

# Valine Accumulation by $\alpha$ -Aminobutyric Acid-Resistant Mutants of *Serratia marcescens*

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$\alpha$ -Aminobutyric acid, norvaline, and norleucine, which are analogues of branched-chain amino acids, inhibited the growth of *Serratia marcescens*. The inhibitory effect of these three analogues was counteracted by branched-chain amino acids. A number of mutants resistant to these analogues were isolated.  $\alpha$ -Aminobutyric acid-resistant (*abu-r*) mutants markedly accumulated L-valine in the culture medium, but the other analogue-resistant mutants did not. Acetohydroxy acid synthetase, which seems to be rate-limiting for the biosynthesis of L-valine, was derepressed in *abu-r* mutants. One of the *abu-r* mutants, no. 140, which accumulated over 8 mg of L-valine per ml, had about a 20-fold increase in the enzyme level. Most of the *abu-r* mutants had acetohydroxy acid synthetase activity which was sensitive to feedback inhibition by L-valine to the same extent as in the parent strain. However, the enzyme of two of *abu-r* mutants was less sensitive to L-valine, and one of the two was the best valine accumulator.

Since Adelberg and Cohen (1, 3) observed that mutants resistant to thienylalanine, *p*-fluorophenylalanine, and norleucine excreted L-phenylalanine, L-tyrosine, and L-methionine, respectively, it has been reported that several amino acid analogue-resistant mutants excreted the corresponding amino acids and lacked regulatory mechanisms for amino acid biosynthesis (5). Therefore, we intended to utilize amino acid analogue-resistant mutants for amino acid production, and succeeded in isoleucine and valine production (presented in part at the 39th Annual Meeting of the Agricultural Chemical Society, Japan, 1968). Recently, accumulation of L-threonine and L-lysine by analogue-resistant mutants has been also reported (22, 23).

In our studies on L-isoleucine accumulation from D-threonine by *Serratia marcescens* (12-14), it was observed that the addition of L-valine strongly inhibited L-isoleucine accumulation and caused  $\alpha$ -aminobutyric acid formation in the culture medium. A similar result was observed with addition of L-leucine. These phenomena were considered to be due to either feedback inhibition or repression by L-valine and L-leucine of acetohydroxy acid (AHA) synthetase, which is a bifunctional enzyme involved in the formation both L-isoleucine and L-valine (see Fig. 1). By using mutants in which AHA synthetase would be released from feedback inhibition, repression, or both, it might be expected that L-isoleucine accu-

mulation would markedly increase and that L-valine would be accumulated in the culture medium devoid of D-threonine.

A number of mutants resistant to  $\alpha$ -aminobutyric acid, norvaline, and norleucine, which antagonized branched-chain amino acids, were isolated. This paper reports that  $\alpha$ -aminobutyric acid-resistant (*abu-r*) mutants markedly accumulated L-valine and were derepressed for AHA synthetase.

## MATERIALS AND METHODS

**Organisms.** The organisms used in this study were *S. marcescens* no. 1 (12) and mutants resistant to  $\alpha$ -aminobutyric acid, norvaline, and norleucine, which were derived from *S. marcescens* no. 1 as described below.

**Isolation of amino acid analogue-resistant mutants.** The cells of *S. marcescens* no. 1 were irradiated with ultraviolet light or treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and cultured at 30 C for 48 hr on agar plates containing the minimal medium of Davis and Mingioli (4), modified by omitting the citrate and increasing the glucose to 0.5%, plus amino acid analogues (DL- $\alpha$ -aminobutyric acid, DL-norvaline, and DL-norleucine) at a concentration of 10 or 20 mg/ml. Large colonies were isolated as amino acid analogue-resistant mutants.

**Media and cultural conditions.** Testing for antagonism and growth of resistant mutants was performed in test tubes (13 by 103 mm) containing 2 ml of the medium. The cultures in late log or early stationary phase in the minimal medium were diluted with the fresh minimal medium supplemented with or without

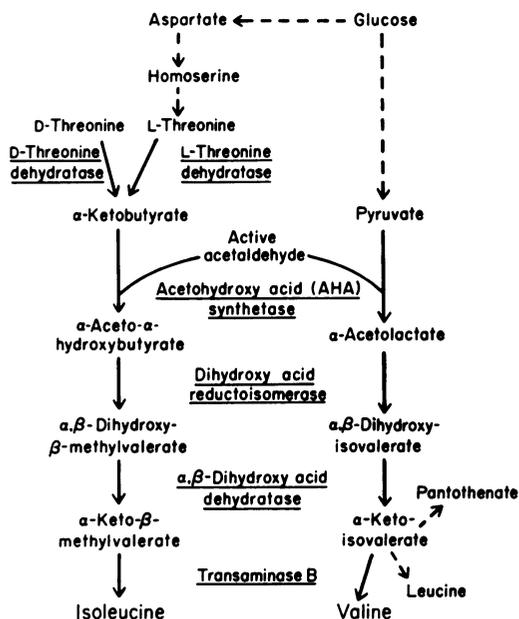


FIG. 1. Metabolic pathway of isoleucine-valine biosynthesis.

branched-chain amino acids and analogues. Incubation was at 30 C with shaking. The medium for L-valine accumulation contained 2% glucose, 10% dextrin (obtained from Matsutani Chemical Co., Ltd.), 1% urea, 0.1%  $K_2HPO_4$ , 0.05%  $MgSO_4 \cdot 7H_2O$ , and 2%  $CaCO_3$ . Glucose plus dextrin were autoclaved separately and added aseptically to the remaining components of the medium. The organisms were grown in 500-ml shaking flasks containing 15 ml of the medium at 30 C for 48 hr with reciprocal shaking (140 rev/min, 8-cm stroke).

**Analytical methods.** L-Valine was measured micro-biologically by using *Leuconostoc mesenteroides* P-60. In growth experiments, the growth was measured turbidimetrically at 660 nm with a Hitachi electric photometer (EPO-B type). An optical density of 0.10 corresponded to 160  $\mu$ g of dry cells per ml. In experiments of L-valine accumulation, the growth was estimated by measuring the optical density at 660 nm of fermentation broth diluted 1:10 with 0.1 N HCl to dissolve  $CaCO_3$  in the broth, and is expressed as dry cell weight calculated from a standard curve.

**Preparation of cell-free extracts and assay of enzyme activities.** The cells cultivated for 24 hr in the medium for L-valine accumulation were harvested by centrifugation and washed twice with 0.05 M potassium phosphate buffer (pH 8.0). The cells were suspended in the same buffer containing 30% glycerin to stabilize AHA synthetase (M. Kisumi and H. E. Umbarger, unpublished data) and disrupted at below 10 C with a sonic oscillator (Toyoriko model UD-5D-5, 10 KHz, 100 w) for 4 min. The sonically treated suspension was centrifuged at  $15,000 \times g$  for 30 min at 0 C, and the supernatant fluid was used as cell-free extract for the assay of enzymes. Protein in cell-free extracts was determined by the method of Lowry et al. (16).

The reaction mixture for the assay of AHA synthetase contained 40  $\mu$ moles of sodium pyruvate, 10  $\mu$ moles of  $MgCl_2$ , 0.2  $\mu$ mole of thiamine pyrophosphate, 0.05  $\mu$ mole of flavine adenine dinucleotide, 100  $\mu$ moles of potassium phosphate buffer (pH 6.0 or 8.0) and cell-free extract (0.5 to 1.0 mg of protein) in a total volume of 1.0 ml. The incubation was carried out at 37 C for 15 min.  $\alpha$ -Acetylacetyl-CoA formed was determined as acetoin by the method of Westerfeld (27) after heating in the presence of  $H_2SO_4$ . Unless otherwise noted, AHA synthetase refers to the enzyme with an optimum at pH 8.0. The reaction mixture for the assay of L-threonine dehydratase contained 20  $\mu$ moles of L-threonine, 0.02  $\mu$ mole of pyridoxal phosphate, 100  $\mu$ moles of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 9.0), and cell-free extracts (0.5 to 1.0 mg of protein) in a total volume of 1.0 ml. The incubation was carried out at 37 C for 15 min.  $\alpha$ -Keto-butyrate formed was determined by the method of Friedemann and Haugen (7). Specific activities of enzymes are expressed as micromoles of products formed per milligram of protein per minute.

## RESULTS

**Antagonism between branched-chain amino acids and their analogues.** For the accumulation of amino acids, it may be useful to use mutants which lack certain metabolic regulatory mechanisms of amino acid biosynthesis. Adelberg devised a method for obtaining regulatory mutants by selecting for amino acid analogue-resistant strains (1). The effect of analogues of branched-chain amino acids on the growth of *S. marcescens* and the reversal of growth inhibition by the corresponding amino acids were examined.  $\alpha$ -Aminobutyric acid, norvaline, and norleucine were used as representative analogues.

As shown in Fig. 2,  $\alpha$ -aminobutyric acid was inhibitory to the growth of *S. marcescens*. This growth inhibition was partially antagonized by L-valine and completely by L-valine plus L-leucine. A transitory growth inhibition by norvaline was slightly overcome by any single branched-chain amino acid and more effectively by the mixture of three amino acids. Growth inhibition due to norleucine was also slightly reversed by L-isoleucine, L-valine, or L-leucine, and partially by a mixture of them. The possibility exists that biosynthetic enzymes of branched-chain amino acids are released either from feedback inhibition or from repression in mutants resistant to these analogues. Therefore, a number of resistant mutants were isolated by the method described above.

**Growth of analogue-resistant mutants.** Analogue-resistant mutants were grown in the medium with or without analogues. The growth curves of *abu-r* mutant no. 140, norvaline-resistant (*nva-r*) mutant no. 14, and norleucine-resistant (*nle-r*) mutant no. 141 are shown as typ-

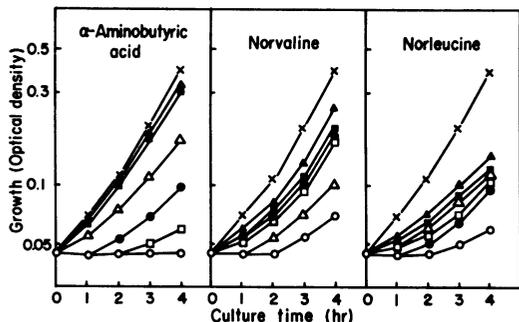


FIG. 2. Antagonism between branched-chain amino acids and their analogues in *Serratia marcescens*. Analogues and amino acids were added to the minimal medium at a concentration of  $2 \times 10^{-2}$  M and  $10^{-3}$  M, respectively, except the controls. Symbols: x, minimal medium; o, minimal medium containing the analogue; (DL-form); □, same, containing the analogue and L-isoleucine; △, same, containing the analogue and L-valine; ●, same, containing the analogue and L-leucine; ■, same, containing the analogue, L-valine, and L-leucine; ▲, same, containing the analogue, L-isoleucine, L-valine, and L-leucine.

ical examples in Fig. 3. Some *abu-r* mutants, e.g., no. 140, were very resistant to  $\alpha$ -aminobutyric acid, whereas the other *abu-r* mutants were only slightly resistant to this analogue. In addition, considerable cross-resistance to these three analogues was observed in some resistant mutants.

**L-Valine accumulation by analogue-resistant mutants.** Kinoshita et al. (11) and Hayashibe et al. (9) have reported that *S. marcescens* excreted low concentrations of L-valine into the culture medium. The parent strain of *S. marcescens* no. 1 hardly accumulated L-valine in the culture medium, as shown in Table 1. Ten representative *abu-r* mutants which differed in resistance level were tested for L-valine accumulation. Mutants *abu-r* no. 44 and 139, which showed only slight resistance to  $\alpha$ -aminobutyric acid, hardly accumulated L-valine. All the highly resistant mutants accumulated large amounts of L-valine. For example, no. 140, 5, and 9 accumulated over 8 mg of L-valine per ml in the medium when cultured for 48 hr. No. 117, which was isolated as a *nva-r* mutant, was simultaneously resistant to  $\alpha$ -aminobutyric acid and accumulated L-valine.

Mutants *nva-r* and *nle-r* were also examined for L-valine accumulation. The results are shown in Table 2. Only no. 43, mutant *nva-r*, accumulated L-valine. Though Karlström reported that *nva-r* mutants of *Escherichia coli* K-12 excreted L-valine in the medium (10), *nva-r* mutants of *S. marcescens* hardly accumulated L-valine.

**AHA synthetase levels in analogue-resistant**

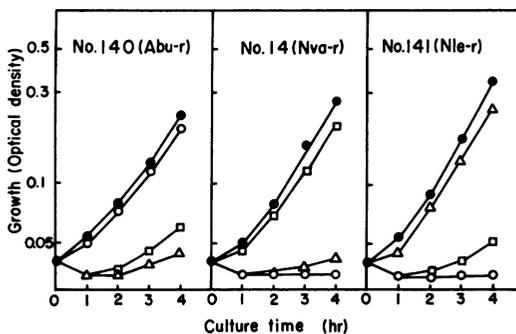


FIG. 3. Growth of three analogue-resistant mutants in the presence and absence of analogues. Symbols: ●, minimal medium; ○, same, containing  $8 \times 10^{-2}$  M DL- $\alpha$ -aminobutyric acid; □, same, containing  $8 \times 10^{-2}$  M DL-norvaline; △, same, containing  $8 \times 10^{-2}$  M DL-norleucine.

TABLE 1. Accumulation of L-valine and levels of acetohydroxy acid synthetase in  $\alpha$ -aminobutyric acid-resistant mutants

Strain (no.)	Growth <sup>a</sup>		L-Valine accumulated (mg/ml)		Acetohydroxy acid synthetase <sup>b</sup>	
	24 hr	48 hr	24 hr	48 hr	pH 6.0	pH 8.0
Parent	12.1	17.2	0.2	0.6	0.11	0.043
44	11.5	16.0	0	0.5	0.13	0.043
139	12.7	16.8	0	0.9	0.23	0.060
117	13.0	18.1	0.6	2.0	0.11	0.15
131	8.4	14.3	0.9	2.4	0.26	0.24
7	10.3	20.0	2.7	4.2	0.20	0.23
106	8.2	24.6	0.9	6.8	0.16	0.43
130-1	10.0	24.6	3.5	7.2	0.12	0.53
140	7.1	16.3	0.9	8.1	0.18	0.73
5	11.5	18.1	3.1	8.9	0.11	0.28
9	10.6	20.3	3.8	9.6	0.11	0.41

<sup>a</sup> Dry cell weight (mg/ml).

<sup>b</sup> Specific activity of acetohydroxy acid synthetase was determined at pH 6.0 and 8.0 with cell-free extracts prepared from cells cultured for 24 hr and is expressed as micromoles of  $\alpha$ -acetolactate formed per milligram of protein per minute.

**mutants.** It has been reported that several bacteria possess two distinct AHA synthetases, one with an optimum at pH 8 and the other with an optimum at pH 6 (8, 19). It is also known that the pH 8 AHA synthetase, which is feedback-inhibited by L-valine, is involved in the biosynthesis of L-isoleucine and L-valine and that the pH 6 enzyme serves to divert glucose catabolism from acidic to neutral products. Ramakrishnan and Adelberg (21), moreover, demonstrated that the pH 8 AHA synthetase might be rate-limiting for the biosynthesis of L-valine. On the other hand, Udaka and Kinoshita (26) described the optimal pH for AHA synthetase of L-valine-accumulating bacteria as being near pH 5.5 and showed that the activity was not feedback-in-

TABLE 2. Accumulation of L-valine and levels of acetohydroxy acid synthetase in norvaline- and norleucine-resistant mutants

Strain (no.)	Growth <sup>a</sup>		L-Valine accumulated (mg/ml)		Acetohydroxy acid synthetase <sup>b</sup>	
	24 hr	48 hr	24 hr	48 hr	pH 6.0	pH 8.0
Parent	12.1	17.2	0.2	0.6	0.11	0.043
14 ( <i>nva-r</i> ) <sup>c</sup>	3.3	16.3	0	0.2	0.13	0.043
42 ( <i>nva-r</i> ) <sup>c</sup>	9.7	16.3	0	0.2	0.032	0.082
43 ( <i>nva-r</i> ) <sup>c</sup>	12.7	24.6	0.6	1.3	0.39	0.060
45 ( <i>nva-r</i> ) <sup>c</sup>	1.6	1.9	0	0		
20 ( <i>nle-r</i> ) <sup>d</sup>	10.6	15.0	0.5	0.5	0.038	0.057
23 ( <i>nle-r</i> ) <sup>d</sup>	12.7	17.7	0	0	0.038	0.033
122 ( <i>nle-r</i> ) <sup>d</sup>	12.7	28.6	0.2	0.5	0.077	0.027
141 ( <i>nle-r</i> ) <sup>d</sup>	14.3	19.0	0	0.2	0.13	0.043

<sup>a</sup> Dry cell weight (mg/ml).

<sup>b</sup> Specific activity of acetohydroxy acid synthetase was determined at pH 6.0 and 8.0 with cell-free extracts prepared from cells cultured for 24 hr and is expressed as micromoles of  $\alpha$ -acetolactate formed per milligram of protein per minute.

<sup>c</sup> Norvaline-resistant mutant.

<sup>d</sup> Norleucine-resistant mutant.

hibited by L-valine. To investigate the mechanism for L-valine accumulation by *abu-r* mutants, AHA synthetase levels were determined at pH 8.0 and 6.0.

A correlation was observed between pH 8 AHA synthetase levels and L-valine accumulation (Table 1). One of the best accumulators, no. 140, had a 20-fold increased level of this enzyme as compared with the parent strain. It should be noted that no. 5 and 9, which had lower levels than no. 140, accumulated a larger amount of L-valine. This will be explained below. Levels of this enzyme in *nva-r* and *nle-r* mutants were as low as that in the parent strain (Table 2).

Levels of the pH 6 AHA synthetase in analogue-resistant mutants were varied (Tables 2 and 3). No. 43 had the highest level and some *abu-r* mutants had higher levels than the parent strain. However, no correlation was observed between pH 6 AHA synthetase level and L-valine accumulation.

**Feedback inhibition of AHA synthetase by L-valine.** To test whether L-valine accumulation by *abu-r* mutants might also be due to insensitivity of AHA synthetase activity to L-valine, feedback inhibition of this enzyme was examined. As shown in Table 3,  $10^{-3}$  M L-valine inhibited by about 40% of the enzyme activities of the parent strain, no. 130-1 and 140. There seems to be no marked difference in the sensitivity of AHA synthetase activity to L-valine among these three strains. Most of the *abu-r* mutants tested had AHA synthetase as sensitive to feedback inhibition by L-valine as the parent strain. As stated above, no. 5 and 9 accumulated a larger amount

TABLE 3. Feedback inhibition of acetohydroxy acid synthetase by L-valine in the parent strain and  $\alpha$ -aminobutyric acid-resistant mutants<sup>a</sup>

Strain (no.)	Inhibition (%)		
	L-Valine added (M)		
	$10^{-3}$	$10^{-2}$	$10^{-1}$
Parent	42	46	53
130-1	43	57	70
140	42	53	73
5	16	21	23
9	7	6	8

<sup>a</sup> Activity of acetohydroxy acid synthetase was determined at pH 8.0 with cell-free extracts prepared from cells cultured for 24 hr.

of L-valine than no. 130-1 and 140, although the former mutants had a lower level of AHA synthetase than the latter. As indicated in Table 3, the enzymes of no. 5 and 9 were feedback-inhibited to a lesser extent than those of the other strains. Notably, the enzyme of no. 9 was hardly inhibited even by high concentrations of L-valine. It was, therefore, considered that no. 9 was the best accumulator among *abu-r* mutants because of the lack of both control mechanisms: feedback inhibition and repression.

**Effect of pH on the activity of AHA synthetase.** The effect of pH on the activity of AHA synthetase was examined, using the parent strain, *abu-r* mutant no. 140, and *nva-r* mutant no. 43, because the ratios of the activity at pH 6 to that at pH 8 were different among many strains. As shown in Fig. 4, the activity of the parent strain at pH 6.0 was higher than that at pH 8.0, and the pH 6 activity was not feedback-inhibited by  $10^{-2}$  M L-valine. In the *abu-r* mutant, there was much greater activity at pH 8.0. Accordingly, the resistance to  $\alpha$ -aminobutyric acid is considered to alter the formation of only the pH 8 AHA synthetase. In no. 43, which hardly accumulated L-valine, the activity at pH 6.0 was much higher than that at pH 8.0. These results also indicate that two distinct AHA synthetases exist in *S. marcescens*, as recognized in *Aerobacter aerogenes* (8) and *E. coli* (19), and that the pH 8 enzyme is considered to participate in L-valine formation.

**Coordinately derepressed levels of L-threonine dehydratase and AHA synthetase in *abu-r* mutants.** L-Threonine dehydratase is considered to be the rate-limiting enzyme for L-isoleucine biosynthesis (20). It was of interest to examine whether L-threonine dehydratase is derepressed in *abu-r* mutants. As shown in Fig. 5, the levels of L-threonine dehydratase were parallel with those of AHA synthetase in *abu-r* mutants. One

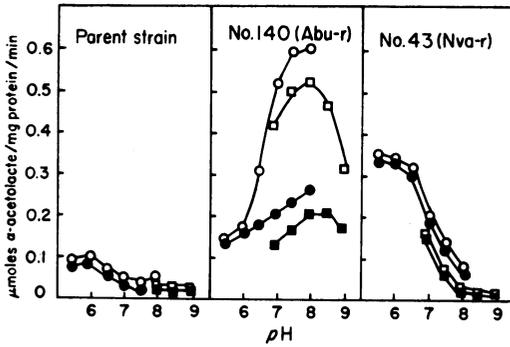


FIG. 4. Effect of pH on the activity of acetohydroxy acid synthetase in the parent strain and two analogue-resistant mutants. Activity was determined by using cell-free extracts prepared from cells cultured for 24 hr. Symbols: ○, potassium phosphate buffer without L-valine; ●, potassium phosphate buffer with  $10^{-2}$  M L-valine; □, Tris-hydrochloride buffer without L-valine; ■, Tris-hydrochloride buffer with  $10^{-2}$  M L-valine.

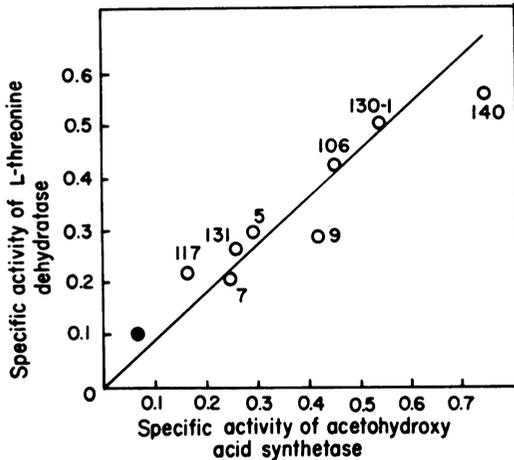


FIG. 5. Coordinately derepressed levels of L-threonine dehydratase and acetohydroxy acid synthetase (pH 8.0) in  $\alpha$ -aminobutyric acid-resistant mutants. Specific activities were determined by using cell-free extracts prepared from cells cultured for 24 hr and are expressed as micromoles of product formed per milligram of protein per minute. The numbers refer to the numbers of  $\alpha$ -aminobutyric acid-resistant mutants. Symbols: ●, parent strain; ○,  $\alpha$ -aminobutyric acid-resistant mutants.

of the best L-valine accumulators, no. 140, which had the highest AHA synthetase level, also had the most increased level of L-threonine dehydratase. However, *abu-r* mutants did not accumulate L-isoleucine in the medium either with or without L-threonine. This is evidently in agreement with our finding that L-threonine dehydratase was almost completely inhibited by low concentrations of L-isoleucine in the mutants as well as in

the parent strain. The levels of this enzyme in *nva-r* and *nle-r* mutants were the same as that in the parent strain.

**Changes during L-valine accumulation.** Typical changes during L-valine accumulation by no. 140 are shown in Fig. 6. L-Valine accumulation paralleled with the growth and reached a maximum at 48 hr. A high level of the pH 8 AHA synthetase was evident from 18 to 36 hr, during the log phase of growth. The intense L-valine accumulation during this period may be attributed to the high level of the enzyme. Besides L-valine, acetoin derived from  $\alpha$ -acetolactate, presumably formed by the pH 6 AHA synthetase, and a small amount of L-leucine were observed in the culture medium.

Other *abu-r* mutants showed the same pattern of changes. Mutant *nva-r*, no. 43, which had a high level of the pH 6 AHA synthetase, formed a large amount of acetoin (ca. 10 mg/ml) in the medium without L-valine when cultured for 48 hr.

## DISCUSSION

L-Valine production by wild strains and auxotrophs of several bacteria has been reported (17, 24, 26). However, there has been no report of a marked accumulation of L-valine by mutants resistant to analogues of L-valine. The *abu-r* mutants of *S. marcescens* described in this paper accumulated large amounts of L-valine. To elucidate the mechanism of this L-valine accumulation, levels and feedback inhibition of AHA synthetase were examined. It was found that many *abu-r* mutants were derepressed for this enzyme. AHA synthetase of most of the *abu-r* mutants was as sensitive to feedback inhibition by L-valine as that of the parent strain. However, the

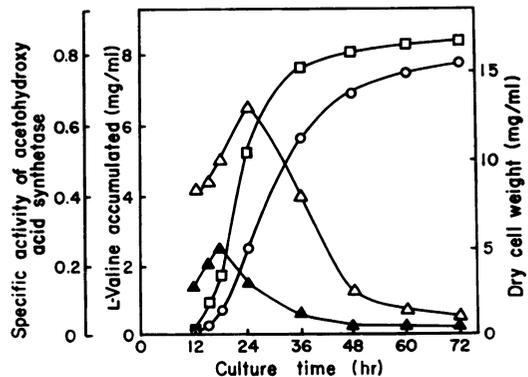


FIG. 6. Changes of L-valine accumulation and acetohydroxy acid synthetase level in  $\alpha$ -aminobutyric acid-resistant mutant no. 140. Symbols: ○, growth (dry cell weight); □, L-valine accumulated; △, specific activity of acetohydroxy acid synthetase at pH 8.0; ▲, same, at pH 6.0.

intensity of feedback inhibition was not as great as that of *E. coli* described by Leavitt and Umbarger (15). On the basis of these facts, it may be concluded that L-valine accumulation by *S. marcescens* mutants is due to both the derepressed level and weak feedback inhibition of AHA synthetase. Two of the *abu-r* mutants had a desensitized AHA synthetase, as well as increased levels of the enzyme. These mutants were the best accumulators among *abu-r* mutants tested, presumably because of the lack of both control mechanisms: repression and feedback inhibition.

The *abu-r* mutants of *S. marcescens* might be compared with the *abu-r* and valine-resistant (*val-r*) mutants of *E. coli* K-12 described by Ramakrishnan and Adelberg (20). In an *abu-r* mutant of *E. coli*, AHA synthetase was derepressed (21) and in *val-r* mutants of *E. coli*, three of the isoleucine-valine enzymes, i.e., L-threonine dehydratase, dihydroxy acid dehydratase, and transaminase B, were coordinately derepressed (20). The *val-r* mutants were found to excrete a detectable amount of L-isoleucine, but it was not revealed whether the *abu-r* mutant excreted any branched-chain amino acid. Their genetic studies showed that the three structural genes governing L-threonine dehydratase, dihydroxy acid dehydratase, and transaminase B are controlled by operator A, whereas a structural gene governing AHA synthetase is controlled by operator B (21). On the other hand, the *abu-r* mutation in *S. marcescens* caused the coordinate derepression of AHA synthetase, L-threonine dehydratase, and transaminase B (*manuscript in preparation*). It has also been found that these enzymes of an isoleucine-valine auxotroph of *S. marcescens* were multivalently repressed by L-isoleucine, L-valine, and L-leucine. The AHA synthetase level was roughly parallel to those of L-threonine dehydratase and transaminase B under various conditions in this auxotroph (presented at the 17th Symposium on Amino Acid and Nucleic Acid, Tokyo, Japan, 1968). These facts suggest that a single operator may control the three structural genes which govern these enzymes. Moreover, according to the suggestion that the derepression of four enzymes leading to the isoleucine-valine biosynthesis in *abu-r* and *val-r* mutants of *E. coli* was caused by the mutation in the operator loci (20, 21), there exists the possibility that the *abu-r* mutation in *S. marcescens* might alter a single operator controlling synthesis of AHA synthetase, L-threonine dehydratase, and transaminase B.

Calvo et al. (2) recently showed that a majority of trifluoroleucine-resistant mutants of *Salmonella typhimurium* had constitutive levels of isoleucine, valine, and leucine biosynthetic enzymes. In the present study, growth inhibition

by  $\alpha$ -aminobutyric acid of *S. marcescens* was reversed not only by L-valine but also by L-leucine. Moreover, it has been found that leaky revertants of an isoleucine auxotroph derived from an *abu-r* mutant accumulated large amounts of L-leucine in the culture medium (presented at the Annual Meeting of the Society of Fermentation Technology, Osaka, Japan, 1969). These facts cannot exclude the possibility that some *abu-r* mutations in *S. marcescens* are the alterations of a regulator gene controlling synthesis of all enzymes forming the three branched-chain amino acids.

It is also known that several amino acid analogues inhibit the growth of bacteria by blocking the transfer of normal amino acids to transfer ribonucleic acid (tRNA) and that analogue-resistant mutants which have altered aminoacyl-tRNA synthetases are derepressed for the enzymes involved in the biosynthesis of amino acids (18). Freundlich and Clarke (6) reported that the competitive inhibition by  $\alpha$ -aminobutyric acid of valyl-tRNA synthetase may be one of the reasons for growth inhibition by this analogue. Szentirmai et al. (25) also demonstrated that the alteration of isoleucyl-tRNA in thiaisleucine-resistant mutants is accompanied by the derepression of the isoleucine-valine enzymes. These observations do not rule out the possibility that some *abu-r* mutation in *S. marcescens* might be alterations of the structural gene for valyl-tRNA synthetase.

Accordingly, further genetic analysis will be necessary for determining whether the *abu-r* mutation is located in the operator locus, in the regulator locus, or in the valyl-tRNA synthetase locus. In any case, it may be concluded that *abu-r* mutants of *S. marcescens* accumulate L-valine on account of genetic derepression of isoleucine-valine enzymes.

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