigation has been the isolation of azetidine-2-carboxylic acid in large quantity from the rhizome of *Polygonatum multiflorum*, which can be readily obtained in England.

EXPERIMENTAL

Chromatographic techniques. All paper chromatograms were run on Whatman no. 3MM chromatographic-grade filter-paper sheets under conditions which were essentially those used by Consden, Gordon & Martin (1944). The solvents used were water-saturated phenol with NH_3 (Dent, 1947), butanol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948), 70% (v/v) propanol, and the upper phase of an ethyl acetate–pyridine–water mixture (2:1:2, by vol.). A 0·1% (w/v) solution of ninhydrin in 95% (v/v) ethanol was used as the spray reagent.

Extraction of the Polygonatum *rhizome.* Fresh rhizome (40 lb.) was washed and coarsely minced. It was then macerated in an Atomix blendor (M.S.E. Ltd., London) with sufficient 95% (v/v) ethanol to give a final ethanol concentration of 70% (v/v). The mush was left at room temperature for 2 weeks with occassional stirring to allow complete extraction. The mush was filtered, and the residue pressed to obtain the maximum yield of extract. The combined extract (36 l.) was then evaporated in a continuous rotary vacuum evaporator (Flash Evaporator, Lab. Glass and Instruments Corp., New York, 31) at 50° to about 1·5 l. Little ethanol remained in the concentrated extract at this stage.

Fractionation of the extract on cation-exchange-resin columns. The extract was diluted to 10 l. and then run through a column (depth 100 cm., diam. 7 cm.) of Zeo-Karb 215 in the H^+ form (60–80 mesh) at 1 l./hr. The column was then washed with 30 l. of water to elute all non-cationic substances. Finally the amino acids were displaced by 12 l. of 0·5 N-NH_3 solution without fractionation. This procedure was to remove metallic cations since previous experience had shown that fractionation of amino acids in plant extracts on Zeo-Karb 215 columns was often poor, if attempted before the removal of such ions.

The eluate containing the amino acids was again concentrated to 10 l. and adjusted to pH 5. It was then applied at the rate of 1 l./hr. to the same Zeo-Karb column after regeneration with 2 N-HCl. The column was washed with 15 l. of water and elution with aq. 0·5 N-NH_3 was commenced at the rate of about 100 ml./hr. When amino acids appeared in the eluate, collection of 17 ml. fractions was commenced. In all 750 fractions were taken. The first 30 fractions contained only aspartic acid; the next 430 contained azetidine-2-carboxylic acid, with smaller amounts of aspartic acid and glutamic acid. These 430 fractions were pooled to give eluate A, from which the imino acid was isolated. Fractions 460–505 contained the hydroxyamino acids serine and threonine in addition to the previous acids, and homoserine appeared in fractions 506–534. Neutral amino acids then began to appear in the eluate. Fractions 615–667 were pooled as eluate B, which contained both neutral and basic amino acids, together with asparagine and γ-aminobutyric acid. Fractions 668–750 contained mainly lysine and arginine with smaller amounts of other amino acids.

Isolation of azetidine-2-carboxylic acid from eluate A. This eluate (7·3 l.) was concentrated *in vacuo* at 50° to about 250 ml. The concentrate was decolorized by treatment with absorbent charcoal, adjusted to pH 7 by addition of 10 N-NH_3 and evaporated *in vacuo* to about 100 ml. When the solution was kept at about −10°, 18·9 g. of azetidine-2-carboxylic acid crystallized out. Successive concentration and crystallization yielded 40 g. of the imino acid. The ammonium salts of glutamic and aspartic acids remained in solution. The azetidine-2-carboxylic acid was dissolved in the minimum quantity of hot water and boiling ethanol added to the point of crystallization. On cooling, 33 g. of white crystals of the L-imino acid were obtained.

Chromatographic identification of aspartic acid and homoserine. A small amount of crude aspartic acid was obtained by concentration and crystallization of fractions 1–30. Crude homoserine was obtained as a sticky residue from a portion of the pooled fractions 506–534 by separation from the other amino acids by paper chromatography. The preparations of aspartic acid and homoserine were identified by co-chromatography with authentic samples, in all four solvent systems.

Isolation of $\alpha\gamma$-diaminobutyric acid from eluate B. This eluate was concentrated *in vacuo* to 500 ml. and then applied at the rate of 80 ml./hr. to a column (depth 42 cm., diam. 2·5 cm.) of Dowex 50-X4 (100–200 mesh) in the H^+ form. The column was then washed with 2 l. of water and the amino acids eluted with 0·2 N-NH_3 solution. Eighty fractions of 17 ml. were collected. Fractions 72–76 contained diaminobutyric acid and an unidentified amino acid in about equal amounts, together with traces of glycine and valine. Fractions 77–80 contained mainly the unidentified amino acid with smaller amounts of diaminobutyric acid.

Fractions 72–76 were evaporated *in vacuo* to a syrup. This was dissolved to give 3 ml. of solution and 1 ml. was applied as a streak across one edge of each of three sheets of washed Whatman no. 3MM filter paper (24 in. × 24 in.). The chromatograms were developed in the butanol–acetic acid mixture for 1 week; a good separation of diaminobutyric acid from the slower-moving unknown substance was effected; each component was eluted separately from the chromatograms.

The eluate containing the diaminobutyric acid was decolorized by treatment with charcoal and evaporated *in vacuo*; a few drops of conc. HCl were added before final drying. The residue (61 mg.) was dissolved in the minimum volume of hot water and a hot mixture of equal volumes of conc. HCl and acetic acid was added. After crystallization at 0°, 28 mg. of crystals, m.p. 195–198° (decomp.), was collected. The mixed m.p. with an authentic sample of L-diaminobutyric acid dihydrochloride (m.p. 194–197°, decomp.) prepared by the method of Adamson (1939) was

197–198° (decomp.). (All m.p.'s are corrected.) (Found: C, 24·7; H, 6·4; N, 14·3; Cl, 37·6. Calc. for $C_4H_{10}O_2N_2$,2HCl: C, 25·1; H, 6·3; N, 14·7; Cl, 37·2%).

The isolate was inseparable from diaminobutyric acid when run on chromatograms developed in all four solvents listed above. After a portion of the isolate had been mixed with a sample of [^{14}C]diaminobutyric acid (prepared from [^{14}C]glutamic acid by a micro modification of the method of Adamson, 1939), the mixture was chromatographed on papers developed in phenol–aq. NH_3 and butanol–acetic acid. Radioautographs from the dry chromatograms were compared with the chromatograms after development with ninhydrin. The radioactive areas coincided with those of the ninhydrin-reacting material.

The isolate was converted into azetidine-2-carboxylic acid and α-hydroxy-γ-aminobutyric acid (Fowden, 1956) by mixing 5 mg. with 14 mg. of solid $AgNO_2$ and 0·15 ml. of 10 N-HCl. The mixture was kept in a boiling-water bath for 45 min.; it was then diluted to 1 ml. with water, and the precipitated AgCl removed by centrifuging. The supernatant was evaporated to dryness several times to remove excess of HCl. The residue was dissolved finally in 0·5 ml. of water, solid $Ba(OH)_2,8H_2O$ added to give a 0·5 N-solution, and the mixture heated at 100° for 30 min. Barium was precipitated as sulphate and any excess of H_2SO_4 was neutralized by addition of solid $BaCO_3$. After centrifuging, the clear liquid was evaporated to dryness and the residue redissolved in a small volume of water. Measured portions were used to prepare two-dimensional chromatograms, which were developed in phenol–aq. NH_3, followed by butanol–acetic acid. Development with ninhydrin revealed a brown spot that was inseparable from added azetidine-2-carboxylic acid and a purple spot coincident with added γ-amino-α-hydroxybutyric acid. The conversion into these two products is specific for diaminobutyric acid as a starting material.

DISCUSSION

The concentrations of the free amino acids present in the rhizome of *Polygonatum* are small in relation to the concentration of azetidine-2-carboxylic acid; the imino acid usually accounted for 75% or more of the total non-protein nitrogen of the tissue. Few steps are involved in the isolation procedure and so the preparation of the imino acid in this way has a definite advantage over chemical synthesis from glutamic acid (Fowden, 1956). The synthesis gives either D- or DL-azetidine-2-carboxylic acid, which for biological purposes is less useful than the L-form obtained by isolation.

The fact that homoserine occurs in *Polygonatum* is of interest since it may be implicated in azetidine-2-carboxylic acid metabolism. First isolated from peas (Miettinen, Kari, Moisio, Alfthan & Virtanen, 1953), homoserine has later been found in many other plants (Virtanen, 1955*b*). The present authors also (unpublished observations) have shown that it is a common constituent of higher plants, although normally only in small amounts relative to those of other free amino acids. This finding is not unexpected since homoserine is known to be an intermediate in the biosynthesis of threonine in yeast (Watanabe, Konishi & Shimura, 1955).

The isolation and characterization of αγ-diaminobutyric acid as a natural product of higher plants adds another amino acid to the very considerable list of new non-protein acids isolated by chromatographic methods during the last decade. Previously diaminobutyric acid had been known only as a metabolic product of certain micro-organisms, being present in several peptides possessing antibiotic properties; these include the polymyxins (Catch, Jones & Wilkinson, 1949), circulin (Peterson & Reineke, 1949), and colistin (Oda & Ueda, 1954). Zacharius, Pollard & Steward (1955) suggested that it may be present in potato tuber, from chromatographic evidence only.

The new diamino acid is a member of the series which includes ornithine and lysine. Of the three acids, only lysine is known as a constituent of proteins from plants. The presence of ornithine in plants has only recently been established and the free base does not seem to have been isolated yet in sufficient quantities for strict chemical identification to be made. Coleman (1958) has isolated and chemically identified it as a 2:4-dinitrophenyl derivative from extracts of sulphur-deficient flax plants. Kasting & Delwiche (1957) have prepared from barley leaves, wheat roots and water-melon seedlings small amounts of a substance with chromatographic properties and specific colour reactions identical with those of ornithine. The identification of this series of three basic amino acids now means that each known imino acid of plants (azetidine-2-carboxylic acid, proline, and pipecolic acid) is matched by a naturally occurring diamino acid that may act as a precursor in their biosynthesis. Ornithine is established as a precursor of proline (Vogel, 1955), and lysine of pipecolic acid (Grobbelaar & Steward, 1953; Lowy, 1953).

SUMMARY

1. The amino acids in a 70% ethanol extract of 40 lb. of the rhizome of *Polygonatum multiflorum* (Solomon's seal) were fractionated on ion-exchange resin columns and by paper chromatography.

2. Azetidine-2-carboxylic acid (40 g.) was isolated. Evidence was also obtained for the presence of aspartic acid and homoserine.

3. Diaminobutyric acid was isolated in small amount as its dihydrochloride. This represents the first unequivocal isolation of this diamino acid from a higher plant material.

This investigation was carried out while one of us (M. B.) held a Postgraduate Research Studentship of the University of London. The continuous rotary vacuum evaporator was purchased with the help of a grant from the Central Research Funds Committee of the University of London.

REFERENCES

Adamson, D. W. (1939). *J. chem. Soc.* p. 1564.
Catch, J. R., Jones, T. G. S. & Wilkinson, S. (1949). *Ann. N.Y. Acad. Sci.* **51**, 917.
Coleman, R. G. (1958). *Nature, Lond.*, **181**, 776.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Dent, C. E. (1947). *Biochem. J.* **41**, 327.
Fowden, L. (1955). *Nature, Lond.*, **176**, 347.
Fowden, L. (1956). *Biochem. J.* **64**, 323.
Fowden, L. & Steward, F. C. (1957). *Ann. Bot., N.S.*, **21**, 53.
Grobbelaar, N. & Steward, F. C. (1953). *J. Amer. chem. Soc.* **75**, 4341.
Kasting, R. & Delwiche, C. C. (1957). *Plant Physiol.* **32**, 471.
Lowy, P. H. (1953). *Arch. Biochem. Biophys.* **47**, 228.
Miettinen, J. K., Kari, S., Moisio, T., Alfthan, M. & Virtanen, A. I. (1953). *Suomen Kemistilehti (Acta chem. fenn.)*, B**2**, 26.
Oda, T. & Ueda, F. (1954). *J. pharm. Soc. Japan*, **74**, 1246.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Peterson, D. H. & Reineke, L. M. (1949). *J. biol. Chem.* **181**, 95.
Virtanen, A. I. (1955*a*). *Nature, Lond.*, **176**, 984.
Virtanen, A. I. (1955*b*). *Angew. Chem.* **67**, 381.
Vogel, H. J. (1955). In *Amino Acid Metabolism*, p. 335. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Watanabe, Y., Konishi, S. & Shimura, K. (1955). *J. Biochem., Tokyo*, **42**, 837.
Zacharius, R. M., Cathey, H. M. & Steward, F. C. (1957). *Ann. Bot., N.S.*, **21**, 193.
Zacharius, R. M., Pollard, J. K. & Steward, F. C. (1955). *Ann. Acad. Sci. fenn. (Chem.)*, A II, **60**, 321.

Some Observations on a Hydroxypipecolic Acid from Thrift (*Armeria maritima*)

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In recent years, knowledge of the free amino and imino acid components of plants has expanded rapidly (for reviews, see Virtanen, 1956, 1957; Steward & Pollard, 1957; Fowden, 1958). More than thirty new acids, additional to the twenty or so present in proteins, are known as products of higher-plant metabolism. Imino acids are among these newer compounds, and several contain a piperidine ring. Pipecolic acid (piperidine-2-carboxylic acid) is the most commonly found member of this group, being particularly characteristic of the legumes (Zacharius, Thompson & Steward, 1954), although it occurs in members of many other families (Morrison, 1953). Baikiain ($\Delta^{4:5}$-dehydropipecolic acid), which occurs far less frequently in plants than pipecolic acid, was first isolated from *Baikiaea plurijuga* (Rhodesian teak), in which it occurs in relatively large amounts in both the seeds (Grobbelaar, Pollard & Steward, 1955) and the wood (King, King & Warwick, 1950). Hyde (1955) considers it to be a constituent of the developing pea seed. 5-Hydroxypipecolic acid was isolated from a palm (*Rhapis flabelliformis*) by Virtanen & Kari (1954), and from *Baikiaea* by Grobbelaar *et al.* (1955). It is also present in the fruit of the date palm, and accompanies pipecolic acid in several legume seeds, including the pea (Hyde, 1955). Another hydroxypipecolic acid, considered to be 4-hydroxypipecolic acid, was isolated from *Acacia pentadena* by Virtanen & Kari (1955). In *Acacia* the two hydroxypipecolic acids co-existed, but in *Albizzia lophantha* only the presumed 4-hydroxy compound was present.

The present investigation reports the isolation of a hydroxypipecolic acid from thrift (*Armeria maritima*), a member of the family Plumbaginaceae. This substance is identical with that isolated from *Acacia*. However, evidence will be presented to suggest that these compounds may be 3-hydroxypipecolic acid, and not the 4-hydroxy derivative as suggested by Virtanen & Kari (1955).

EXPERIMENTAL

All melting points are corrected.

Paper chromatography. The method and solvent mixtures were as described by Fowden & Bryant (1958).

Synthesis of hydroxypipecolic acids

4-Hydroxypipecolic acid. This was obtained in small yield by catalytic hydrogenation of 4-hydroxypicolinic acid (4-hydroxypyridine-2-carboxylic acid), m.p. 263° (decomp.), which was prepared by the method of Meyer & Graf (1928), who gave m.p. 254–255° (decomp.). 4-Hydroxypicolinic acid (1·1 g.) was dissolved in 20 ml. of water and converted into its hydrochloride by the addition of the calculated amount of HCl. PtO (0·2 g.) was added and a slow stream of H_2 was passed through the mixture in a water bath at 90° for 12 hr. After 6 hr. the catalyst was replaced by a further 0·2 g. of PtO. After final removal of the Pt, the mixture was evaporated to about 5 ml., when much unchanged 4-hydroxypicolinic acid hydrochloride