Azetidine-2-carboxylic Acid: a New Cyclic Imino Acid Occurring in Plants

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In recent years the paper-chromatographic technique has been used extensively to study the soluble nitrogenous compounds of plants. In this way many new amino and imino acids have been recognized, and some of the newly characterized substances have been shown to be major constituents of certain plant species, e.g. γ -methyleneglutamine and γ -methyleneglutamic acid in groundnuts, *Arachis hypogaea* (Done & Fowden, 1952); γ -hydroxyglutamic acid in *Phlox decussata* (Virtanen & Hietala, 1955a); γ -hydroxy- γ -methylglutamic acid in the maidenhair fern, *Adiantum pedatum* (Grobelaar, Pollard & Steward, 1955); γ -hydroxy- α -aminopimelic acid in the fern *Asplenium septentrionale* (Virtanen, Uksila & Matikkala, 1954); 4:5-dehydropiperidine-2-carboxylic acid (baikiaïn) in Rhodesian teak, *Baikiaea plurijura* (King, King & Warwick, 1950); 5-hydroxypiperidine-2-carboxylic acid in the palm, *Rhapis flabelliformis* (Virtanen & Kari, 1954), and in *Baikiaea* (Grobelaar *et al.* 1955); 4-hydroxypiperidine-2-carboxylic acid in *Acacia pentadena* (Virtanen & Kari, 1955). Many of these substances are derived from either glutamic acid or piperidine-2-carboxylic acid.

This paper describes the isolation and identification of a new imino acid, azetidine-2-carboxylic acid, which occurs as an important constituent of many plants belonging to the Liliaceae. The structure of this substance and the atom-numbering system used in the text are as shown in (I)

No previous reference to the compound occurs in the chemical literature, although the free base, azetidine, was described in the latter part of the nineteenth century. It is also the first time that any simple derivative of this four-atom ring system has been described as occurring in plants, except for an earlier brief account of the present work (Fowden, 1955).

RESULTS

Isolation

The new substance was first encountered during a limited survey of plant materials for the presence of γ -methyleneglutamine and γ -methyleneglutamic acid. Two-dimensional chromatograms (run first in phenol and then in butanol-acetic acid) of 70% (v/v) ethanol extracts of fresh leaves of *Convallaria majalis* (lily-of-the-valley) showed the presence of a substance that reacted with ninhydrin to give a brown-coloured spot in a position similar to that occupied by γ -methyleneglutamine, which also gives a brownish ninhydrin colour. Although, like γ -methyleneglutamine, the compound was labile to strong mineral acid, chromatography for longer periods of time, with phenol as solvent, effected a separation between the two substances. Since no other substance possessing similar chromatographic properties had been described, the *Convallaria* compound, hereafter termed Z, was considered to be a new plant constituent, and a larger-scale isolation was made by using the combined techniques of ion-exchange and preparative paper chromatography.

Two separate isolations of Z were made, one with Zeo-Karb 215 for the primary amino acid fractionation followed by purification on washed Whatman no. 3 filter-paper chromatograms, and the second with Dowex 50 for the primary fractionation. Z was finally recrystallized from hot 95% (v/v) methanol, and its purity checked by chromatography in solvents A, D, E and F (see Experimental section). This material was optically active, $[\alpha]_D^{20} - 108^\circ$.

Chemical properties

Elementary analysis of Z suggested an empirical formula $C_4H_7O_2N$, and determination of its molecular weight by the method of Barger (1904) gave a value of about 100. Thus the molecular formula was probably identical with the empirical formula. The hydrogen atoms in this formula are too few in number for it to represent a saturated open-chain amino acid. Two feasible, alternative types of compound, however, fit the formula. The first is an aminobutyric acid with a single C:C unsaturation, and the second is an azetidine-type ring system

with a carboxylic acid group substituent on one of the ring carbon atoms. If *Z* were of the first type, it should be readily hydrogenated catalytically to the corresponding saturated aminobutyric acid. In practice *Z* could not be altered by hydrogenation. Support for the alternative azetidine-type structure was obtained when *Z* was shown to contain an imino nitrogen atom by a test reported to be specific for the secondary amine group.

The most striking property of *Z* was its instability to treatment with mineral acids. Several hydrolysis products were detected on two-dimensional chromatograms (solvent *A* followed by solvent *D*) when *Z* was hydrolysed with 6*N* hydrochloric acid. Fig. 1 is a diagrammatic representation of a typical chromatogram. *S*1 (Fig. 1) was identified as homoserine by its chromatographic position and colour, its characteristic lactone formation under acid conditions (streak 1*a*) and its oxidation to aspartic acid. Two other products reacted with ninhydrin to give yellow-coloured spots (*S*2 and *S*3 on Fig. 1), whose colour later changed through a brownish hue to purple. Their initial ninhydrin colour might indicate unsaturation, and the fact that they could be catalytically hydrogenated to γ - and α -aminobutyric acids respectively seemed evidence in support of this idea. However, treatment of the compounds with aqueous or ethanolic ammonia gave $\alpha\gamma$ -diaminobutyric acid as the main product. This suggested an alternative explanation for the colour reactions and products formed on hydrogenation, namely that two chloroaminobutyric acids (γ -amino- α -chlorobutyric acid, *S*2, and α -amino- γ -chlorobutyric acid,

*S*3) were arising by treatment of *Z* with hydrochloric acid. If an azetidine-type ring structure for *Z* is assumed, such hydrolytic behaviour would be in accordance with the known manner of breakdown of the free base, azetidine, on hydrolysis with hydrochloric acid, when 1-amino-3-hydroxypropane and 1-amino-3-chloropropane are the products (Howard & Marekwald, 1899). γ -Amino- α -chlorobutyric acid was later synthesized and shown to have chromatographic properties identical with those of spot *S*2 on Fig. 1.

The fourth logical breakdown product, γ -amino- α -hydroxybutyric acid, was not detected on two-dimensional chromatograms run with solvents *A* and *D*. The reason for this later became apparent when the substance was synthesized and chromatographed; in phenol and butanol-acetic acid it has *R_f* values identical with those of homoserine. In collidine-lutidine, however, γ -amino- α -hydroxybutyric acid flows a little slower than homoserine. When the products of hydrolysis by hydrochloric acid of a larger amount of *Z* were re-examined on phenol/collidine-lutidine chromatograms (see Fig. 2), a small amount of a substance (*S*4) inseparable from added γ -amino- α -hydroxybutyric acid was shown to be present. This substance was oxidized by acidic potassium permanganate solution to give β -alanine.

When 3*N* sulphuric acid or 70 % (w/v) hydriodic acid was used for hydrolysis, the breakdown products were different. Although homoserine was a product in all cases, the nature of the other products as indicated by chromatography differed

Fig. 1. Positions on a two-dimensional chromatogram developed with phenol (*A*) and butanol-acetic acid (*D*) of the substances produced by hydrolysis of *Z* with hydrochloric acid. *S*1, homoserine; *S*1*a*, homoserine lactone; *S*2, γ -amino- α -chlorobutyric acid; *S*3, α -amino- γ -chlorobutyric acid. The position occupied by *Z* (before hydrolysis) is indicated by the dotted area; + marks the position alanine would occupy on a chromatogram run in these solvents.

Fig. 2. Positions on a two-dimensional chromatogram, developed with phenol (*A*) and collidine-lutidine (*B*), of the substances produced by hydrolysis of *Z* with hydrochloric acid. *S*1, homoserine; *S*1*a*, homoserine lactone; *S*2, γ -amino- α -chlorobutyric acid; *S*3, α -amino- γ -chlorobutyric acid; *S*4, γ -amino- α -hydroxybutyric acid. The position occupied by *Z* (before hydrolysis) is indicated by the dotted area; + marks the position alanine would occupy on a chromatogram run in these solvents.

Synthesis

The foregoing evidence strongly suggested that Z was azetidine-2-carboxylic acid. To confirm this conclusion azetidine-2-carboxylic acid was synthesized by two methods, the final stage in each being the removal of a molecule of hydrogen halide from an γ -amino- α -halogenobutyric acid under the

(a) Synthesis .

(b) Degradation

Fig. 3. Summary of synthetic and degradative stages for azetidine-2-carboxylic acid.

influence of barium hydroxide. Optically inactive azetidine-2-carboxylic acid was obtained in small yield from γ -aminobutyric acid by α -bromination, followed by removal of hydrogen bromide from the intermediate γ -amino- α -bromobutyric acid and ring closure by treatment with 0.5N barium hydroxide solution. An optically active azetidine-2-carboxylic acid, $[\alpha]_D^{20} + 102^\circ$, was obtained by treatment of α - γ -diaminobutyric acid dihydrochloride with a mixture of nitrous and hydrochloric acids to yield γ -amino- α -chlorobutyric acid, followed by elimination of hydrogen chloride and cyclization by treatment with barium hydroxide. This second synthesis was based on that of proline from ornithine (Hamilton, 1952), where a similar inversion of optical rotation occurred.

Both synthetic azetidine-2-carboxylic acid samples were shown to be identical with Z by chromatography (all solvents) and by the identical nature of their degradation products after hydrolysis with hydrochloric acid with those obtained from Z. The infrared absorption spectrum of the synthetic material ($[\alpha]_D^{20} + 120^\circ$) was identical with that of Z.

The synthetic and degradative steps described above for azetidine-2-carboxylic acid can be summarized as in Fig. 3.

Distribution

Azetidine-2-carboxylic acid would appear to be mainly restricted to plants belonging to the family Liliaceae with a less frequent occurrence in the

members of the families Agavaceae and Amaryllidaceae. An account of the qualitative occurrence of this and other recently discovered amino acids in many plants of these families is given by Fowden & Steward (1956), where the taxonomic implications of a study of their distribution are discussed. Quantitative estimates of the amounts of various amino acids, particularly azetidine-2-carboxylic acid, in plant materials were made by using, in a slightly modified form, the method of Thompson, Zacharius & Steward (1951) and Thompson & Steward (1951). All quantitative assays were made on two-dimensional chromatograms run first in phenol at pH 5.5 followed by the one-phase butanol-acetic acid mixture. The data obtained are presented in Table 1, where the amounts of α -amino or -imino nitrogen are expressed as a percentage of the total amino or imino nitrogen present in all the acids. Azetidine-2-carboxylic acid is present in widely different amounts in different species.

DISCUSSION

The identification of azetidine-2-carboxylic acid as a natural product has added to the known types of heterocyclic ring systems that can occur in plants. The ring has been reported to occur in a natural product on one previous occasion by Stoll, Renz & Brack (1951), who showed that it was present in the antibiotic nocardamin, where it formed a part of a more complex structure. The isolation of this lower

Table 1. *Relative amounts of various amino and imino acids present in some plants belonging to the families Liliaceae and Agavaceae*

The amount of α -amino or -imino nitrogen of each amino acid is expressed as a percentage of the total amino and imino nitrogen present in all the acids.

	Aspartic acid	Glutamic acid	Serine + Glycine	Asparagine	Threonine	Alanine	Glutamine	Lysine	Azetidine-2-carboxylic acid	Proline	Valine	γ -Aminobutyric acid	Other amino acids
<i>Convallaria majalis</i> (leaf)	3.3	5.4	1.0	1.5	0.2	2.4	7.3	0.1	75.7	1.8	0.1	1.2	0.1
<i>C. majalis</i> (seed)	0.8	5.0	1.0	—	0.3	1.4	0.2	—	70.1	1.8	1.3	1.2	15.4
<i>Maianthemum canadense</i> (leaf)	1.2	7.8	3.6	3.9	0.5	4.8	8.2	0.1	58.9	0.1	1.0	1.8	8.1
<i>M. canadense</i> (seed)	3.1	2.9	3.7	2.1	1.9	3.7	13.6	—	54.7	4.3	1.9	5.9	2.0
<i>Polygonatum</i> sp. (leaf)	3.3	8.1	3.5	1.7	0.4	3.5	16.7	0.1	58.0	8.2	1.4	0.8	1.2
<i>Rohdea japonica</i> (leaf)	0.4	3.9	4.5	1.9	0.7	5.7	0.7	0.1	66.5	1.1	0.6	7.4	0.2
<i>Ruscus aculeatus</i> (leaf)	0.9	6.4	4.1	—	1.8	27.9	1.4	1.3	38.7	0.7	1.6	13.9	1.4
<i>Bowiea volubilis</i> (stem)	1.7	4.6	2.0	0.5	0.5	17.1	1.0	1.8	61.7	—	0.5	7.9	0.5
<i>Liriope muscari</i> (leaf)	0.5	2.5	5.1	—	1.1	13.9	1.2	0.5	33.6	21.8	0.6	18.7	0.5
<i>L. spicata</i> (seed)	1.7	4.2	4.7	3.2	0.5	9.3	11.3	0.7	36.1	4.4	0.9	4.5	0.2
<i>Danae racemosa</i> (seed)	0.7	0.4	1.8	1.7	1.5	4.6	18.3	0.8	56.9	8.2	0.7	3.0	1.3
<i>Littonia modesta</i> (seed)	6.4	5.9	5.6	10.9	2.3	10.0	15.1	1.1	21.6	5.7	2.8	4.3	8.2
<i>Hosta sieboldiana</i> (seed)	5.0	12.5	4.1	10.5	1.5	8.2	23.2	0.4	11.1	6.4	3.7	3.6	9.9
<i>Dracaena fragrans</i> (leaf)	3.5	14.8	4.3	0.3	1.3	11.3	3.8	1.8	34.6	11.0	0.3	12.5	0.6
<i>D. sanderiana</i> (leaf)	0.2	1.3	1.9	—	0.5	7.4	10.4	0.5	61.5	10.9	0.2	5.1	0.2
<i>D. godseffiana</i> (leaf)	1.6	9.1	22.1	0.3	1.1	13.0	19.3	0.3	16.4	3.1	0.3	3.7	9.8

analogue of proline follows closely on the recent recognition of piperidine-2-carboxylic acid (pipecolic acid), the next higher analogue of proline, as a plant constituent (Zacharius, Thompson & Steward, 1952; Morrison, 1952). While present knowledge would indicate that proline is the most widely distributed of the three cyclic imino acids throughout the plant kingdom, pipecolic acid is also found to be present in species of many plant families and is often most abundant in seeds (Zacharius, Thompson & Steward, 1954). But of the three substances it is azetidine-2-carboxylic acid that appears capable of being accumulated by plants to the greatest degree, e.g. about 3 g./kg. fresh weight in *Convallaria*, *Polygonatum* and *Rohdea* leaves (see Table 1). The seeds of *Convallaria* contain all three imino acids.

Recently the presence in plants of allohydroxyproline (obtained from *Santalum album* by Radhakrishnan & Giri, 1954) and 5-hydroxypipecolic acid (obtained from *Rhapis flabelliformis* by Virtanen & Kari, 1954; and from *Baikiaea plurijura* by Grobbelaar *et al.* 1955) have been confirmed by isolation. Now that the presence of a third simple heterocyclic ring is established, the possibility exists that hydroxyazetidine-2-carboxylic acids may eventually also be shown to be plant constituents.

The complex way in which azetidine-2-carboxylic acid broke down when treated with concentrated hydrochloric acid to give four products at first suggested that Z might be a peptide-like compound. However, no biuret reaction was obtained, and the determined molecular weight proved this idea to be impossible. Virtanen & Linko (1955) reported that *Polygonatum* contained large amounts of a new compound of imino acid type. The properties they described for this substance were identical with those found for azetidine-2-carboxylic acid, with the exception of their published molecular weight of 303. Virtanen & Linko originally suggested that their compound had a more complex cyclic structure, but Virtanen (1955) later concluded that his

original molecular weight was in error and that the substance isolated from *Polygonatum* was in fact azetidine-2-carboxylic acid.

The natures and relative quantities of the amino acids produced when azetidine-2-carboxylic acid broke down in the presence of hydrochloric acid do not give a clear indication of the character of the primary steps of the degradation process. Hydrolytic additive fission of the ring may accompany similar fission by hydrogen chloride, addition of water occurring largely across the 1:4-linkage and addition of hydrogen chloride preferentially at the 1:2-linkage, or the primary fission may result only in hydrogen chloride addition, followed by varying degrees of hydrolysis of the two chloroaminobutyric acids produced. If the latter type of fission occurred, it would follow that the γ -chloro acid was more readily hydrolysed under these conditions than was the α -chloro acid, since homoserine was found in the final products in much larger quantities than γ -amino- α -hydroxybutyric acid. Several pieces of indirect evidence relating to the ease of replacement of the γ -chloro atom by hydroxyl have been obtained. When an attempt was made to synthesize S3 by condensation of 1:2-dichloroethane with the diethyl ester of α -acetamidomalonic acid, although the initial condensation step was very incomplete, the product formed when 6N hydrochloric acid was used to effect hydrolysis and decarboxylation of the intermediate ester was mainly homoserine and its lactone, together with only small amounts of α -amino- γ -chlorobutyric acid. Also, when the preparation of azetidine-2-carboxylic acid was attempted by treating $\alpha\gamma$ -dibromobutyric acid (prepared by bromination of γ -butyrolactone; Plieninger, 1950) with concentrated aqueous ammonia, the product again consisted mainly of homoserine, only traces of azetidine-2-carboxylic acid being formed. Lastly, treatment of S3 (α -amino- γ -chlorobutyric acid) with concentrated ammonia solution preferentially replaced the γ -chloro atom by an hydroxyl group rather than by an amino group, whereas similar treatment of S2

(γ -amino- α -chlorobutyric acid) gave α γ -diaminobutyric acid and only a trace of γ -amino- α -hydroxybutyric acid.

Whenever a new nitrogenous compound is found to occur naturally in significant amounts and its structure has been established, it becomes possible to speculate on its biogenesis and the ways in which it might be concerned with the metabolism of related compounds. By analogy with the biogenesis of proline from glutamic acid or ornithine via glutamic- γ -semialdehyde in micro-organisms (Vogel & Davis, 1952; Vogel, 1953; Fincham, 1953; Vogel & Bonner, 1954), and that of pipercolic acid from lysine in animals and plants (Grobelaar & Steward, 1953; Lowy, 1953; Rothstein & Miller, 1953; Schweet, 1954), it might be supposed that azetidine-2-carboxylic acid arises in a similar manner from one or more of the amino acids aspartic acid, homoserine and diaminobutyric acid. All three possible precursors have been detected in plants, and the details of the conversion of aspartic acid into homoserine have recently been established by Black & Wright (1955*a, b, c*) with liver preparations. Thus possible pathways leading to the biosynthesis of azetidine-2-carboxylic acid are as shown in the diagram on p. 327.

Experiments are planned to test for the presence in plants of some of these interconversions.

EXPERIMENTAL

Chromatographic techniques. All paper chromatograms were run on Whatman no. 3 chromatographic grade filter paper under conditions which were essentially those of Consden, Gordon & Martin (1944). The solvents used included: (A) water-saturated phenol used after adjustment to pH 5.5 (measured with a glass electrode) by addition of NaOH (Steward, Zacharius & Pollard, 1955); (B) water-saturated phenol with NH_3 soln. (Dent, 1947); (C) the *n*-butanol-acetic acid-water mixture (4:1:5, by vol.) of Partridge (1948); (D) a one-phase solvent consisting of 450 ml. of *n*-butanol, 50 ml. of acetic acid and 145 ml. of water; (E) the collidine-lutidine mixture of Dent (1947), and (F) 70% (v/v) *n*-propanol solution. A 0.1% (w/v) solution of ninhydrin dissolved either in 95% (v/v) ethanol or *n*-butanol saturated with water was used as the spraying agent.

Isolation of Z. Fresh *Convallaria* leaves (1.85 kg.), collected just before the plants flowered, were macerated in small batches with a total volume of 4 l. of 70% (v/v) ethanol in an Atomix blender. The mush was pressed through muslin and the filtrate was centrifuged to remove the finer debris. It was then evaporated to small bulk (250 ml.) *in vacuo* at 50° to remove most of the ethanol, diluted to 750 ml. with water, and extracted twice with 500 ml. portions of CHCl_3 to remove the leaf pigments. The aqueous layer, further diluted to 1.2 l., was shaken overnight with 50 g. of acetic acid-treated charcoal (British Drug Houses Ltd., absorbent grade) to absorb tyrosine and phenylalanine, and remaining colouring matter (Partridge, 1949). The final filtrate was applied to a Zeo-Karb 215 column (resin mesh 80–100).

The column used contained a packed resin bed, depth 700 mm., diameter 35 mm. The treated extract was applied to the column at a rate of 200 ml./hr., all the amino acids being absorbed. The column was washed with 2.5 l. of water, followed by elution of the amino acids with a solution of 0.15N- NH_3 soln. (2.8 l.); 20 ml. fractions of the eluate were collected. Z was present in fractions nos. 37–75, together with neutral and hydroxyamino acids. Fractions containing Z in appreciable amounts (nos. 44–63) were pooled (vol. 400 ml.) and evaporated to dryness at 50°; they yielded a partially crystalline residue I (1.81 g.).

Further purification of Z was achieved by means of paper chromatography. Sheets of Whatman no. 3 filter paper (22 in. $\times$ 18 in.) were washed with the following sequence of reagents: 0.5N-HCl, water, 0.5N-NaOH, water. They were then dried at 40°. Residue I was dissolved in water to give 4 ml. of final solution, and 1 ml. was applied as a continuous band across the 18 in. dimension of each of four such washed sheets of paper. The chromatograms were run in solvent B for 24 hr. and dried at 40°. The areas containing Z were cut out after its position on each chromatogram had been determined by ninhydrin development of three narrow location strips cut along the direction of solvent flow. Z was eluted from the areas of paper by macerating them in water, and the filtrate was evaporated to dryness at 50° to yield a residue II (0.54 g.).

Residue II was dissolved in water to give 2 ml. of final solution, and 1 ml. was applied to each of two washed sheets of paper. The chromatograms were developed in solvent C for 48 hr., and Z was located and isolated as described above. The residue III obtained one vaporation (250 mg.) was a buff-coloured, crystalline material, and consisted of Z apparently free of other amino acids. It was recrystallized from 95% (v/v) methanol and yielded a first crop of 151 mg. of pure Z.

A second isolation was made from 2.2 kg. of *Convallaria* leaves, grown the following year, with columns of Dowex 50, $\times$ 4 (mesh 100–200). The leaf extract, treated as described above before application to the Zeo-Karb, was first applied to a column containing a packed resin bed, depth 420 mm., diameter 30 mm. After absorption of the amino acids and thorough washing of the column, the amino acids were eluted with *n*- NH_3 soln., no fractionation of the eluate (vol. 500 ml.) being made. The eluate containing the amino acids was diluted to 1.1 l. and reappplied to a two-stage column (upper 375 mm. $\times$ 30 mm., lower 250 mm. $\times$ 20 mm.) at a rate of 60 ml./hr. The column was washed with 3 l. of water and the amino acids were eluted with 0.15N- NH_3 soln.; 20 ml. fractions of the eluate (total vol. 1.1 l.) were collected. Z was eluted from the column after aspartic and glutamic acids and before serine and threonine. Fractions containing Z (nos. 14–26) were pooled and evaporated to give a residue (0.92 g.) containing glutamic acid, serine and threonine as impurities. The impure Z was purified as above by chromatography on four sheets of washed paper with phenol as solvent. Chromatographically pure Z (520 mg.) was obtained, which yielded 350 mg. of recrystallized material.

Physical properties of Z. The substance did not melt, but on heating began to discolour at about 200° and continued to darken until 310°, when heating was stopped. It had $[\alpha]_D^{20} - 108^\circ$ in water (*c*, 3.6). (Found: C, 47.6; H, 6.8; N, 13.4. $\text{C}_4\text{H}_7\text{O}_2\text{N}$ requires C, 47.5; H, 6.9; N, 13.8%.) Determination of the molecular weight of Z was made by the method of Barger (1904), aqueous proline solutions of known

concentration being used as the comparative standards. The method as used, whilst not capable of yielding a result of extreme accuracy, indicated that Z had a molecular weight of about 100. It therefore established that the empirical and molecular formulae for Z were identical. Z was very soluble in both cold and hot water, but virtually insoluble in absolute ethanol.

Presence of imino N in Z. Feigl (1954) describes a specific spot test for the secondary amine group. Z (1 mg.) was dissolved in 0.25 ml. of water, and a drop of this solution was mixed with a drop of the test reagent (1% (w/v) sodium nitroprusside solution to which 10% by volume of acetaldehyde had been added). Finally a drop of 2% (w/v) Na_2CO_3 solution was added with stirring to the mixture, when a strong blue colour, characteristically given by compounds containing imino groups, developed rapidly. When proline was tested under the same conditions the reaction appeared slightly more sensitive, but the α -amino acids tested gave no reaction.

Lability of Z to acid. In testing for the possible presence of an amide group in Z, the compound was heated with N-HCl for 3 hr. at 100° . Several reaction products, in addition to much unchanged Z, were detected chromatographically (indicating that Z was somewhat unstable to acid). More vigorous acid hydrolysis with 6N-HCl for 24 hr. at 100° completely decomposed Z to yield products in the positions shown in Fig. 1 on two-dimensional chromatograms developed with solvents A and D. S1 was probably homoserine, the streak S1a being due to the gradual decomposition, during the development with phenol, of homoserine lactone, produced from homoserine under the conditions of acid hydrolysis. Synthetic homoserine, after heating with 6N-HCl as above, showed identical chromatographic behaviour, producing spots in the positions of those of S1 and S1a.

To obtain information regarding the nature of the compounds S2 and S3 (Fig. 1), 100 mg. of Z was hydrolysed with 6N-HCl, and after repeated evaporation of the hydrolysate to remove HCl the residue was dissolved in 1 ml. of water and applied as a band across a sheet of washed Whatman no. 3 paper. The chromatogram was developed for 40 hr. in *n*-butanol-acetic acid (solvent D) and, after drying, the positions of the hydrolysis products were determined by the location-strip technique. The products S2 and S3 were well resolved and were isolated from the paper in a manner similar to that described for Z. S1 (homoserine) and S1a (homoserine lactone, which had a slightly higher R_F than the parent compound) were not isolated separately.

The purity of the materials isolated was checked by chromatography in phenol (A), butanol-acetic acid (D) and collidine-lutidine (E). Whilst S2 and S3 were free of other ninhydrin-reacting substances, chromatograms developed with solvent E showed that the S1/S1a complex contained small amounts of a further ninhydrin-reactive substance S4 (see Fig. 2). The isolated S1 plus S1a was therefore further purified on a paper chromatogram run for 36 hr. in collidine-lutidine, from which separate fractions of S1 plus S1a and S4 were isolated.

Nature of S1 and S1a. S1a was converted into a substance which behaved like S1 on paper chromatograms by treatment with 10N- NH_3 soln. at 100° . Such treatment converts homoserine lactone into homoserine.

S1 was inseparable from homoserine when the two substances were chromatographed together on paper chromatograms developed with solvents B, C, E and F.

S1 was slowly oxidized by a solution of 1% (w/v) KMnO_4 in 20% (w/v) H_2SO_4 to a substance chromatographically identical with aspartic acid.

These observations support the idea that S1 was homoserine, and S1a was homoserine lactone.

Identity of S2. S2, dissolved in 0.5N acetic acid was readily converted into γ -aminobutyric acid by treatment with H_2 at a pressure of 50 lb./sq.in. in the presence of Adams's PtO_2 catalyst. The γ -aminobutyric acid was identified chromatographically by comparison with an authentic sample of synthetic material. It had m.p. 191° , and the mixed m.p. with an authentic sample (m.p. 191°) was 189° .

When heated with 10N- NH_3 soln. in a sealed ampoule at 100° for 3-4 hr., S2 gave mainly α -diaminobutyric acid, together with traces of azetidine-2-carboxylic acid and γ -amino- α -hydroxybutyric acid. Each product was recognized by chromatographic comparison with synthetic material; the characteristic ninhydrin colour responses of α -diaminobutyric acid (red-brown) and azetidine-2-carboxylic acid (brown) aided in their identification.

S2 was inseparable from synthetic γ -amino- α -chlorobutyric acid when the two substances were chromatographed together in solvents A, D and E. The synthetic material reacted in the same way as S2 when hydrogenated or treated with NH_3 soln.

These observations support the idea that S2 was γ -amino- α -chlorobutyric acid.

Identity of S3. When hydrogenated under the conditions described above, S3 gave α -aminobutyric acid (identified chromatographically). When treated with aqueous 10N- NH_3 soln., some α -diaminobutyric acid was formed together with larger amounts of homoserine. From these facts and the similarities of the ninhydrin colours of S2 and S3, it is inferred that S3 was α -amino- γ -chlorobutyric acid. No satisfactory method of synthesizing this compound has been found (see Discussion).

Identity of S4. S4 was shown to be inseparable from synthetic γ -amino- α -hydroxybutyric acid when the two substances were chromatographed together in solvents A, D and E. S4 was oxidized far more readily than homoserine by 1% (w/v) of KMnO_4 in 20% (w/v) of H_2SO_4 , giving a product chromatographically indistinguishable from β -alanine. These observations support the idea that S4 was γ -amino- α -hydroxybutyric acid.

Under the conditions used for the hydrolysis of Z with HCl, homoserine and γ -amino- α -chlorobutyric acid were the main reaction products, being formed in approximately equal amounts, and γ -amino- α -hydroxybutyric acid and α -amino- γ -chlorobutyric acid were produced in much smaller amounts.

Other hydrolytic reagents. Hydrolysis of Z with 3N- H_2SO_4 at 100° for 24 hr. yielded homoserine, and two other products which moved very slowly on paper chromatograms in solvent D and gave a normal purple colour with ninhydrin. Their identities were not studied further.

Treatment of Z with 70% (w/v) HI and a trace of red P at 140 - 150° for 4 hr. gave homoserine, homoserine lactone and γ -aminobutyric acid as the main products together with small amounts of α -aminobutyric acid (all substances recognized chromatographically).

Z was apparently unchanged after treatment with 5N- $\text{Ba}(\text{OH})_2$ solution at 100° for 24 hr.

Synthesis of γ -amino- α -chlorobutyric acid. In the synthesis of this substance use was made of the fact that the α -amino group of an amino acid is far more readily replaced by another substituent (e.g. OH or Cl) than are amino groups on other carbon atoms.

L- α -Diaminobutyric acid dihydrochloride (225 mg.), prepared from L-glutamic acid by the method of Adamson (1939), was dissolved in 10 ml. of 10N-HCl in a Kjeldahl flask. Finely powdered AgNO_3 (0.75 g.) was then added to 8 ml. of ice-cold N-HCl, and the mixture filtered through glass wool into the solution of diaminobutyric acid. This solution was then boiled for 1 hr., 6N-HCl solution being added as necessary. The contents of the flask were then evaporated to dryness three times to remove HCl. The residue consisted of γ -amino- α -chlorobutyric acid, together with unchanged diaminobutyric acid. These two substances were readily separated on a paper chromatogram developed with solvent *D* for 40 hr. *γ -Amino- α -chlorobutyric acid* was isolated from the chromatogram by procedures described above, and purified by repeated precipitation from concentrated aqueous solutions by the addition of ethanol. Yield 30 mg., m.p. 171°. (Found: C, 35.2; H, 5.7; N, 9.9; Cl, 25.5. $\text{C}_4\text{H}_8\text{O}_2\text{NCl}$ requires C, 34.9; H, 5.8; N, 10.2; Cl, 25.8%). The optical configuration of the product was not determined, although the conditions used probably caused racemization to occur (compare Hamilton, 1952).

Synthesis of γ -amino- α -hydroxybutyric acid. This substance was prepared from α -diaminobutyric acid under conditions normally used for the replacement of an α -amino group by an OH group. Silver nitrite (0.75 g.) was added to 10 ml. of N-H₂SO₄ with stirring. The mixture was filtered through glass wool into a solution of 225 mg. of L- α -diaminobutyric acid dihydrochloride dissolved in 10 ml. of water. After standing for 30 min. most of the Ag^+ and SO_4^{2-} ions were removed by addition of hot $\text{Ba}(\text{OH})_2$, and solid BaCO_3 was added to complete the precipitation of Ag_2O and BaSO_4 . The filtrate was then evaporated to dryness; the residue consisted of unchanged diaminobutyric acid and γ -amino- α -hydroxybutyric acid. These two substances were separated on a chromatogram developed in solvent *D* for 40 hr., and the γ -amino- α -hydroxybutyric acid was isolated free of other ninhydrin-reactive substances. It was very soluble in water, and the syrup obtained only slowly crystallized when desiccated. Recrystallization of the small quantity obtained (35 mg.) was not achieved.

γ -Amino- α -hydroxybutyric acid has been prepared by decarboxylation of γ -hydroxyglutamic acid with *Escherichia coli* by Virtanen & Hietala (1955b), who observed chromatographic properties for their material similar to those recorded on Fig. 1 for *S*₄.

Synthesis of azetidine-2-carboxylic acid. (a) *From γ -aminobutyric acid.* γ -Aminobutyric acid (200 mg.) was mixed with 0.1 ml. of Br_2 and a trace of red P and heated in a sealed ampoule at 100° for 12 hr. A small portion of the reddish brown viscous product was dissolved in water and the solution chromatographically examined. The γ -aminobutyric acid had been largely converted into a substance moving slightly slower than the parent compound in butanol-acetic acid (solvent *D*), and which reacted with ninhydrin to give a yellowish brown colour which slowly changed to purple over 24 hr. This intermediate, although not separately isolated, was almost certainly γ -amino- α -bromobutyric acid, by the similarity of its properties (e.g. colour behaviour with ninhydrin; reaction with conc. NH_3

soln. to give α -diaminobutyric acid) to those of γ -amino- α -chlorobutyric acid, and the fact that halogenation normally occurs at the carbon atom adjacent to a carboxyl group. The remaining product of bromination was dissolved in 10 ml. of water, and 10 ml. of hot N-Ba(OH)₂ was added. The mixture was refluxed for 20 min. and the barium was precipitated by the addition of the theoretical quantity of H₂SO₄. Bromide was removed from the filtrate by stirring in freshly prepared Ag₂CO₃, and after a further filtration silver was removed by H₂S. The final filtrate was evaporated to dryness; the residue consisted of azetidine-2-carboxylic acid, some unchanged γ -aminobutyric acid and a trace of γ -amino- α -hydroxybutyric acid. The azetidine-2-carboxylic acid was purified by chromatography on a sheet of Whatman no. 3 paper developed in solvent *D* for 48 hr. The isolated material was recrystallized from 95% (v/v) methanol to yield 38 mg. of optically inactive azetidine-2-carboxylic acid. On heating, the crystals gradually darkened in colour above 200° without melting.

(b) *From L- α -diaminobutyric acid dihydrochloride* (prepared as Adamson, 1939). L-Diaminobutyric acid dihydrochloride (5 g.) was dissolved in 150 ml. of 10N-HCl in a 400 ml. beaker placed in a water bath at 60°. Finely powdered AgNO_3 (10 g.) was then added to 120 ml. of ice-cold N-HCl with stirring. Silver chloride was removed by filtration through glass wool, the filtrate being run into the beaker containing the diaminobutyric acid solution. The mixture was kept at 60° for 20 min. and then evaporated rapidly *in vacuo* at 50° on a rotary evaporator. The residue was found by paper chromatography to consist of γ -amino- α -chlorobutyric acid, together with some unchanged diaminobutyric acid and a small amount of γ -amino- α -hydroxybutyric acid. The isolation of the chloroamino acid was not attempted at this stage, but the residue was dissolved in 100 ml. of water and 100 ml. of hot N-Ba(OH)₂ was added. The mixture was refluxed for 20 min. and the azetidine-2-carboxylic acid formed was isolated by the procedure used in method (a) above. The yield of recrystallized material was 1.3 g. It had $[\alpha]_D^{20} + 102^\circ$ in water (c, 3.6). The substance did not melt but slowly darkened in colour when heated above 200°. (Found: C, 47.5; H, 6.9; N, 13.5. $\text{C}_4\text{H}_7\text{O}_2\text{N}$ requires C, 47.5; H, 6.9; N, 13.8%). The molecular weight by Barger's method was about 100.

The optical activity of the final product was dependent upon the temperature and the time of heating of the diaminobutyric acid with the HNO₃ and HCl mixture in the first stage of the preparation. When a temperature of 70° was used for 40 min., the azetidine-2-carboxylic acid obtained had $[\alpha]_D^{20} + 79^\circ$, and boiling for 90 min. gave an optically inactive product.

Identity of Z with synthetic azetidine-2-carboxylic acid. The two substances showed identical chromatographic behaviours in solvents *A*, *D*, *E* and *F*, and reacted with ninhydrin under normal conditions (chromatograms heated to about 80°) to give characteristic brown-coloured spots. When the chromatograms were sprayed with ninhydrin and allowed to stand at laboratory temperature to develop colour, the substances reacted slowly to give a more normal colour (purple), which later changed to brown on heating.

The infrared spectra of the natural material (*Z*) and the optically active synthetic compound ($[\alpha]_D^{20} + 102^\circ$) were identical (Fig. 4). The infrared spectrum of synthetic optically inactive azetidine-2-carboxylic acid was quite different. Marked differences have been found to occur

Fig. 4. Infrared spectra of azetidine-2-carboxylic acid. A, natural, (-) form; B, synthetic, (+) form; C, synthetic (racemic). All spectra were determined with Nujol mulls of the imino acid.

between the spectra of other active and racemic compounds (amino acids including γ -methyleneglutamic acid and γ -methyleneglutamine, Wailes, Whiting & Fowden, 1954; proline, Hamilton, 1952; pipercolic acid, personal communication from Professor F. C. Steward, Cornell University).

All spectra were determined on mulls prepared by intimately grinding the samples with Nujol (liquid paraffin).

Quantitative extraction of amino acids from plant materials. Plant material was macerated with ethanol to give a final ethanol concentration of 70% (v/v). The extraction was repeated until virtually complete extraction of soluble nitrogenous compounds had been achieved. The combined extracts were evaporated to dryness at laboratory temperature and the residues redissolved in small volumes of water. Each solution was twice extracted with an equal volume of CHCl_3 to remove pigments, and the aqueous layers were re-evaporated. The residues were finally dissolved in water so that 1 ml. of each solution contained the soluble substances present originally in 5 g. of fresh leaf material or in 2.5 g. of air-dry seed material. Portions (30 μl .) of all extracts were used in the preparation of the quantitative chromatograms.

SUMMARY

1. A new cyclic imino acid, azetidine-2-carboxylic acid, was isolated from leaves of *Convallaria majalis* (lily-of-the-valley), and its structure confirmed by comparison with synthetic material.

2. DL-Azetidine-2-carboxylic acid was synthesized from γ -aminobutyric acid; optically active azetidine-2-carboxylic acid [(+)-form] was synthesized from L- α -diaminobutyric acid.

3. Four products resulted when azetidine-2-carboxylic acid was treated with 6N hydrochloric acid. These products were identified as homoserine, γ -amino- α -hydroxybutyric acid, γ -amino- α -chlorobutyric acid and α -amino- γ -chlorobutyric acid, the nature of the first three being confirmed by comparison with synthetic material.

4. The amounts of azetidine-2-carboxylic acid and other amino acids present in certain members of the Liliaceae and Agavaceae were determined. The

new imino acid was a major soluble nitrogenous constituent of several of the plants.

5. Possible steps involved in the biogenesis of azetidine-2-carboxylic acid have been briefly discussed.

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REFERENCES

- Adamson, D. W. (1939). *J. chem. Soc.* p. 1564.
 Barger, G. (1904). *J. chem. Soc.* p. 286.
 Black, S. & Wright, N. G. (1955a). *J. biol. Chem.* **213**, 27.
 Black, S. & Wright, N. G. (1955b). *J. biol. Chem.* **213**, 39.
 Black, S. & Wright, N. G. (1955c). *J. biol. Chem.* **213**, 51.
 Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
 Dent, C. E. (1947). *Biochem. J.* **41**, 327.
 Done, J. & Fowden, L. (1952). *Biochem. J.* **51**, 451.
 Feigl, F. (1954). *Spot Tests. II. Organic Applications*, p. 189. New York: Elsevier.
 Fincham, J. R. S. (1953). *Biochem. J.* **53**, 313.
 Fowden, L. (1955). *Nature, Lond.*, **176**, 347.
 Fowden, L. & Steward, F. C. (1956). *Ann. Bot., Lond.* (in the Press).
 Grobbelaar, N., Pollard, J. K. & Steward, F. C. (1955). *Nature, Lond.*, **175**, 703.
 Grobbelaar, N. & Steward, F. C. (1953). *J. Amer. chem. Soc.* **75**, 4341.
 Hamilton, P. B. (1952). *J. biol. Chem.* **198**, 587.
 Howard, C. H. & Marekwald, W. (1899). *Ber. dtsch. chem. Ges.* **32**, 2031.
 King, F. E., King, T. J. & Warwick, A. J. (1950). *J. chem. Soc.* p. 3590.
 Lowy, P. H. (1953). *Arch. Biochem. Biophys.* **47**, 228.
 Morrison, R. I. (1952). *Biochem. J.* **50**, xiv.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
 Plieninger, H. (1950). *Ber. dtsch. chem. Ges.* **83**, 265.
 Radhakrishnan, A. N. & Giri, K. V. (1954). *Biochem. J.* **58**, 57.
 Rothstein, M. & Miller, L. L. (1953). *J. Amer. chem. Soc.* **75**, 4371.
 Schweet, R. S. (1954). *J. biol. Chem.* **208**, 603.
 Steward, F. C., Zacharius, R. M. & Pollard, J. K. (1955). *Ann. Acad. Sci. Fenn. A. II*, **60**, 321.
 Stoll, A., Renz, J. & Brack, A. (1951). *Helv. chim. acta*, **34**, 862.
 Thompson, J. F. & Steward, F. C. (1951). *Plant Physiol.* **26**, 421.
 Thompson, J. F., Zacharius, R. M. & Steward, F. C. (1951). *Plant Physiol.* **26**, 375.
 Virtanen, A. I. (1955). *Nature, Lond.*, **176**, 984.
 Virtanen, A. I. & Hietala, P. K. (1955a). *Acta chem. scand.* **9**, 175.

- Virtanen, A. I. & Hietala, P. K. (1955b). *Acta chem. scand.* **9**, 549.
- Virtanen, A. I. & Kari, S. (1954). *Acta chem. scand.* **8**, 1290.
- Virtanen, A. I. & Kari, S. (1955). *Acta chem. scand.* **9**, 170.
- Virtanen, A. I. & Linko, P. (1955). *Acta chem. scand.* **9**, 551.
- Virtanen, A. I., Uksila, E. & Matikkala, E. J. (1954). *Acta chem. scand.* **8**, 1091.
- Vogel, H. J. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 578.
- Vogel, H. J. & Bonner, D. M. (1954). *Proc. nat. Acad. Sci., Wash.*, **40**, 688.
- Vogel, H. J. & Davis, B. D. (1952). *J. Amer. chem. Soc.* **74**, 109.
- Wailes, P., Whiting, M. C. & Fowden, L. (1954). *Nature, Lond.*, **174**, 130.
- Zacharius, R. M., Thompson, J. F. & Steward, F. C. (1952). *J. Amer. chem. Soc.* **74**, 2949.
- Zacharius, R. M., Thompson, J. F. & Steward, F. C. (1954). *J. Amer. chem. Soc.* **76**, 2908.

The Composition of Isolated Cerebral Tissues: Ascorbic Acid and Cozymase

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Isolated cerebral tissues in simple, oxygenated glucose salines reproduce many characteristics of the tissue *in situ*. In particular, energy-rich compounds are in part resynthesized, and this discovery encouraged search for *in vitro* counterparts to excitation. Metabolic responses to electrical stimulation were indeed found (McIlwain, 1951) but were not transmitted to the same extent as in the tissue *in vivo* (McIlwain, Gerard & Ochs, 1952; McIlwain, unpublished work). While this lack may be the result of factors other than chemical ones, it appeared pertinent to examine in more detail the composition of the isolated tissue and to attempt to make good any deficiencies found in it. Previous relevant work includes that of Stern, Eggleston, Hems & Krebs (1949) and Krebs, Eggleston & Turner (1951) on glutamate and K^+ ions; of LeBaron (1955) and McIlwain & Tresize (1956) on glycogen, lactate and glucose, and of Thomas & McIlwain (1956) on Cl^- ions. In the present paper the ascorbic acid and cozymase of rat and guinea-pig cerebral cortex are studied from this point of view.

Ascorbic acid appeared of special interest, for it is at high level in the brain. Concentrations in the hypophysis almost equal those of the adrenal, and in the cerebrospinal fluid of mammals are about twice those of blood plasma (see Lloyd & Sinclair, 1953). Dehydroascorbic acid on injection markedly affects the central nervous system (Patterson & Mastin, 1951). Cozymase has already been studied in separated cerebral tissues in this laboratory (Gore, Ibbott & McIlwain, 1950) and present observations were made to see whether its level changed with

induced changes in tissue metabolism. With both ascorbic acid and cozymase, the present observations were limited to the total quantity of substance without differentiating between oxidized and reduced forms.

EXPERIMENTAL

Tissues. The tissues were from adult rats and guinea pigs. Albino rats of about 300 g. were used, which had been maintained on rat cubes, diet no. 41, obtained from the Associated London Flour Millers. Guinea pigs weighing 400–500 g. had been fed on diet no. 18 from the same source. Animals were stunned by a blow on the neck and were bled. The brain was removed and opened, and large slices 0.35 mm. thick were prepared quickly (see McIlwain, 1951). These were weighed after draining from saline, some fitted to electrodes, and placed in the saline of the experimental vessel. Salines were those of Rodnight & McIlwain (1954), quick-transfer electrodes and procedures those of Heald & McIlwain (1956), and the grid electrodes, type H of Ayres & McIlwain (1953), wound with enamelled silver wire. This last paper also gives details of electrical pulses, manometric vessels and procedures. Incubation was at 37.5°, in manometric vessels (except when stated otherwise), in 3.5 ml. of saline. This was usually oxygenated; in anaerobic experiments N_2 or $N_2 + CO_2$ was used with yellow phosphorus in a centre well.

Estimations. Lactic acid was determined according to Barker & Summerson (1941). For ascorbic acid estimation the tissue and a sample of saline were placed in 4% (w/v) trichloroacetic acid and the method of Roe & Kuether (1943) was applied. Total cozymase was determined with apozymase (Axelrod & Elvehjem, 1939) after extraction with hot buffered nicotinamide (Gore *et al.* 1950). A specimen of cozymase from Sigma Chemical Co. was used as substrate and as standard in the apozymase assay. Spectrophotometric determination showed it to be of 75% purity.

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