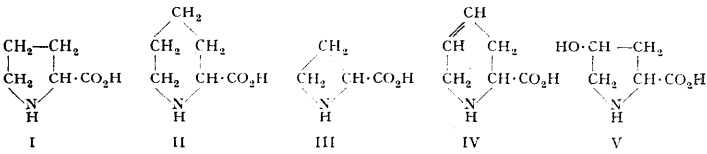
SC 2220

Replacement of proline by azetidine-2-carboxylic acid during biosynthesis of protein

Previous studies on structural analogues of natural amino acids which can be incorporated into proteins during biosynthesis have not revealed an effective proline analogue[1]. Two proline (I) homologues, L-pipecolic acid (II) and L-azetidine-2-carboxylic acid (III), occur naturally; several derivatives of these imino acids, including $\Delta^{4,5}$-dehydro-L-pipecolic acid (baikiain, IV) and 4-hydroxy-L-proline (V), are also natural products[2,3]. Tests have been made to ascertain whether any of these substances can be incorporated into bacterial and higher-plant proteins.



I II III IV V

The imino acids were tested for growth-inhibitory activity on cultures of *Escherichia coli* growing exponentially in C medium containing 0.4% glucose[4]. Only L-azetidine-2-carboxylic acid was growth-inhibitory; inhibition was observed immediately after the addition of this homologue to the culture medium at a final concentration of 100 μg/ml (Fig. 1). No inhibition was observed when DL-proline (final concn., 100 μg/ml) was added to the culture at the same time as the azetidine-2-carboxylic acid.

The kinetics of the growth inhibition shown in Fig. 1 are reminiscent of the effects on growth of other analogues known to be incorporated into proteins[1,5]. Therefore crude protein was isolated from bacterial samples (about 50 mg dry wt.), harvested

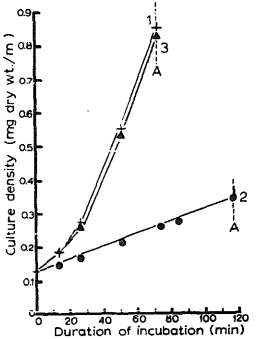


Fig. 1. Effect of azetidine-2-carboxylic acid on growth of *E. coli* in CG medium. Additions (μg/ml): Culture 1, nil; Culture 2, L-azetidine-2-carboxylic acid, 100; Culture 3, L-azetidine-2-carboxylic acid, 100, + DL-proline, 100. A, point of sampling for protein analyses.

from Culture 2 after 4-h incubation with azetidine-2-carboxylic acid, and from Cultures 1 and 3 after 2 h 25 min (see Fig. 1, point A). The bacterial samples were treated to yield the protein plus cell wall fractions[6], and these were hydrolysed with 5 N $Ba(OH)_2$ for 18 h at 100°. (The more usual method for protein hydrolysis, using 6 N HCl, was impracticable because azetidine-2-carboxylic acid is completely destroyed by treatment with strong mineral acid[7].) The presence of azetidine-2-carboxylic acid in hydrolysates was sought by two-dimensional paper chromatography using 75 % (w/w) phenol in the presence of ammonia as the first solvent, followed by butan-1-ol–acetic acid–water (90:10:29, v/v/v). Quantitative determinations of azetidine-2-carboxylic acid after separation on chromatograms were made with the ninhydrin reagent of YEMM AND COCKING, following the procedure described earlier[9]; proline assays were performed by a similar technique, but the acid-ninhydrin reagent (pH 1) of CHINARD[10] was used to obtain higher specificity and sensitivity.

TABLE I

REPLACEMENT OF PROLINE BY AZETIDINE-2-CARBOXYLIC ACID IN PROTEIN OF *Escherichia coli*

Culture No.	*Medium composition CG med...m[4] + DL-proline (μg/ml)*	*+ L-azetidine-2-carboxylic acid (μg/ml)*	*Composition of protein fraction: Proline content (μg/mg protein)*	*Azetidine-2-carboxylic acid content (μg/mg protein)*	*Proline replaced[*] (%)*
1	Nil	Nil	26.8	Nil	Nil
2	Nil	100	18.4	6.2	26.5
3	100	100	28.0	Nil	Nil

[*] For details of calculation: see text.

The results of these incorporation experiments are given in Table I. The proline content of the protein isolated from the culture supplied 100 μg/ml azetidine-2-carboxylic acid alone was about 30 % lower than that determined for the protein from the control cells; the reduced proline content (difference 8.4 μg/mg) was compensated by the presence of a nearly equivalent amount of azetidine-2-carboxylic acid (6.2 μg/mg, equivalent to 7.1 μg/ml proline). Assuming that the protein content of cells produced before and after the addition of azetidine-2-carboxylic acid was the same, then azetidine-2-carboxylic acid replaced nearly 50 % of proline residues when protein was synthesised in the presence of the analogue. No azetidine-2-carboxylic acid was detected in the protein isolated from cells receiving proline together with analogue.

Azetidine-2-carboxylic acid inhibits the growth of seedlings of mung bean (*Phaseolus aureus*). The imino acid was supplied dissolved in water imbibed by seeds during the first day of germination. When increasing amounts of azetidine-2-carboxylic acid were supplied, inhibition was progressively more marked, and 2 mg/g dry wt. of seeds generally proved to be a lethal concentration. Protein fractions were isolated from the radicles of seedlings after 3 days of growth; these fractions were free of cell-wall material, and had higher total nitrogen and proline contents than those determined for the cruder bacterial preparations. Table II shows the proline and azetidine-2-carboxylic acid levels observed. The azetidine-2-carboxylic acid

content of the newly synthesised protein molecules of the radicles was calculated assuming that no turnover of protein originally present in the embryonic radicles occurred. At the higher level of supply, the analogue replaced nearly all the proline residues of new protein molecules.

TABLE II

REPLACEMENT OF PROLINE BY AZETIDINE-2-CARBOXYLIC ACID IN PROTEINS OF MUNG BEAN

*Azetidine-2-carboxylic acid supplied (mg/50 seeds)**	*Fresh wt. of radicles (g)*	*Composition of protein fraction*		*Proline replaced (%) in*	
		Proline (μg/mg protein)	*Azetidine-2-carboxylic acid (μg/mg protein)*	*Total radicle protein*	*Newly-synthesized protein*
0	5.67	42.0	0	0	0
1	1.13	38.0	5.0 (5.7)**	14	21
3	0.31	29.7	11.7 (13.3)**	31	95

* 50 seeds equivalent to 2.25 g dry wt.
** Figures in parentheses are equivalent amounts of proline.

Replacement of proline by azetidine-2-carboxylic acid may have an effect on protein structure not normally associated with the incorporation of structural analogues. Neither proline nor azetidine-2-carboxylic acid is accommodated in the normal α-helical structure of a polypeptide, and the presence of such a residue causes the axis of the α-helix to bend[12,13]. However, stereochemical considerations (see ref. 14 for relevant structures) suggest that an azetidine-2-carboxylic acid residue will turn the helix through an angle about 15° smaller than proline. This could lead to altered tertiary structure.

Department of Botany, University College London, (Great Britain) L. FOWDEN

National Institute for Medical Research, Mill Hill, London, (Great Britain) M. H. RICHMOND

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