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Title THE EFFECT OF AZETIDINE-2-CARBOXYLIC ACID ON PROLINE BIOSYNTHESIS IN ESCHERICHIA COLI

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The effect of the proline analogue, L-azetidine-2-carboxylic acid, on proline biosynthesis in *Escherichia coli* has been studied. The analogue inhibits the formation of proline precursor by acting as a false feedback inhibitor. Analysis of kinetic constants in whole-cell preparations indicate that the inhibition is noncompetitive. Comparative inhibitor constants for proline and azetidine-2-carboxylic acid are 5×10^{-7} M and 4×10^{-5} M, respectively. The latter constant has been corrected for the amount of inhibitor found in the bacterial cell. The structural specificity of the control mechanism is discussed.

THE EFFECT OF AZETIDINE-2-CARBOXYLIC ACID ON PROLINE BIOSYNTHESIS IN *ESCHERICHIA COLI*

by

FAITH IRENE SMITH

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APPROVED:

Redacted for Privacy

Assistant Professor of Chemistry
In Charge of Major

Redacted for Privacy

Chairman of Department of Chemistry

Redacted for Privacy

Dean of Graduate School

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DEDICATION

This thesis is dedicated to my mother and to the memory of my father.

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THE EFFECT OF AZETIDINE-2-CARBOXYLIC ACID ON PROLINE BIOSYNTHESIS IN ESCHERICHIA COLI

INTRODUCTION

Amino acid synthesis in microorganisms is regulated by at least two distinct mechanisms: (a) end-product inhibition of one or more specific enzymes in a biosynthetic pathway and (b) repression of the synthesis of enzymes in the pathway. End-product inhibition has been reported by Umbarger (25) among others. Vogel (28) and Jacob and Monod (12) are only a few of those who have demonstrated repression. Studies of a number of amino acid analogues have indicated that many analogues are capable of operating the first of these biosynthetic control mechanisms and a few, the second. Results of recent investigations have been reviewed by Cohen (8) and Richmond (19). The work with analogues has been useful in elucidating the mechanisms of the control reactions, particularly, the specificities required in the chemical structure. The present work examines the control mechanism involved in the synthesis of the amino acid proline by use of the analogue azetidine-2-carboxylic acid.

BACKGROUND

Vogel and Davis (30) and Strecker (22) have examined the synthesis of proline in Escherichia coli using proline-requiring mutants. One strain, 55-1, isolated by the first group, excreted a compound identified as glutamic γ-semialdehyde (GSA) into the growth medium. A second strain, 55-25, used this compound or its spontaneous cyclization product, Δ^1 pyrroline-5-carboxylic acid (PC) to fulfill its proline requirement. A third strain, 22-64, responded to glutamic acid, GSA or proline. These results suggested that proline is synthesized from glutamic acid by way of GSA or PC.

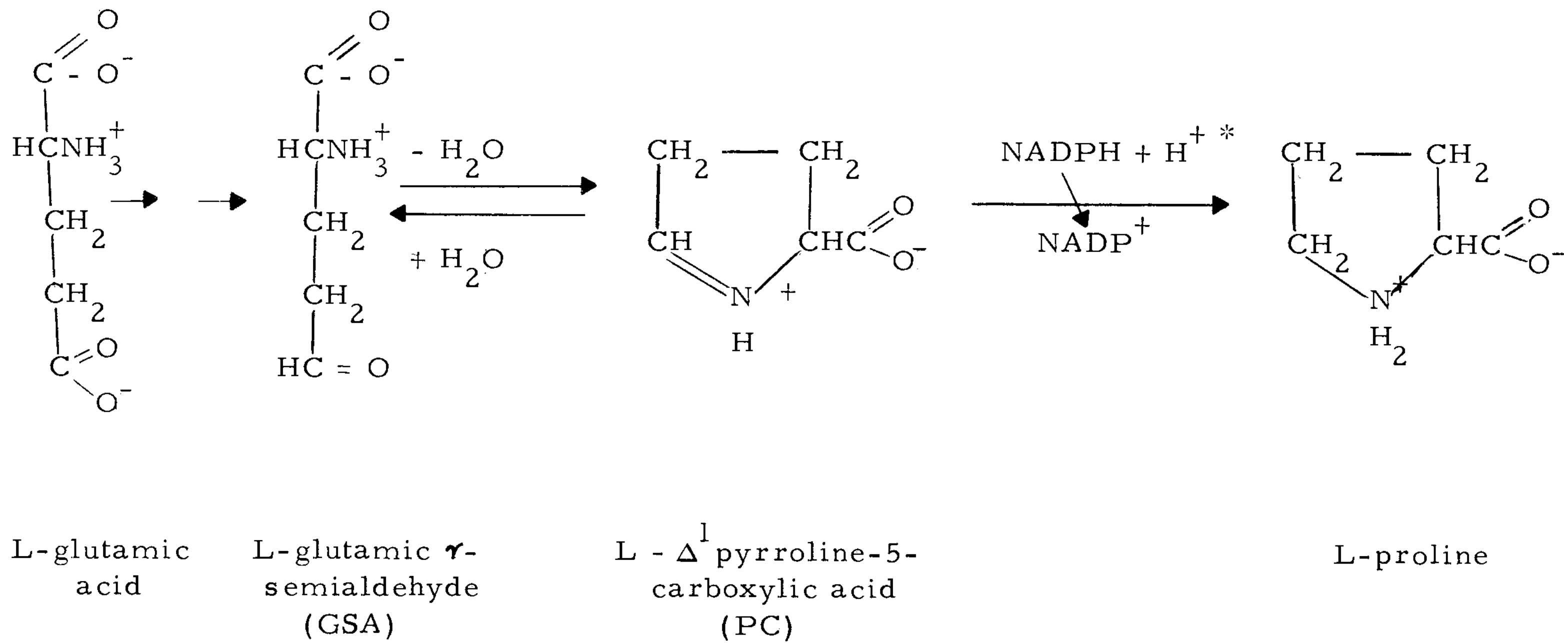
The intermediate position of GSA has also been demonstrated by Abelson et al. (1) through isotopic competition studies. Glucose labeled with carbon 14 and non-labeled GSA were supplied to the growth medium of wild type E. coli. Of the amino acids isolated from the cells, only proline showed no radioactivity.

Piesach and Strecker (18) have identified the enzyme, PC reductase, capable of converting PC to proline in the presence of reduced triphosphopyridine nucleotide (NADPH). A correlation was found between mutants of E. coli which lacked this enzyme and those which were dependent upon proline for growth. Attempts to identify the enzyme catalyzing the reduction of glutamic to GSA have been unsuccessful. Strecker (22) suggested that an unstable

intermediate may be involved at this point. The known reactions leading to proline synthesis are shown in Figure 1.

Strecker (22) and Baich and Pierson (3) have investigated the reduction of glutamic acid to GSA in the *E. coli* mutant 55-1. Both groups found that proline inhibited this step in the intact cells. Quantitatively, the production of GSA in resting cells was immediately halted in the presence of as little as 8.7×10^{-6} M proline. Further evidence of control of the first reduction step was shown by the observation that no proline was excreted when wild type *E. coli* was grown in the presence of excess glutamic acid. Proline was excreted when this strain was grown in the presence of GSA. Only the first reduction step appeared to be controlled since PC reductase was neither inhibited nor repressed by proline. The results of these studies indicated that proline synthesis was subject to at least end-product inhibition at the first reduction stage while further conversion to proline was apparently unrestrained.

Tristram and Thurston (24) have also demonstrated end-product inhibition of the reduction of glutamic acid to GSA in cells of Vpro-1, a proline-requiring mutant apparently genetically similar to 55-1. Complete, but transitory, inhibition of GSA production was found in the presence of 1.5×10^{-7} M proline. The duration of inhibition was dependent on the amount of proline added. No recovery from inhibition of GSA production was observed in the presence of 3.0×10^{-4} M



* Catalyzed by Δ^1 pyrroline-5-carboxylic acid reductase (PC reductase).

Figure 1. Proline Biosynthetic Pathway in E. coli.

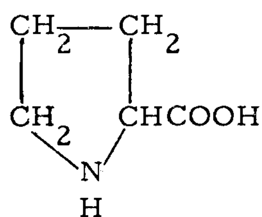
proline. These results are not in quantitative agreement with those of Baich and Pierson (3) given above. The difference in the concentration of proline required for inhibition may be due to the low amount of proline relative to the number of bacterial cells present in the system used by Tristram and Thurston (24). These authors employed a concentration of cells (2.5 mg/ml, dry weight) ten times that used by Baich and Pierson (0.25 mg/ml, dry weight) (3). The number of bacterial cells used by each group was calculated from the dry weight of bacteria by the method of Roberts, et al. (20). The ratio of proline molecules to bacterial cells was approximately 2×10^{10} in the system of Baich and Pierson (3) at a concentration of 8.7×10^{-6} M proline, and only 4×10^{7} in the system of Tristram and Thurston (24) at a concentration of 1.5×10^{-7} M proline. The escape from inhibition in the latter system could possibly be attributed to utilization of proline.

The enzyme catalyzing the reduction of glutamic acid to GSA may also be subject to repression, or a decrease in its rate of synthesis, by proline. Tristram and Thurston (24) found that the rate of production of GSA by cells of Vpro-1 grown in media containing excess proline was between 19 percent and 35 percent of that of cells grown in limiting amounts of proline. These results suggested that repression was in operation at the first reduction step. However, repression of this enzyme is difficult to

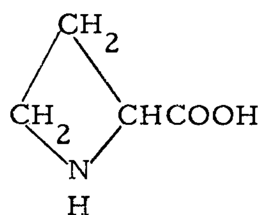
demonstrate directly since the enzyme has not been obtained in a cell-free system.

A number of amino acid analogues are capable of substituting for the corresponding amino acid in causing end-product inhibition. Such analogues are known as false feedback inhibitors. Some of these analogues have the additional property of replacing the amino acid in protein with a consequent effect on the conformation of the polypeptide structure.

The most effective analogues normally possess a chemical structure closely related to that of the natural compound. An analogue of proline, azetidine-2-carboxylic acid (A-2-C), with a four-membered amino ring as shown below, was isolated by



L-proline



L-azetidine-2-carboxylic acid

Figure 2. Structures of L-proline and L-azetidine-2-carboxylic acid.

Fowden (9) and Virtanen and Linko (27) from ethanolic extracts of certain members of the Liliaceae (lily-of-the-valley) and Agavaceae.

This analogue is capable of entering the *E. coli* cell. Proline itself is taken into the cell by the action of a specific energy-dependent transport mechanism (permease) as shown by Britten, Roberts, and French (5). Neale and Tristram (17), through isotopic competition studies, have demonstrated that the proline permease also has a low affinity for A-2-C. Only the L-isomer was concentrated.

The properties of A-2-C as a growth inhibitor in carrot tissue, mung bean seedlings, and *E. coli* have been described by Steward, *et al.* (21), Fowden (10), and Fowden and Richmond (11). Only the L-isomer was found to be active. Growth inhibition by A-2-C has been attributed to the replacement of proline by this compound in newly synthesized proteins. Fowden and Richmond (11) found that approximately 50 percent of the proline residues were replaced when *E. coli* was grown in the presence of 1.0×10^{-3} M A-2-C. The effect of A-2-C on growth was reversed by simultaneous addition of proline and none of the analogue was detected in protein from cells grown in the presence of both A-2-C (1.0×10^{-3} M) and proline (8.7×10^{-4} M).

Incorporation of an amino acid into protein involves the formation of a bond between the amino acid and a specific transfer-ribonucleic acid (s-RNA). Midgley and Wild (16) found that A-2-C was capable of forming this bond with prolyl s-RNA at a rate lower than that of proline. Thus, the analogue could be incorporated into

protein; but only in the absence of significant amounts of the amino acid.

Replacement of proline by A-2-C has an effect on protein structure not usually associated with the incorporation of a structural analogue since neither proline nor A-2-C is accommodated in the normal α-helix structure of a polypeptide. The presence of either residue causes the axis of the α-helix to bend. Through stereochemical considerations, Fowden and Richmond (11) have suggested that an A-2-C residue would turn the helix through an angle approximately 15° smaller than proline. This change of angle would lead to an altered structure for a polypeptide. Decreased growth could then be attributed to the presence of functionally impaired protein.

In view of the effects of A-2-C upon growth of *E. coli*, it was of interest to investigate the effects of the analogue upon proline biosynthesis in this organism. Because of its similarity to proline, it was considered likely that A-2-C[1/] would have an inhibiting effect on the synthesis of this amino acid. While this work was in progress, Tristram and Thurston (24) published a preliminary study showing that the presence of 8.0×10^{-4} M A-2-C does inhibit the production of proline precursor in *E. coli*. In the present work the inhibiting effect of A-2-C has been investigated quantitatively

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[1/] A-2-C refers to the L-isomer unless otherwise designated.

through studies of proline synthesis in various strains of *E. coli* in the presence and absence of the analogue. The type of inhibition has been estimated by kinetic analysis of whole-cell preparations.

METHODS AND MATERIALS

Bacterial Strains

Strains of *E. coli* used in these studies were obtained from stock cultures maintained in this laboratory. The strains were identified as follows: W, wild type; 55-1, a mutant of wild type lacking PC reductase; W-2, wild type lacking the ability to synthesize GSA; WPl, wild type deficient in control of proline synthesis; and WPl-30, a mutant of WPl lacking PC reductase and deficient in control of proline synthesis. The nutritional requirements and presumed genetic compositions (3) of the strains are given in Table 1.

Strain 55-1, with no PC reductase, could excrete GSA into the medium only in the absence of proline. Strains WPl and WPl-30, having deficient control of the proline pathway, excreted proline or GSA, respectively; the latter, even when the medium was supplemented with proline.

Strains 55-1 and W-2 were obtained from Dr. D. F. Bacon of the Institution of Microbiology, Rutgers. Strains WPl and WPl-30 were isolated in this laboratory.

Table I. Nutritional Requirements and Presumed Genetic Compositions of Bacterial Strains

Name	Description	Nutritional Requirements	Presumed Genetic Composition*
W	Wild type	None	Control +, Pro_1+, Pro_2+
55-1	W, lacking PC reductase	Proline	Control +, Pro_1+, Pro_2-
W-2	W, lacking ability to synthesize GSA	Proline, GSA	Control +, Pro_1-, Pro_2+
WP1	W, deficient in control of proline synthesis	None	Control -, Pro_1+, Pro_2+
WP1-30	WP1, lacking PC reductase	Proline	Control -, Pro_1+, Pro_2-

* Baich and Pierson (3).

Culture Conditions

Stock cultures of *E. coli* were maintained at 4° C on Difco nutrient agar. The agar was inoculated and the bacteria allowed to grow overnight at 37° C before refrigeration. Stock cultures were maintained by transfer to fresh media every three to four weeks. Proline auxotrophs were routinely examined for their inability to grow on proline-deficient agar.

Inocula were obtained from overnight cultures of cells taken from nutrient agar plates. Growth medium stock solution consisted of the following: K_2HPO_4, 500 g; $MgSO_4$, 10 g; $NaNH_4HPO_4$, 175 g; citric acid, 100 g; $CHCl_3$ (preservative), 2 cc; all dissolved in 670 ml water (29). The stock solution was diluted 1 to 50 and autoclaved prior to use. The carbon source was glucose at a concentration of 0.5% (2.8×10^{-2} M). The medium was enriched with L-proline, final concentration 8.7×10^{-4} M, whenever required. Stationary cultures were grown at 37° C in a constant-temperature water bath. Cell mass was determined by measuring turbidity in the Klett colorimeter using the No. 66 filter.

Cells to be used in excretion experiments were grown in the following manner. Measured aliquots of overnight cultures were transferred to 200 ml of growth media in 500 ml Erlenmeyer flasks. The cells were grown to early log phase, corresponding to 50 Klett

Units, and harvested by centrifugation in a Servall refrigerated centrifuge. The bacterial pellet was washed twice with 0.1 M phosphate buffer, pH 7.0. The cells were stored overnight at 4° C in pellet form under 10 ml of the buffer. Cells maintained in this way exhibited uniform excretion of GSA for at least 48 hours.

Growth Curves

Growth curves were obtained under conditions indicated above. Cells from 50 ml of an overnight culture were harvested, washed twice, and resuspended in 0.1 M phosphate buffer. A measured aliquot of cells was added to 10 ml of growth medium in a 250 ml Erlenmeyer flask with a Klett tube side-arm. Turbidity was measured at intervals.

Measurement of Glutamic γ-semialdehyde and Proline

Glutamic γ-semialdehyde and proline were determined by microbiological assay. A stock solution of GSA, prepared according to Strecker (23), was analyzed by the method of Albrecht and Vogel (2). Measured aliquots of this solution were reacted with 0.5 ml of *o*-aminobenzaldehyde (4 mg/ml) in the presence of 1.0 ml 3.6 M sodium acetate. Optical density was read at 440 mμ in a Beckman Model DB spectrophotometer, using a cell with a 1-cm light path. Results are given in Figure 3. Measured

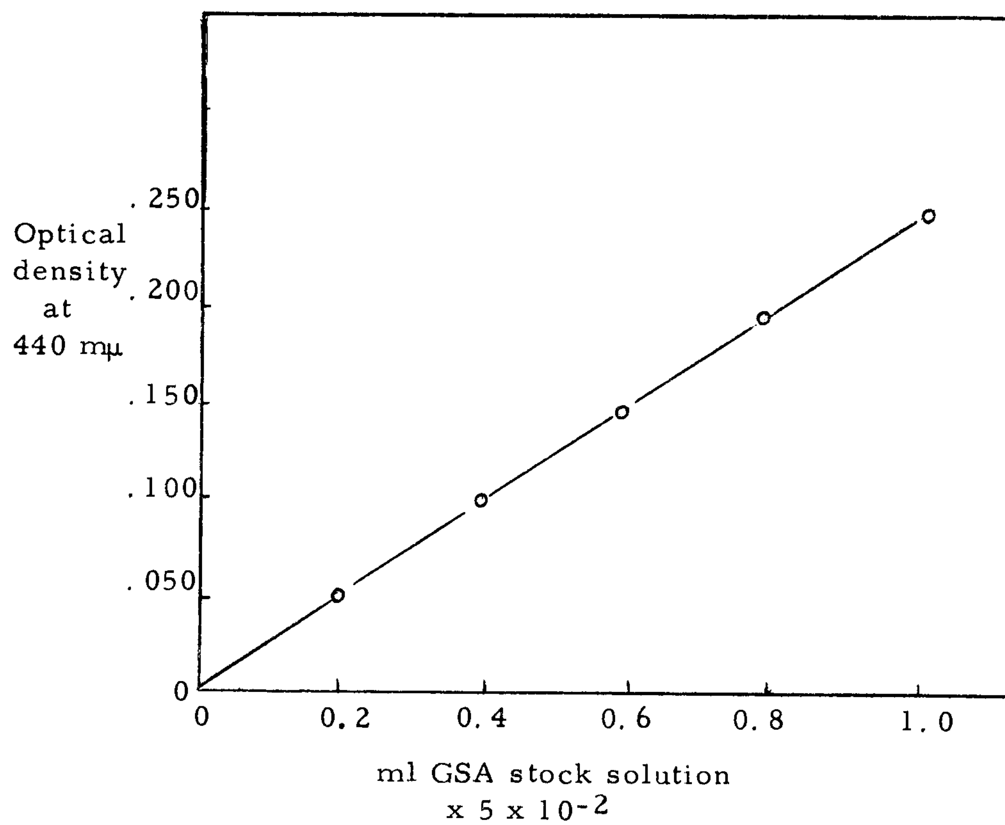

Figure 3. Reaction of GSA with o-Aminobenzaldehyde.

volumes of the stock solution were also used in a microbiological assay for proline using cells of strain W-2, a strain which responds to both GSA and proline. Growth response with proline, as shown in Figure 4, was compared to that with GSA. A standard curve, given in Figure 5, relates optical density at 440 mμ to micromolar concentration of GSA determined by microbiological assay. One micromole of GSA measured under these conditions gives an absorbancy of 0.055 at 440 mμ.

Glutamic γ-semialdehyde Production and Excretion

Glutamic γ-semialdehyde production by resting cells was measured at 37° C. The chilled bacterial pellet obtained from washed cells was dispersed in the overlying buffer. The resulting suspension was allowed to warm to room temperature for approximately 30 minutes. During this interval, excretion media -- consisting of 0.5% glucose and 0.02 M phosphate buffer, pH 7.0; final volume, 114 ml -- was prepared and warmed to 37° C in a water bath. Test tubes or Erlenmeyer flasks containing 0.1 ml of varying concentrations of glutamic acid, pH 7.0, with and without A-2-C, pH 7.0, were also warmed. After 30 minutes, 6 ml of cell suspension was added to the flask containing excretion media. The mixture was allowed to equilibrate for five minutes to remove any temperature differences. The reaction was temperature dependent and strict maintenance of temperature at 37° C was necessary to

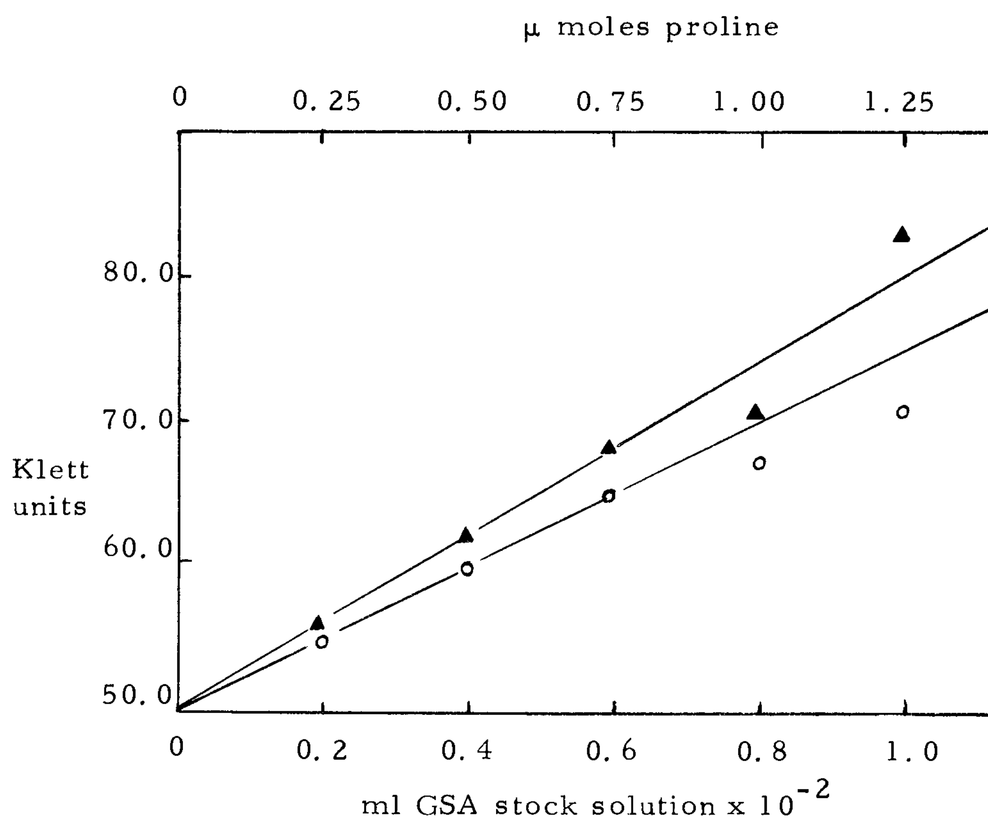

Figure 4. Microbiological Assay of GSA and Proline.
▲, proline; o, GSA

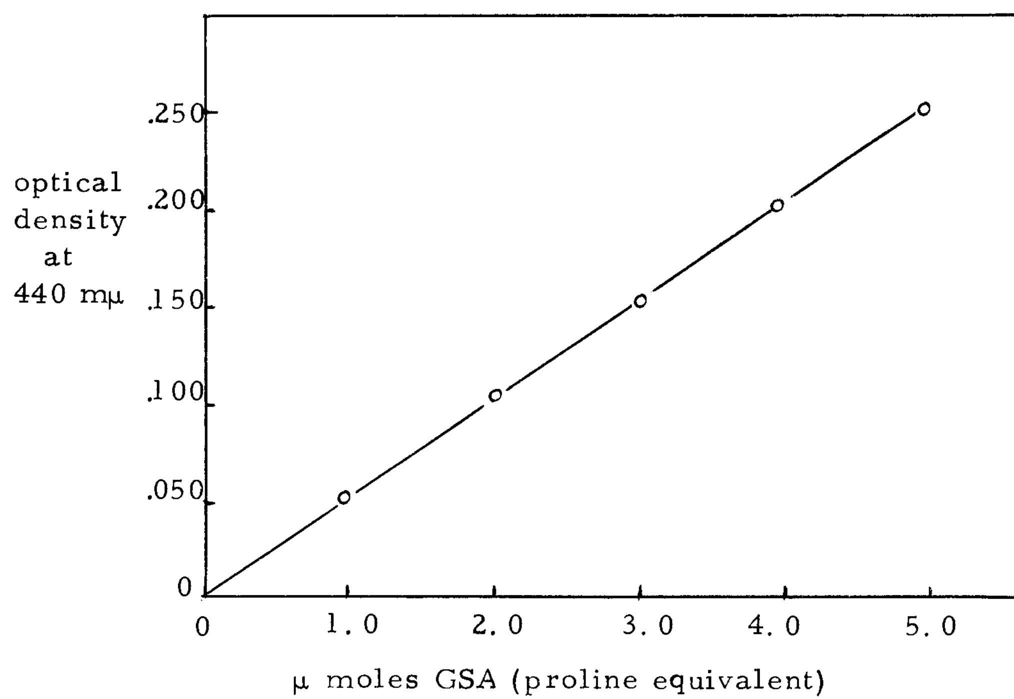

Figure 5. Concentration of GSA (Proline Equivalent)

prevent a decrease in GSA production. After equilibration, 9 ml of the mixture was added to each reaction flask. Two ml samples were withdrawn at intervals and placed in chilled plastic centrifuge tubes containing 0.1 ml of 10% trichloroacetic acid which was used to precipitate cellular protein and to halt GSA excretion. Samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant liquid was poured off and used for determination of GSA content. Protein was measured in each reaction mixture by the method of Lowry, *et al.* (15), with bovine serum albumin as the standard. It was later found that more readily reproducible results could be obtained if the reagents for GSA analysis were added to the samples before centrifugation.

Maximum excretion was observed in the presence of 0.5% glucose and 6.8×10^{-4} M glutamic acid as shown in Table II. The pH was maintained at 7.0 in all experiments.

Table II. Excretion of GSA by Strain 55-1 at Varying Concentrations of Glucose and Glutamic Acid

Glucose (moles/liter)	Glutamic Acid (moles/liter)	GSA after two hours (μm)	Protein (mg)	Specific Activity (μm GSA/mg protein after two hours)
2.8×10^{-2}	6.8×10^{-3}	2.28	0.184	12.4
2.8×10^{-2}	6.8×10^{-4}	2.28	0.138	16.5
1.1×10^{-2}	6.8×10^{-3}	2.40	0.172	14.0

Permeability Studies

Permeability of the cells to proline, glutamic acid and azetidine-2-carboxylic acid was determined using radioactive substrates under conditions similar to the excretion experiments. A 20 ml volume of cells in phosphate buffer with glucose was added to a 125 ml Erlenmeyer flask and warmed to 37° C. At the initial time the warmed mixture was poured into a second flask containing only radioactive test material. Two ml samples were withdrawn at intervals and placed in chilled centrifuge tubes. The samples were centrifuged at 15,000 rpm for 10 minutes. Supernatant liquid was decanted and the cell pellet was washed twice with 1.0 ml volumes of 0.1 M phosphate buffer. The cells were redispersed in another 1.0 ml volume of buffer. Radioactivity and protein were determined in a 0.5 ml aliquot of each sample.

Measurement of Radioactivity

Radioactivity was measured in a Packard Tri-Carb Model 3003 liquid scintillation counter. Counting solution consisted of 14.5 ml: 4.5 ml of a mixture of 100 ml ethanolamine plus 350 ml ethanol; and 10 ml of a mixture of 6 g 2,5-diphenyloxazole (PPO), 300 mg p-bis[2-(5-phenyloxazolyl)] - benzene (POPOP) dissolved in 414 ml of methanol and brought to one liter with

toluene. Volumes up to 0.5 ml water could be counted with this solution. The counting efficiency was 86.3% (6). Samples of 0.5 ml of cells dispersed in phosphate buffer were added to the scintillator solution and chilled before counting. Maximum counts were obtained at 18% gain with window settings of 500-1000 divisions.

Materials

L-proline was obtained from General Biochemicals. L-azetidine-2-carboxylic acid was obtained from CalBiochem. The analogue was checked for proline contamination by thin-layer chromatography using a pyridine-acetic acid-water solvent system (50: 35: 15) (v/v) (4). Isatin spray (0.2% in acetone) was used for development. Under these conditions 0.1% proline could be detected. No contamination was observed at this level.

L-proline-uniformly labeled-C^{14} was obtained from New England Nuclear Corporation and examined for impurities by paper chromatography using a butanol-acetic acid-water solvent system (4: 1 : 1) (v/v) (4). The radioactive peak was located with a Biospan Model 1002 Actigraph III paper-strip counter. No contamination was detected in this preparation. DL-azetidine-2-carboxylic acid-4-C^{14} was obtained from CalBiochem and examined in the same manner. A secondary peak was observed at an Rf value slightly greater than that of the major peak. The center of

the primary peak was cut from the chromatogram, eluted with water, and examined a second time. No further contamination was detected. L-glutamic acid-uniformly labeled-C^{14} was obtained from Sigma Corporation. This material had been previously purified by paper chromatography according to Krishnaswamy, Pamiljans and Meister (13) followed by column chromatography with charcoal and celite by a method developed in this laboratory (6). The amounts of the first two radioactive compounds were determined by dry weight measurement. The amount of radioactive glutamic acid was determined by the quantitative ninhydrin method of Yemm and Cocking (31).

RESULTS AND DISCUSSION

Effect of Azetidine-2- carboxylic Acid on Growth

In order to estimate the overall effect of A-2-C on *E. coli*, experiments designed to determine the effect of this compound on the growth rate of strain W were conducted. The concentration of proline was varied in the presence of 1.0×10^{-4} M A-2-C. The resulting growth curves are shown in Figure 6.

Under the conditions of the experiment, a generation time of 72 minutes was found both in the presence and absence of 8.7×10^{-5} M proline in the absence of inhibitor. These results indicate that strain W is capable of synthesizing sufficient proline for growth since additional proline did not affect the growth rate. However, a marked decrease of growth rate was noted in the presence of the analogue alone as shown in Table III. Simultaneous addition of small amounts of proline, relative to the amount of A-2-C present, significantly reduced inhibition. An equal weight of proline (8.7×10^{-5} M) completely reversed the effect of the inhibitor. These data are in agreement with those of Fowden and Richmond (11) who found similar inhibition of the growth rate of *E. coli* in the presence of A-2-C.

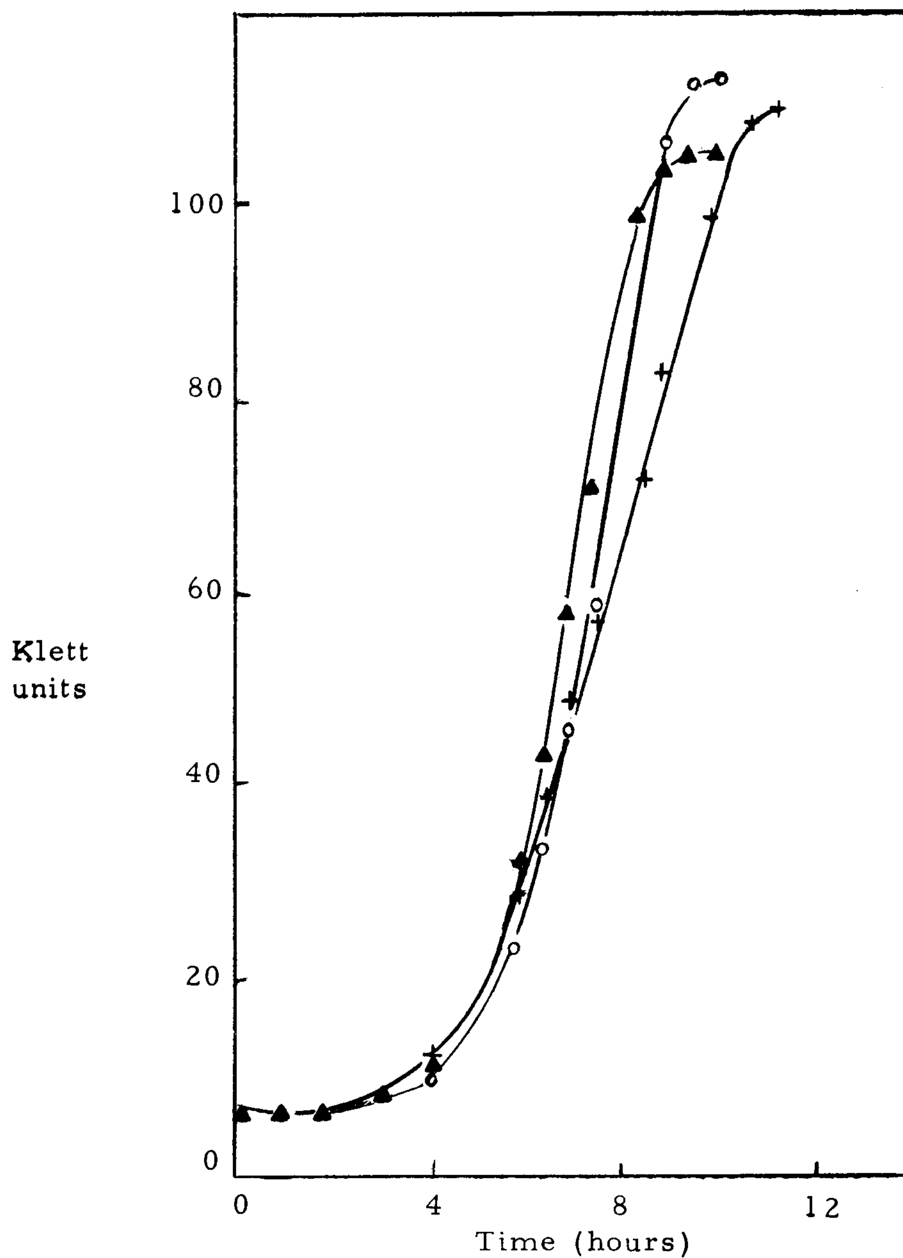

Figure 6A. Effect of A-2-C on Growth of Strain W in the Presence of Varying Concentrations of Proline. Concentrations in M/L x 10^{-5}.

▲; 0, A-2-C; 0, proline and
0, A-2-C; 8.7, proline
o; 10.0 A-2-C; 8.7, proline
+; 10.0 A-2-C; 8.7 x 10^{-1}, proline

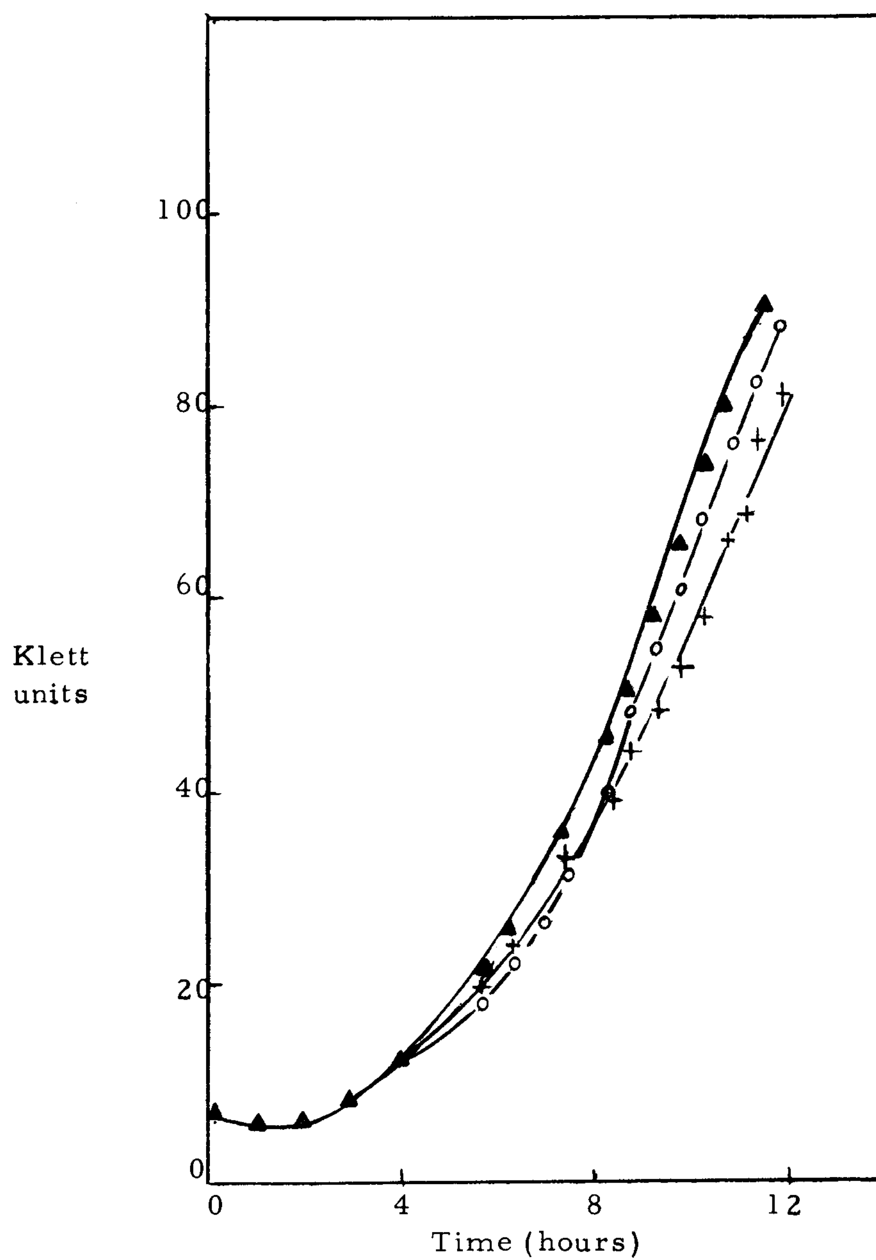

Figure 6B. Effect of A-2-C on Growth of Strain W in the Presence of Varying Concentrations of Proline. Concentrations in M/L x 10^{-5}.

▲; 10.0, A-2-C; 8.7 x 10^{-2} proline
o; 10.0, A-2-C; 4.4 x 10^{-2} proline
+; 10.0, A-2-C; 0, proline

Table III. Effect of A-2-C on Growth of Strain W in the Presence of Varying Concentrations of Proline.

Proline (moles/liter)	A-2-C (moles/liter)	Generation Time (minutes)	Inhibition of Growth (%)
0	0	72	0
8.7×10^{-5}	0	72	0
8.7×10^{-5}	1.0×10^{-4}	73	0
8.7×10^{-6}	1.0×10^{-4}	138	47.0
8.7×10^{-7}	1.0×10^{-4}	159	54.0
4.4×10^{-7}	1.0×10^{-4}	168	56.5
0	1.0×10^{-4}	246	70.4

In contrast to the results with strain W, addition of A-2-C to cells of strain WPl gave no inhibition of growth. In all cases a mean generation time of 75 minutes was obtained as shown in Table IV. The lack of effect of A-2-C indicates the presence of sufficient proline to overcome inhibition.

Strain WPl is apparently isogenic with the wild type strain W except that it is unable to control its proline biosynthesis and excretes an excess of the amino acid into the medium. Baich and Pierson (3) found that cells of WPl excreted 0.22 mg of proline (2×10^{-3} moles) per mg of bacteria (402 Klett Units) when grown in minimal medium. On the basis of these findings, it was calculated that a concentration of 3×10^{-5} M proline was present in the medium at the beginning of growth of WPl in the current work. This concentration of proline was sufficient to reverse inhibition of an external concentration of

A-2-C over three times as large (10×10^{-5} M).

The fact that small amounts of proline counter the effect of A-2-C and that the wild type strain W is normally able to make this much proline suggests that A-2-C inhibits biosynthesis of this amino acid.

Table IV. Effect of A-2-C on Growth of Strain WPl In the Presence of Varying Concentrations of Proline.

Proline (moles/liter)	A-2-C (moles/liter)	Generation Time (minutes)
0	0	73
8.7×10^{-5}	0	73
8.7×10^{-5}	1.0×10^{-4}	78
8.7×10^{-6}	1.0×10^{-4}	78
8.7×10^{-7}	1.0×10^{-4}	75
4.4×10^{-7}	1.0×10^{-4}	75
0	1.0×10^{-4}	73

Effect of Azetidine-2- carboxylic Acid on Excretion of Glutamic γ- semialdehyde

In view of the effects of A-2-C on the growth of strains W and WPl, it was considered of interest to investigate the effect of A-2-C on the reduction of glutamic acid to GSA to determine possible inhibition of this step by the analogue. Strains 55-1 and WPl-30 were used for this study since these strains excrete GSA into the medium.

Strecker (22) and Baich and Pierson (3) have shown that the

excretion of GSA in resting cells of strain 55-1 is very sensitive to the presence of proline. Excretion is halted in the presence of as little as 8.7×10^{-6} M proline in the medium. Similar inhibition by proline has been found by Tristram and Thurston (24) in strain Vpro-1. These inhibiting effects have been attributed to control by feedback inhibition of the enzyme reducing glutamic acid to GSA.

The data presented in Figure 7 indicate that this enzyme is subject to false feedback inhibition by A-2-C. However, the effect of A-2-C is much less than that of proline. The excretion of GSA by resting cells of 55-1 was slightly decreased in the presence of analogue at an external concentration of 1.0×10^{-5} M. Approximately 38 percent inhibition was obtained at 1.0×10^{-4} M and the excretion of GSA was almost completely inhibited at 1.0×10^{-3} M A-2-C. Glutamic acid substrate was maintained at a constant level of 3.4×10^{-4} M in these experiments.

These results are in agreement with those of Tristram and Thurston (24) who reported an inhibition of 20 to 28 percent in the presence of 8×10^{-4} M A-2-C. These authors used 2.5 mg/ml bacteria (25×10^{8} cells) (20), while only 0.3 mg/ml bacteria (3×10^{8} cells) (20) were used in the present study. The inhibition produced at a concentration of 8.0×10^{-4} M A-2-C in the system of Tristram and Thurston (24) is comparable to that obtained here at 1.0×10^{-4} M.

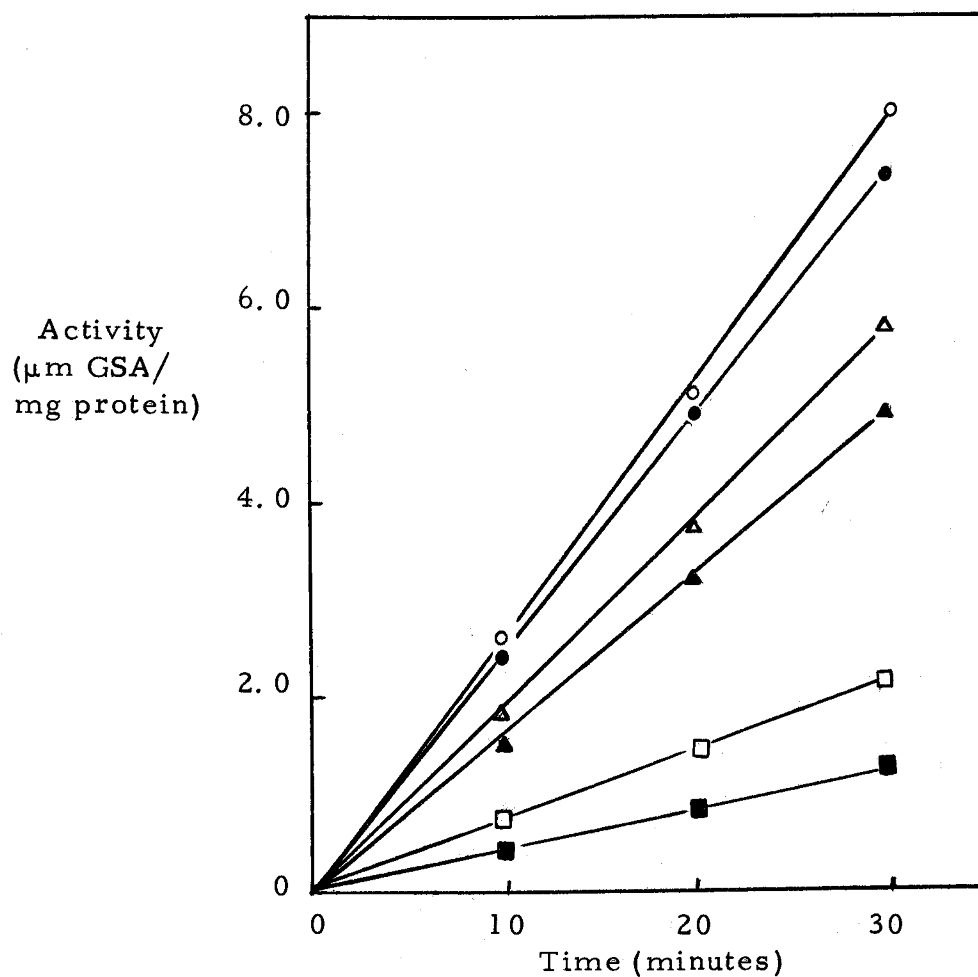

Figure 7. Excretion of GSA by Strain 55-1 in the Presence of Varying Concentrations of A-2-C. Concentrations of A-2-C in M/L x 10^{-5} o, 0; ●, 1; △, 5; ▲, 10; □, 50; ■, 100.

Data from two similar experiments with cells of strain WPl-30 are shown in Table V. Strain WPl-30 has no control of the proline pathway but is otherwise genetically identical to strain 55-1. As may be seen, an external concentration of A-2-C as high as 1×10^{-3} M had no significant effect on GSA production.

Table V. Excretion of GSA by Strain WPl-30 in the Presence of Varying Concentrations of A-2-C.

Concentration of Inhibitor (moles/liter x 10^{-5})	Specific Activity (μm GSA at 30 min/mg protein) Experiment 1	Experiment 2
0	3.74	3.00
1.0	3.95	3.21
5.0	4.16	2.89
10.0	3.95	2.93
50.0	4.25	2.89
100.0	4.16	3.14

Baich and Pierson (3) have reported that production of GSA by strain WPl-30 is also insensitive to the presence of proline at a final concentration of 8.7×10^{-6} M. These authors attribute the lack of sensitivity to a breakdown in control at the enzyme activity level. The data obtained in the current work indicate that A-2-C also has no effect on GSA synthesis in this strain.

Excretion of GSA by strain 55-1 was measured at varying concentrations of glutamic acid in the presence and absence of 1.0×10^{-4} M

A-2-C. The results of several independent experiments are presented in Table VI. The range of these data was large; however, the averages gave a reasonable pattern. The differences in excretion may be due to the physiological condition of the cells since the reaction demands active uptake of glutamic acid. The averages of the excretion as a function of time are shown in Figure 8. It should be noted that the amount of glutamic acid rapidly became limiting below a concentration of 8.7×10^{-5} M. The initial rate of excretion was extrapolated to 30 minutes in each case in which the limit was reached prior to this time. Specific activity, calculated as μm GSA/mg protein, was determined from the values as extrapolated.

A kinetic analysis of the inhibition of GSA excretion in intact cells of 55-1 was made from the above data by the method of Lineweaver and Burk (14) using a double-reciprocal plot as shown in Figure 9. Noncompetitive inhibition by A-2-C was indicated. The Michaelis constant (K_m), a measure of the dissociation of the enzyme-substrate complex, was calculated for glutamic acid in the presence and absence of A-2-C. The K_m of the normal substrate alone was 4.6×10^{-5} M, while that in the presence of A-2-C was 9.6×10^{-5} M. Thus, a decreased affinity of the substrate for the enzyme was indicated in the presence of the analogue.

The double-reciprocal plot of a comparable experiment using 1.7×10^{-7} M proline as the inhibitor is shown in Figure 10.

Table VI. Excretion of GSA by Strain 55-1 at Varying Concentrations of Glutamic Acid in the Presence and Absence of 1.0×10^{-4} M A-2-C.

Experiment No.	Specific Activity (μm GSA/mg protein after 30 min)									
	Without Inhibitor					With Inhibitor				
	Concentration of Glutamic Acid x 10^{-5} M									
	3.4	5.1	6.8	34.0	68.0	3.4	5.1	6.8	34.0	68.0
A	2.09	2.08	3.00	5.33	5.34	0.15	0.20	0.26	0.40	0.45
B	4.46	7.13	7.85	11.44	12.80	2.52	3.61	4.04	6.88	8.24
C	2.16	2.80	3.74	7.02	7.80	0.92	0.99	1.77	2.94	4.09
D	5.95	6.33	7.20	7.97	9.00	1.47	1.92	2.65	4.42	5.13
E	3.26	4.18	5.22	6.76	6.96	1.05	1.43	1.77	3.62	4.14
F	2.64	3.18	3.84	5.87	5.90	0.92	1.41	1.46	2.62	3.02
Average	3.43	4.30	5.14	7.40	7.95	1.17	1.59	1.99	3.48	4.18

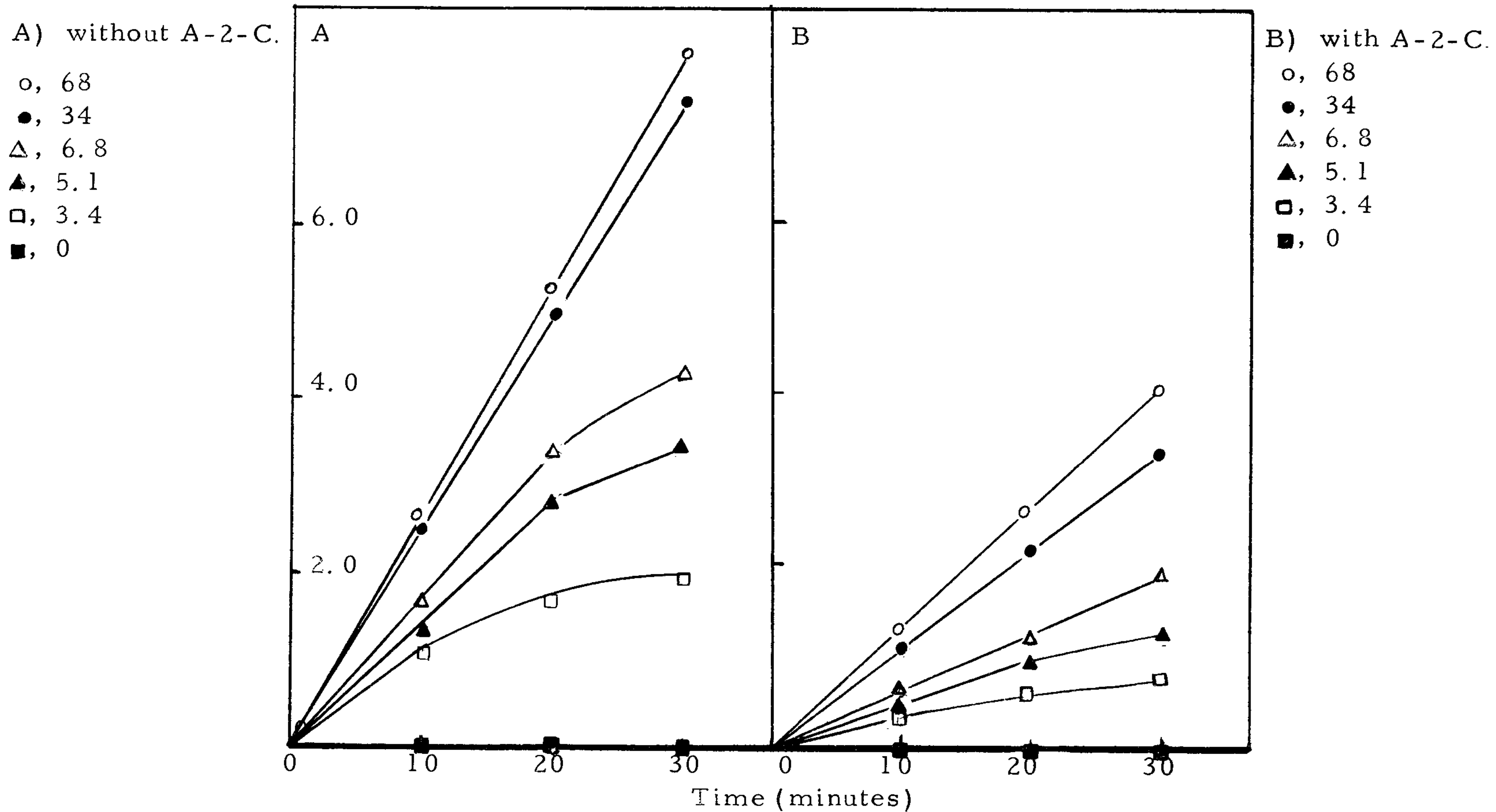

Figure 8. Excretion of GSA by Strain 55-1 at Varying Concentrations of Glutamic Acid in the Presence and Absence of 1.0×10^{-4} M A-2-C. Glutamic Acid Concentrations in M/L $\times 10^{-5}$.

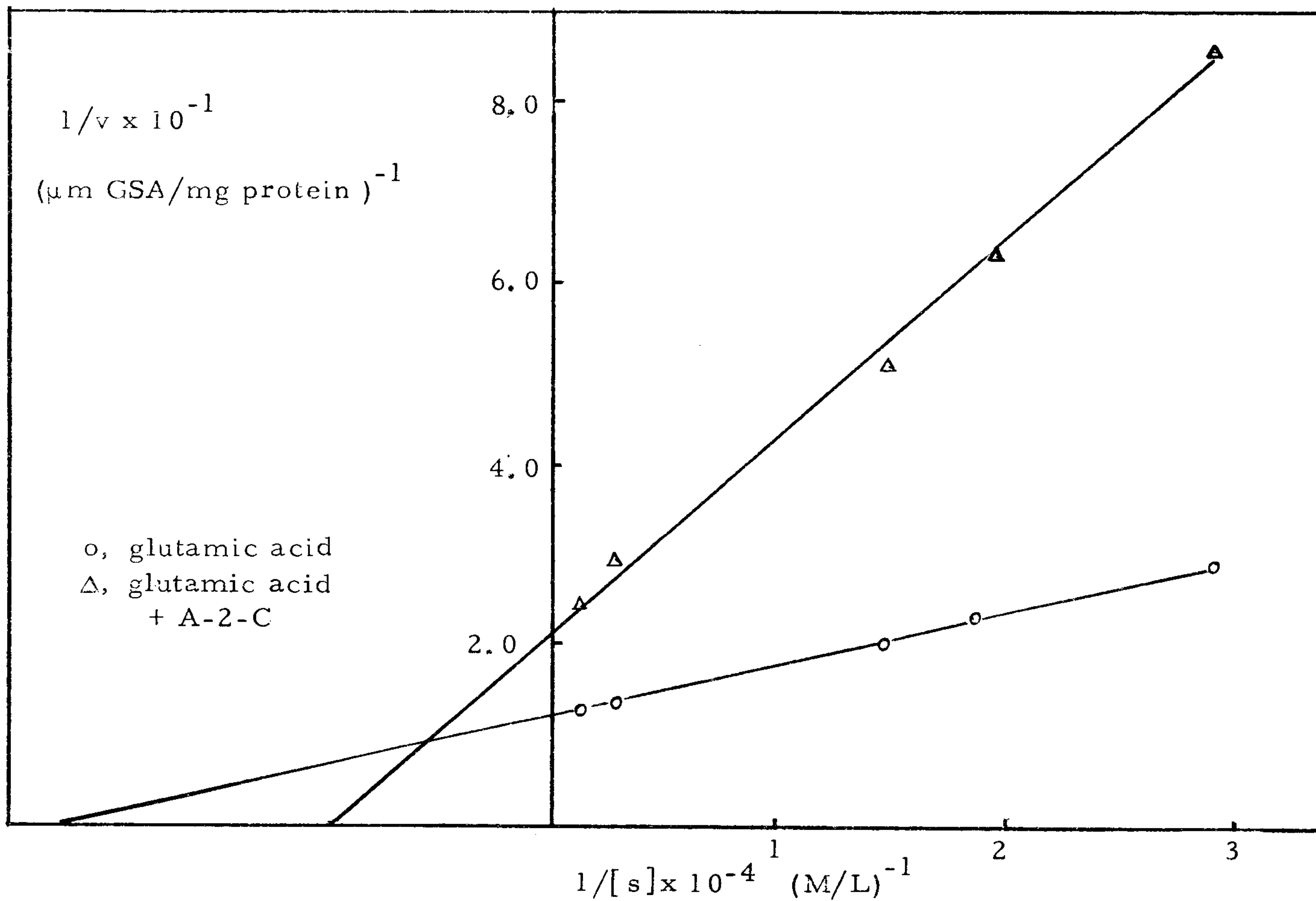

Figure 9. Lineweaver-Burk Analysis of GSA Production from Glutamic Acid in the Presence and Absence of 1.0 x 10^{-4} M-A-2-C.

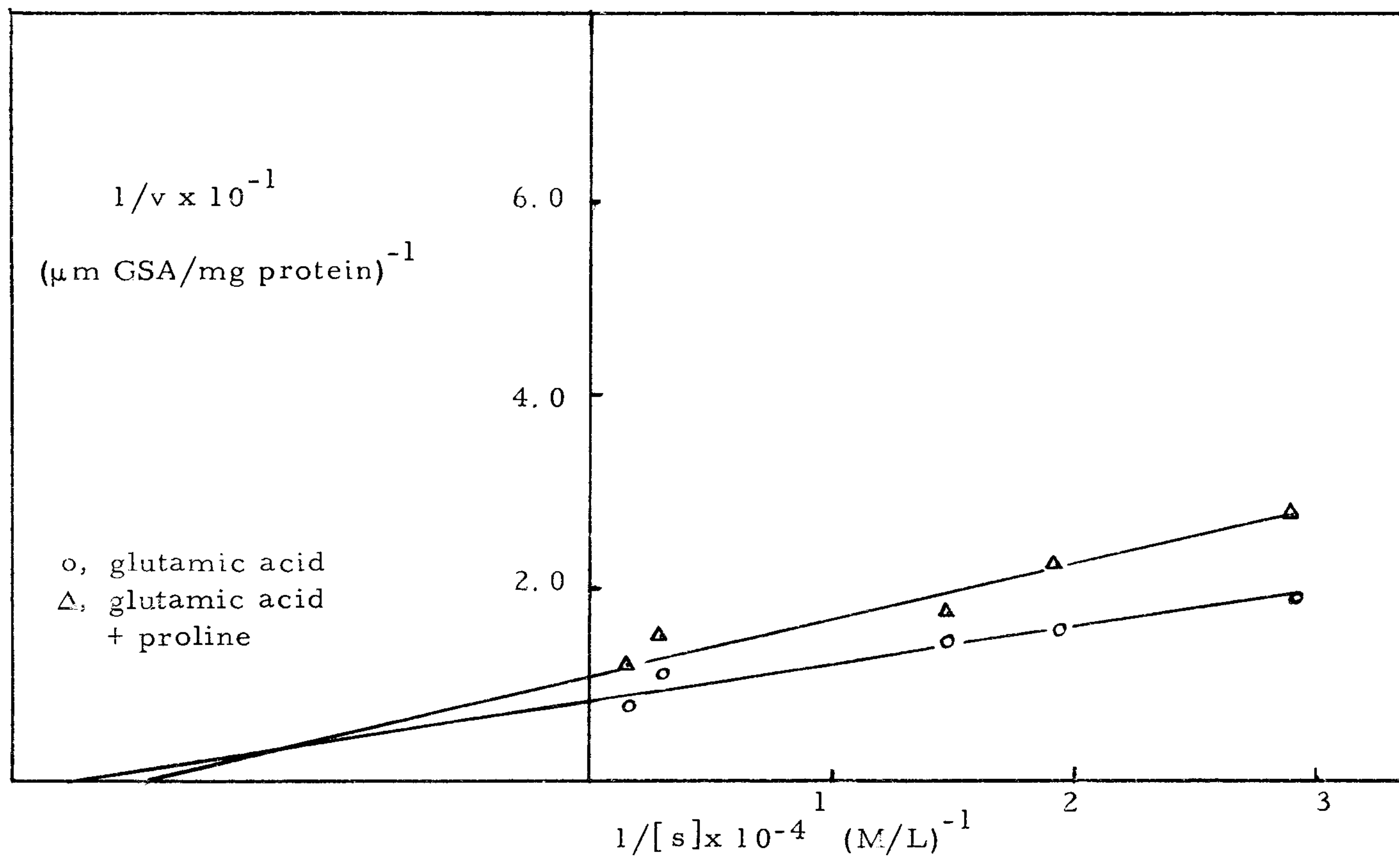

Figure 10. Lineweaver-Burk Analysis of GSA Production from Glutamic Acid in the Presence and Absence of 1.7×10^{-7} M Proline.

Noncompetitive inhibition by proline was indicated. The K_m in the presence of proline was 5.6×10^{-5} M, again indicating decreased binding in the presence of the inhibitor.

The dissociation of the enzyme-inhibitor complex, K_i, was also calculated by the method of Lineweaver and Burk (14). The K_i for A-2-C was 1.3×10^{-4} M while that for proline was 5.3×10^{-7} M. Proline forms a much more stable complex with the enzyme than does A-2-C. Thus, proline is clearly a more effective inhibitor of its own biosynthetic pathway than A-2-C, although the analogue is also capable of controlling this pathway.

Permeability Studies

Because of the marked differences in the inhibitor constants for A-2-C and proline, it was necessary to determine the actual internal concentrations of these compounds since the amount supplied externally to the bacteria need not reflect the concentration within the cells. Thus, it was of interest to determine the permeability of the strains employed in this study to glutamic acid, A-2-C, and proline.

Radioactive substrates were used to assess permeability. The results of one experiment of this type with strain W are shown in Figure 11. The relatively high uptake of glutamic acid was indicative of its ability to function in various metabolic pathways. The uptake

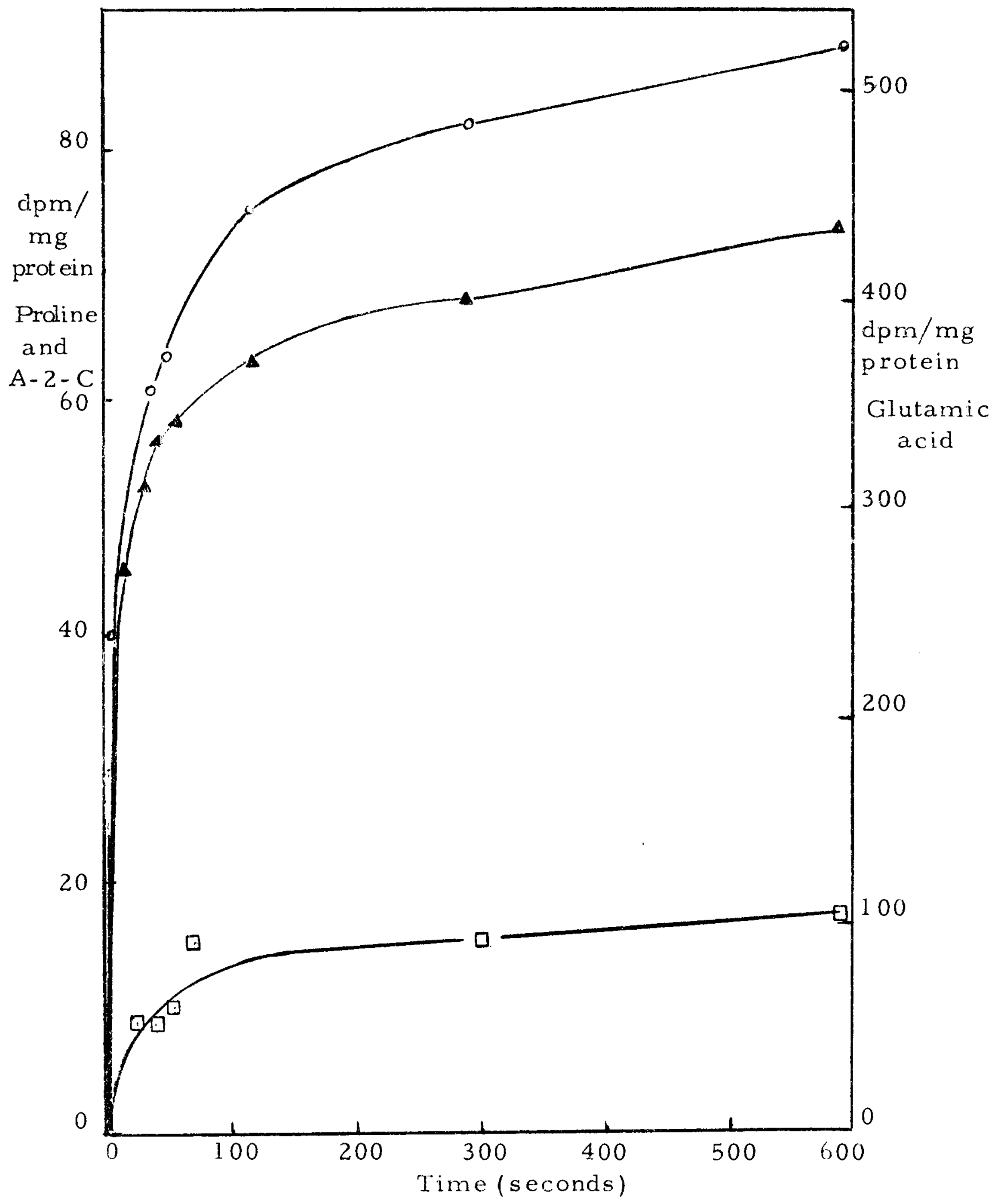

Figure 11. Uptake of Radioactivity by Resting Cells of Strain W.
o, L-glutamic acid - UL - C^{14};
▲, L-proline - UL - C^{14};
□, DL-azetidine - 2 - carboxylic acid - 4 - C^{14}

of proline by this strain was lower denoting a smaller pool of this amino acid within the cells. A measurable amount of A-2-C was also taken into the cells. Similar uptake of another proline analogue, thioproline, has been demonstrated by Unger and DeMoss (26).

The results of permeability studies with all strains may be seen in Table VII. Maximum uptake of radioactivity was observed within approximately two minutes with all substrates. The uptake of glutamic acid was high in all cases. Proline uptake was consistently lower. The uptake of A-2-C was low but relatively uniform in all strains. Uptake of the analogue by strains W, 55-1 and WP1-30 was 10 percent to 23 percent that of proline.

Somewhat unusual results were noted in the experiments with strain WP1. The uptake of A-2-C by this strain appeared to be equal to that of proline. These results may be explained by the fact that WP1 excretes excess proline as demonstrated by Baich and Pierson (3). This proline would effectively dilute the concentration of the radioactive amino acid.

The amounts of A-2-C and proline in cells of strain 55-1 were calculated as 6×10^{-2} μmoles/mg protein and 5×10^{-1} μmoles/mg protein, respectively, in a volume of one ml. These values correspond to internal concentrations of 6×10^{-5} M A-2-C and 5×10^{-4}M proline. Assuming that one-half the amount of A-2-C was the inactive

D-isomer, the above figure gives an effective internal concentration of 3×10^{-5} M, which is three times less than the external concentration of inhibitor (10×10^{-5} M) employed in the excretion experiments. The inhibitor constant (K_i) previously calculated for A-2-C should be corrected by a corresponding amount, from 1.3×10^{-4} M to 4×10^{-5} M. Any effect upon the K_i for proline could not be evaluated since the concentration of proline taken into the cells (5×10^{-4}M) was greater than the external concentration used in the excretion experiments (1.7×10^{-7} M).

The above data indicate that A-2-C is a more effective inhibitor than previously believed since only a limited amount of the material actually enters the bacterial cell.

Table VII. Uptake of Radioactivity by Resting Cells of Strains W, 55-1, WPl, and WPl-30 Utilizing C^{14} Labeled Substrates.

	Specific Activity x 10^4 (dpm/mg protein)*					
	Strain W			Strain 55-1		
Time (sec.)	L-glutamic Acid-UL-C^{14}	L-proline-UL-C^{14}	DL-azetidine-2-carboxylic Acid-4-C^{14}	L-glutamic Acid-UL-C^{14}	L-proline-UL-C^{14}	DL-azetidine-2-carboxylic Acid-2-C^{14}
0	0+	0	0	0	0	0
15	273	46	10	222	73	9
30	348	53	10	268	76	10
45	363	57	11	261	78	10
60	376	58	16	267	85	11
120	448	63	15	290	94	13
300	477	68	15	319	108	13
600	524	73	17	348	113	11
	Strain WPl			Strain WPl-30		
0	0	0	0	0	0	0
15	605	6	7	247	27	5
30	536	6	6	277	32	7
45	563	6	7	317	34	6
60	620	6	7	348	39	6
120	618	7	7	435	46	10
300	760	8	9	396	62	10
600	800	9	10	451	73	11

*Normalized to 1 $\mu c/\mu$mole substrate specific activity.
+ Background.

SUMMARY AND CONCLUSIONS

It is concluded that azetidine-2-carboxylic acid is an effective false feedback inhibitor of proline biosynthesis in E. coli from the following: (a) The analogue inhibits the growth of the wild type strain W. Inhibition is reversed by simultaneous addition of small amounts of proline. Since strain W can normally produce this much proline, these results suggest that A-2-C inhibits biosynthesis of the amino acid. (b) The analogue is capable of inhibiting the production of GSA in strain 55-1. Similar inhibition of GSA production by proline has been attributed to feedback inhibition (3, 21, 23). Thus, the analogue may be regarded as a false feedback inhibitor of the proline pathway.

Analysis of kinetic constants indicates that both A-2-C and proline are noncompetitive inhibitors of glutamic acid. The characteristics of the enzyme utilizing glutamic acid are in keeping with the model of an allosteric protein proposed by Jacob and Monod (12) in which separate sites are available for the action of substrate and inhibitor. However, since whole-cell preparations were used, it is not possible to conclude that the enzyme itself is an allosteric one. Comparison of inhibitor constants shows that proline forms a more stable complex with the enzyme than does the analogue. This result would be expected since natural feedback inhibitors are usually the most effective.

Consideration of A-2-C as a false feedback inhibitor suggests that the action of this analogue may be attributed largely to its structural similarity with proline. This is not to say that ionization of the analogue molecule does not also influence its effectiveness as an inhibitor. As Richmond (19) has stated, an analogue must, of necessity, either produce the same type of ion as the corresponding amino acid or be un-ionized at physiological pH values. There is a lack of information regarding the dissociation constants of the α-carboxyl and imino nitrogen functions of A-2-C. However, these constants must not differ greatly from those of proline since the analogue can substitute for the amino acid not only in blocking proline synthesis, but also in being concentrated by the proline permease (17) and in bonding with prolyl sRNA (16).

Stereochemical analysis (7) indicates that the four-membered imino ring of A-2-C is relatively planar; while the five-membered ring of proline, in addition to containing one more methylene group, is somewhat distorted. Therefore a proline molecule would occupy a significantly larger volume of space than one of the analogue. Any crevice in the enzyme which would admit a proline molecule would also be expected to accept an A-2-C molecule. However, binding of the latter by the enzyme would likely be much weaker than the binding of proline since the analogue molecule would not fill the space available at the reactive site on the enzyme.

Thus, A-2-C would function as an effective inhibitor of proline biosynthesis, although it would be less efficient than the amino acid itself.

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