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AZETIDINE-2-CARBOXYLIC ACID BREAKDOWN BY SOIL MICRO-ORGANISM

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Abstract—An *Agrobacterium* sp. was isolated from a soil extract; the organism could utilize azetidine-2-carboxylic acid as a sole nitrogen source for growth. The organism has been used to study pathways involved in azetidine-2-carboxylic acid degradation. The initial step appears to consist of a hydrolytic ring fission leading to α-hydroxy-γ-aminobutyric acid. This compound subsequently undergoes transamination with pyruvate to yield alanine. Some properties of the enzymes are described.

INTRODUCTION

AZETIDINE-2-CARBOXYLIC acid, the lower imino-acid homologue of proline, occurs in high concentration in many members of the family Liliaceae.[1] Its biosynthesis seems to be a feature peculiar to some members of this family and to a very few species from the allied families, Agavaceae and Amaryllidaceae.

At the present time, little information is available concerning the degradation of the imino acid in liliaceous plants. Indeed, such degradation appears to be very slow and little metabolism of ^{14}C-labelled azetidine-2-carboxylic acid occurred during 48 hr after introduction into excised leaves of *Convallaria majalis*.[2] This extremely slow breakdown is probably responsible in part for accumulations of the imino acid in amounts equivalent to about 3·5 and 6·5 per cent of the dry weight of certain tissues of *Convallaria* and *Polygonatum* plants respectively.[3] This paper presents evidence concerning the degradative pathway operative in a soil micro-organism (*Agrobacterium* sp.) which can utilize azetidine-2-carboxylic acid as its sole source of nitrogen for growth. The details of the initial steps have been established giving a degradative pattern against which a new comparative study may be made using higher plants and labelled metabolites.

Other studies have shown that azetidine-2-carboxylic acid acts as a proline analogue becoming incorporated into protein in place of residues of proline and thereby acting as a lethal agent when supplied to germinating seedlings, *Escherichia coli* and developing chick embryos.[4,5] Liliaceous plants are unaffected by their own toxic product because their proline activating enzyme, unlike that of sensitive species, discriminates against azetidine-2-carboxylic acid by failing to activate it.[6] Observations relevent to activation and incorporation of azetidine-2-carboxylic acid by the resistant species of *Agrobacterium* being used are also recorded.

[1] L. FOWDEN and F. C. STEWARD, *Ann. Bot.* N.S. **21**, 53 (1957).
[2] L. FOWDEN and M. BRYANT, *Biochem. J.* **71**, 210 (1959).
[3] L. FOWDEN, *Biochem. J.* **71**, 643 (1959).
[4] L. FOWDEN and M. H. RICHMOND, *Biochim. Biophys. Acta* **71**, 459 (1963).
[5] L. FOWDEN, *J. Exp. Bot.* **14**, 387 (1963).
[6] P. J. PETERSON and L. FOWDEN, *Nature* **200**, 148 (1963).

RESULTS

The organism used was identified by Dr. A. J. Holding (Edinburgh School of Agriculture) as an *Agrobacterium* closely related to *A. radiobacter*. The methods used for its isolation and identification are outlined in the experimental section. It was chosen for these studies in preference to a number of other isolates all capable of degrading azetidine-2-carboxylic acid because (*a*) it grew rapidly as a single cell culture on a simple glucose–mineral salts medium, (*b*) its growth was not associated with production of mucilage or pigments, and (*c*) it degraded azetidine-2-carboxylic acid very rapidly.

Initial experiments established that the organism must be grown in a medium containing azetidine-2-carboxylic acid (for example medium F, experimental section) if cells are to produce the degradative enzyme system. Later it was shown that only the enzyme catalysing the primary ring-fission step (reaction A, Fig. 1) was adaptive in character: the transaminase (reaction D) required for the next step was present in cells grown in a medium where NH_4 ion formed the only nitrogen source.

Degradation by Whole Cells

Cells grown in medium F were harvested, twice washed with sterile water and suspended in 0·04 M-phosphate buffer, pH 7·0. Azetidine-2-carboxylic acid was added to the dense cell suspension at a final concentration of 1 mg/ml and the mixture was incubated at 27 with shaking. Samples were withdrawn at intervals and pipetted into 2 vol ethanol to stop the reaction. Cell debris was sedimented and portions of the supernatant were examined by paper chromatographic and paper electrophoretic techniques to detect degradation products. An initial breakdown product, identified as α-hydroxy-γ-aminobutyric acid, was recognized after 3 min. Its concentration increased until all the azetidine-2-carboxylic acid had disappeared (about 2 hr) and then decreased. After 10 min alanine was noticed as an additional product: its concentration likewise reached a maximum and then declined during a 4 hr reaction period.

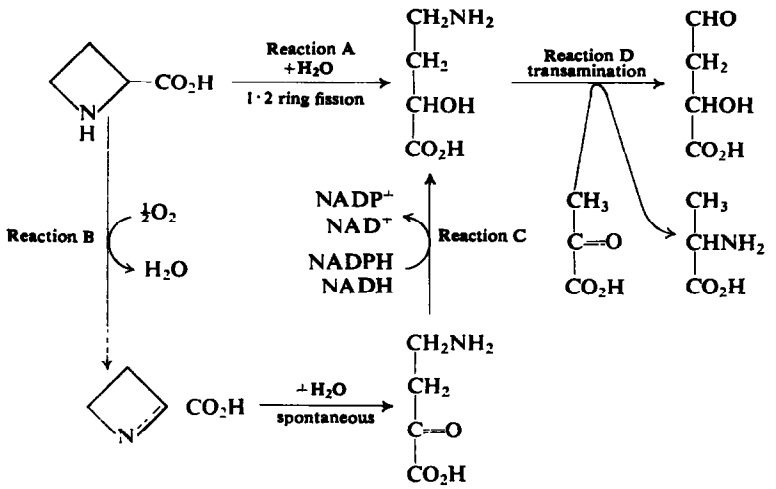
Degradation by Cell-Free Extracts

A heavy cell suspension in 0·04 M-phosphate or 0·1 M-tris-HCl buffer of appropriate pH was subjected to ultrasonic disintegration for 10 min and the supernatant resulting after centrifuging at 20,000 *g* for 30 min was used as a source of degradative enzymes. When the supernatant was incubated with azetidine-2-carboxylic acid under the conditions above, complete disappearance of the compound was again found. α-Hydroxy-γ-aminobutyric acid was the first reaction product observed and alanine appeared subsequently. However, the concentration of the hydroxyamino acid did not noticeably decrease after longer periods of incubation and it always remained considerably in excess of that of alanine.

The ability to degrade azetidine-2-carboxylic acid to α-hydroxy-γ-aminobutyric acid was retained by the supernatant preparation after dialysis overnight at 4 against many changes of buffer. Alanine was not a reaction product when dialysed preparations were used: it was formed, however, when pyruvate was added together with azetidine-2-carboxylic acid.

Reaction Pathways

Alternate pathways illustrated in Fig. 1 may be invoked to explain the observed reaction products.



The most logical route to α-hydroxy-γ-aminobutyric acid would be by a simple hydrolytic cleavage of the heterocyclic ring between $N_{(1)}$ and $C_{(2)}$ (reaction A). Support for this reaction depends mainly upon the fact that no intermediates could be detected during hydroxyamino acid formation by dialysed supernatant and that this conversion was not dependent upon added cofactors. The alternate reaction scheme involves an initial oxidation (reaction B) to yield 1,2-dehydroazetidine-2-carboxylic acid, i.e. a reaction comparable to that catalysed by proline oxidase, followed by spontaneous ring opening and subsequent reduction of the α-oxo-γ-aminobutyric acid (reaction C). A comparison with the analogous reactions involved in the reduction of homologous α-oxo-ω-amino acids by *Neurospora crassa* extracts,[7] suggests that NADH or NADPH would be required for this last reduction step. In practice, additions of NADH and NADPH alone or in combination with α-oxo-γ-aminobutyric acid had no measurable effect upon the rate of formation of α-hydroxy-γ-aminobutyric acid from azetidine-2-carboxylic acid by dialysed supernatant. In the absence of azetidine-2-carboxylic acid, a slight conversion of α-oxo- into α-hydroxy-γ-aminobutyric acid was observed in the presence of NADPH.

Alanine production apparently was dependent upon a transamination (reaction D). Malic-β-semialdehyde would be the associated product of the proposed reaction but some further oxidation to malic acid might be anticipated. Tentative support for these nitrogen-free reaction products was obtained by applying paper chromatographic methods. The anionic fraction from appropriate reaction mixtures separated using Dowex-1 resin contained a trace amount of an organic acid inseparable from malic acid on chromatograms developed in a chloroform–*tert.*-amyl alcohol–formic acid–water mixture.[8] The dinitrophenylhydrazones of keto and aldehydo acids present in reaction mixtures were separated on chromatograms run in butan-1-ol-ammonia mixtures.[9] In addition to strong yellow spots due to the derivative of pyruvic acid, two additional brown spots were noticed. Catalytic hydrogenation of the slower-moving of these two spots gave α-hydroxy-γ-aminobutyric acid (identified chromatographically), the product expected from a dinitrophenylhydrazone of malic-β-semialdehyde.

[7] T. YURA and H. J. VOGEL, *J. Biol. Chem.* **234**, 335 (1959).
[8] L. E. BENTLEY, *Nature* **170**, 847 (1952).
[9] L. FOWDEN and J. A. WEBB, *Biochem. J.* **55**, 548 (1953).

Properties of the Ring-Splitting Enzyme

Portions of a dialysed supernatant were adjusted to pH values in the range 6·7–11·4 (the protein concentration in each preparation was maintained constant) and the ability of each to hydrolyse azetidine-2-carboxylic acid was measured during a 30 min reaction period. The results indicated that the enzyme was active over a wide range of pH values: the usual type of pH-activity curve showed a flat maximum in the range 8·3–10·8, but at pH values outside this range activity fell sharply.

The enzyme showed high specificity for L-azetidine-2-carboxylic acid and no degradation of the higher homologues, L-proline and L-pipecolic acid, occurred. D-Azetidine-2-carboxylic acid was not utilized as a substrate and its presence in reaction mixtures led to a marked inhibition of the rate of breakdown of equimolar amounts of the L-isomer.

The hydrolytic activity was not due to a surface enzyme associated with the cell walls because the debris sedimented after ultrasonic disintegration was unable to degrade the imino acid.

Properties of the Transaminase

The transaminase exhibited high activity in the pH range of 8–10 and most experiments were performed at pH 8·4–8·8.

When α-oxoglutaric acid replaced pyruvic acid in systems containing dialysed supernatant and either azetidine-2-carboxylic acid or α-hydroxy-γ-aminobutyric acid, no glutamic acid was formed. This *in vitro* finding was in agreement with the observation that glutamic acid was not a product when intact cells metabolized azetidine-2-carboxylic acid, although the cells would be expected to possess a metabolic pool of α-oxoglutaric acid.

A number of amino acids in addition to α-hydroxy-γ-aminobutyric acid could donate their amino groups to pyruvate in reactions catalysed by dialysed supernatant obtained from *Agrobacterium* cells grown initially in medium F. Many of the acids contained an ω-amino group and the relative rates at which they transaminated were as follows:

ornithine > γ-aminobutyric acid > α-hydroxy-γ-aminobutyric acid > lysine > α,γ-diaminobutyric acid.

TABLE 1. TRANSAMINATION BETWEEN VARIOUS AMINO ACIDS AND PYRUVIC ACID CATALYZED BY EXTRACTS OF *Agrobacterium* CELLS GROWN IN DIFFERENT MEDIA

Growth conditions	Amino acid substrate	Alanine formed (μg/ml reaction mixture*)	Alanine formed (relative to γAB = 100)
Medium F	αHγAB†	205	36
	γAB	573	100
	αγDAB	66	11
Equimolar γAB replacing A2C in Medium F	αHγAB	186	36
	γAB	511	100
	αγDAB	34	7
Equimolar αγDAB replacing A2C in Medium F	αHγAB	131	29
	γAB	449	100
	αγDAB	50	11

* Reaction mixtures contained: amino acid, 0·05 M; pyruvic acid, 0·05 M; pH 8·8 phosphate, 0·05 M; bacterial protein, 10 mg/ml. Incubation time was 1 hr at 28·5

† Abbreviations: A2C, azetidine-2-carboxylic acid; αHγAB, α-hydroxy-γ-aminobutyric acid; γAB, γ-aminobutyric acid; αγDAB, α,γ-diaminobutyric acid.

The transaminase activity associated with cells seemed to be independent of the nitrogen source used in the growth medium. Azetidine-2-carboxylic acid was certainly not an essential medium constituent for when it was omitted from medium F an extract of the resulting cells catalysed transamination between pyruvate and α-hydroxy-γ-aminobutyric acid at the same rate as a similar extract from cells grown in medium F (alanine production was expressed per mg protein in each extract). The available evidence then favours the idea that the effective transaminase was a constitutive enzyme exhibiting wide amino acid specificity. This is supported by the data in Table 1, which show that when a γ-amino acid (e.g. γ-aminobutyrate or α,γ-diaminobutyrate) replaced azetidine-2-carboxylic acid in a medium based on F, there was no effect upon the relative rates at which different cell extracts could catalyse transamination between γ-aminobutyric acid, α-hydroxy-γ-aminobutyric acid or α,γ-diaminobutyric acid and pyruvic acid. Furthermore, the absolute rates of transamination observed for extracts of equal protein concentration obtained from cells grown under different conditions showed only small differences.

DISCUSSION

Two simple steps are used by *Agrobacterium* cells to introduce the nitrogen atom of azetidine-2-carboxylic acid into alanine. Since this last amino acid forms one of the central, highly dynamic compounds of intermediary nitrogen metabolic processes, the further distribution of the nitrogen into protein amino acids would follow readily. In nature, *Agrobacterium* species, by catalysing these steps, may play a role in a nitrogen cycle which commences with the azetidine-2-carboxylic acid present in senescent vegetative tissues of many liliaceous plants. The experiments reported do not permit crucial comment upon this idea but it is interesting that the source of *Agrobacterium* used was soil collected from around the roots and rhizomes of *C. majalis*.

Presumably, the energy required to break the strained four-atom azetidine ring is considerably less than that necessary to split the stable pyrrolidine ring, and this factor may have influenced the evolution of a simple, one-step hydrolytic process for ring fission of azetidine-2-carboxylic acid in contrast with the more complex oxidative mechanism used by many organisms to degrade proline. The hydrolytic reaction was entirely specific for splitting the $N_{(1)}$–$C_{(2)}$ bond and is in contrast with H^+ catalysed breakdown of azetidine-2-carboxylic acid which led to a mixture of α-hydroxy-γ-aminobutyric acid and homoserine,[10] the latter formed by hydrolytic fission of the $N_{(1)}$–$C_{(4)}$ linkage. No evidence was obtained for a reductive fission of the ring which might be expected to yield α- or γ-aminobutyric acids by direct hydrogenation or γ-aminobutyric acid alone if a Stickland[11] type reaction occurred.

The inability of α-oxoglutaric acid to replace pyruvic acid as an amino group acceptor in the transamination process is reminiscent of other systems involving transamination of non-α-amino acids. For example, transamination of γ-aminobutyric acid catalysed by pea seedlings[12] or yeast[13] extracts preferentially used pyruvate rather than α-oxoglutarate as the acceptor while α-oxoglutarate was quite ineffective as a substrate for the β-alanine–alanine transaminase present in *Pseudomonas*.[14]

Other experiments[15] have established that the proline-activating enzyme of *Agrobacterium*

[10] L. Fowden, *Biochem. J.* **64**, 323 (1956).
[11] L. H. Stickland, *Biochem. J.* **28**, 1746 (1934); **29**, 288, 889, 896 (1935).
[12] R. O. D. Dixon and L. Fowden, *Ann. Bot.* N.S. **25**, 513 (1961).
[13] R. Pietruszko and L. Fowden, *Ann. Bot.* N.S. **25**, 491 (1961).
[14] Y. Nishizuka, M. Takeshita, S. Kumo and O. Hayaishi, *Biochim. Biophys. Acta* **33**, 591 (1959).
[15] P. J. Peterson and L. Fowden, *Biochem J.* In press.

cells is capable of utilizing azetidine-2-carboxylic acid as a substrate, although at lower rates than that observed for proline itself under comparable conditions. However, when protein was isolated from an *Agrobacterium* culture grown in a medium containing azetidine-2-carboxylic acid as sole nitrogen source (125 mg imino acid/500 ml), no trace of the imino acid was detected in an alkaline hydrolysate[5] of the cell protein. This observation differs from a previous finding with mung bean seedlings where non-specific action of the proline activating enzyme was associated with an observed replacement of residues of proline in the plant protein by azetidine-2-carboxylic acid. The ability of intact cells of *Agrobacterium* to degrade azetidine-2-carboxylic acid may provide an explanation for this difference: the location of the hydrolytic enzyme may ensure that the amount of azetidine-2-carboxylic acid penetrating to the sub-cellular site of the proline-activating enzyme is reduced to an insignificant level.

EXPERIMENTAL

Amino Acids

L-Azetidine-2-carboxylic acid was isolated from *Polygonatum multiflorum*.[16] The D-stereoisomer was a gift from Calbiochem.

α-Hydroxy-γ-aminobutyric acid was prepared by treating L-α,γ-diaminobutyric acid with 1·2 equivalents of $NaNO_2$ in 2 N-HCl for 30 min at 100°. Under these conditions the α-amino group was preferentially attacked and replaced by a hydroxyl substituent. Unchanged diaminobutyric acid was removed using a Zeocarb 226 resin column, which only retains basic amino acids, and the α-hydroxy-γ-aminobutyric acid was separated from any dihydroxy acid by absorption on and elution from a Dowex-50 column. It was finally recrystallized from aqueous ethanol.

α-Oxo-γ-aminobutyric acid was a gift from Dr K. Balenović (Zagreb).

Organism

Organisms present in a soil extract capable of utilizing azetidine-2-carboxylic acid as sole nitrogen source were selected by an enrichment culture technique using medium C for growth. After streaking onto agar plates (medium D), individual colonies were sub-cultured on medium C and, after establishing their homogeneity, stock cultures of each organism were maintained on agar slopes in which azetidine-2-carboxylic acid was the only nitrogen source (medium E).

The characteristics of the *Agrobacterium* sp. selected for the present metabolic investigation have been described by Dr. A. J. Holding in a personal communication. The organism is closely related to *A. radiobacter* (family Rhizobiaceae), differing only in one or two minor characteristics. It shows some colonial variation but these variants seem to be stable and can all be considered as one and the same organism. The cells possess mixed shoulder and peritrichous flagella and grow well with NH_4^+ as a sole nitrogen source. They could utilize azetidine-2-carboxylic acid as sole nitrogen and carbon source. Alternate nitrogen sources did not improve growth but better growth was obtained when glucose was a component of the medium.

Growth Media

Medium A: a basal nitrogen-free medium, had the composition: KH_2PO_4, 4·08 g; glucose, 10 g; NaCl, 1 g; $MgSO_4$, 0·1 g; $FeSO_4$, trace; N-NaOH soln, 21 ml and water to 1 l.; final pH

[16] L. FOWDEN and M. BRYANT, *Biochem. J.* **70**, 626 (1958).

about 7·2. Medium B was obtained by addition of NH_4Cl, 2 g/l, and this when solidified by addition of 2% agar gave Medium D.

Medium C used for the selective enrichment culture of organisms degrading the imino acid, consisted of 1 l. Medium A + 1·4 g azetidine-2-carboxylic acid. Further addition of 1% agar gave Medium E.

Medium F, normally used to grow the organism for metabolic studies, was medium A with additions of 150 mg NH_4Cl and 28 mg azetidine-2-carboxylic acid per litre.

All media were autoclaved for 10 min at 10 lb/in^2.

Growth of Experimental Cells

A suitable inoculum of *Agrobacterium* cells, grown in Medium B, was introduced into 500 ml of Medium F and growth proceeded for 24 hr at 27°. Cells were harvested by centrifuging at 15,000 *g*, washed twice, and finally resuspended to give a dense suspension (4 ml) in 0·04 M-phosphate buffer, pH 7·0, if used for whole cell studies.

Cell-free extracts were obtained by ultrasonic disintegration of similar suspensions in appropriate pH buffer solution using a M.S.E.-Mullard apparatus (20 kc/s for 10 min) followed by centrifuging at 20,000 *g* for 30 min. The supernatant used to degrade azetidine-2-carboxylic acid was used either directly or after dialysis against many changes of either 0·04 M-phosphate or 0·1 M-tris-HCl buffer during 16 hr at 4°. The protein content of extracts, determined by the method of Lowry *et al.*,[17] was normally 8–16 mg/ml.

Chromatographic and Electrophoretic Separations

(*a*) Amino acids: the initial product of azetidine-2-carboxylic acid breakdown could not be distinguished from homoserine on paper chromatograms developed either in 75% (w/w) phenol in the presence of ammonia vapour or in butan-1-ol: acetic acid: water (90:10:29, v/v). However, when an ethyl acetate: pyridine: water solvent (2:1:2, v/v, upper phase) was used, the degradation product had $R_{alanine}$ of 0·67. Four-carbon amino acids viewed as possible products of breakdown had the following $R_{alanine}$ values: homoserine, 0·95; α-hydroxy-γ-aminobutyric acid, 0·67; γ-aminobutyric acid, 0·70, and α-aminobutyric acid, 1·37, while azetidine-2-carboxylic acid moved at $R_{alanine}$ 0·90. Confirmation that the degradation product was α-hydroxy-γ-aminobutric acid was obtained by electrophoresis at pH 2·0 (formic acid 61 ml, acetic acid 97 ml, water to 2 l.), when a good separation from all other amino acids tested was effected in 30 min at 60 V/cm.

Alanine formed during transamination reactions was determined after separation on electropherograms at pH 3·4 (formic acid 100 ml, pyridine 10 ml, water to 2 l.) using the ninhydrin-cadmium acetate method of Atfield and Morris.[18]

(*b*) Keto acids: dinitrophenylhydrazone derivatives of keto and aldehydo acids present after transamination between α-hydroxy-γ-aminobutyric and pyruvic acids were prepared by the method of Pietruszko and Fowden.[13] After their separation on paper chromatograms developed in butan-1-ol saturated with 6% (w/v) NH_3, catalytic hydrogenation under slightly acidic conditions in the presence of PtO_2 catalyst yielded the analogous amino acid,[9,19] subsequently identified on ethyl acetate–pyridine–water chromatograms.

[17] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randle, *J. Biol. Chem.* **193**, 265 (1951).
[18] G. N. Atfield and C. J. O. R. Morris, *Biochem. J.* **81**, 606 (1961).
[19] G. H. N. Towers, J. F. Thompson and F. C. Steward, *J. Am. Chem. Soc.* **76**, 2392 (1954).