ISOLEUCINE–VALINE REQUIRING MUTANTS OF *SALMONELLA TYPHIMURIUM*. III: VALINE-SENSITIVE STRAINS[1]

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WE have isolated a number of mutant strains of *Salmonella typhimurium* that display a marked inhibition of growth when valine is added to minimal medium. Attempts to elucidate the nature of the mutation(s) in these valine-sensitive strains have included enzymatic and transductional analyses, as well as growth studies, on four of these strains. The results of these studies are presented and discussed in this report.

MATERIALS AND METHODS

Bacterial strains: The mutant strains were derived from wild-type *S. typhimurium* LT2, using the mutagen N-methyl-N′-nitro-N-nitrosoguanidine and the procedure described by ADELBERG, MANDEL and CHEN (1965). The strains sensitive to valine were detected by replica plating from nutrient agar master plates onto minimal medium (ARMSTRONG and WAGNER 1964) containing 4 μg L-valine per ml. The abbreviation VS (valine sensitive) was arbitrarily selected to designate those strains whose growth is inhibited by the supplementation of minimal medium with valine. Another characteristic shared by the VS strains is a slow rate of growth on minimal medium. Of the 15 strains isolated, four (VS-7, 9, 10, and 13) were selected for the analyses presented in this report. Other *S. typhimurium* strains utilized in this study include: LT2; *ilvA118* (threonine dehydratase deficient); *ilvC8*, *42*, and *66* (reductoisomerase deficient); and *ilvD6*, *18*, and *47* (dihydroxyacid dehydratase deficient) (ARMSTRONG and WAGNER 1964; ELLIOTT and ARMSTRONG 1968).

Cotransduction tests: The procedures utilized can be found in ARMSTRONG and WAGNER (1964). For these studies four VS and two *ilvC* strains were used as donors. In crosses with the VS strains, transduction mixtures were plated on minimal medium and incubated for a total of 48 hr (first 24 hr at 37°C followed by 24 hr at room temperature). Donor recombinants were identified by their small colony size. To avoid feeding of the donors by the background growth in crosses with *ilvA* and *C* strains, phage and cells were mixed in 2.5 ml of molten minimal-medium agar (0.75%) and spread as an overlay on minimal medium plates. This procedure allowed for a more accurate determination of donor recombinants. In crosses with *ilvC42* and *66* as donors, use was made of partial revertants of these strains that can grow suboptimally on a valine supplement. Transduction mixtures were spread on minimal medium plates supplemented with 4 μg of L-valine per ml. After 4 days of incubation at 37°C, the wild-type and donor recombinants were easily identified by the difference in colony size.

Growth studies: Duplicate assay tubes, each containing 3 ml of medium, were used. The supplementations to minimal medium are listed in Tables 3 and 4. Inocula were prepared from nutrient-broth cultures grown overnight at 37°C on a rotary shaker. These cultures were centri-

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fuged at 3000 × *g* for 10 min, and the cells were resuspended in 3 ml physiological saline. A 40-fold dilution, using physiological saline, of the cells was prepared; and one drop (1–2 million cells) of this dilution was used to inoculate each tube. The assay tubes were incubated for 22 hr at 37°C. Amount of growth is recorded as OD units (600 nm, B & L Spectronic 20).

Enzyme assays: Overnight cultures containing 100 ml minimal medium enriched with 1% nutrient broth were grown at 37°C on a rotary shaker. These cultures were added to 1 liter of fresh medium of the same composition, and growth was allowed to continue until an OD value (600 nm) of 0.40 to 0.50 had been attained (usually requiring 4 to 6 hr of growth). Cells were harvested and cell-free extracts were prepared in the manner described by ATKINS and ARMSTRONG (1969). For those studies involving the supplementation of growth medium with α-aceto-α-hydroxybutyrate (AHB), the procedure of ARFIN, RATZKIN and UMBARGER (1969) was employed except that minimal medium was used to grow the VS strains, and incubation after the addition of AHB was continued for 1 hr.

Acetohydroxy acid synthetase activity was assayed at pH 8 according to the procedure described by RADHAKRISHNAN and SNELL (1960). The reaction mixture has been revised to include flavin adenine dinucleotide (3×10^{-5} M). References to the other enzymatic assays are as follows: reductoisomerase (ARMSTRONG and WAGNER 1961); dihydroxyacid dehydratase (MYERS and ADELBERG 1954); transaminase B (COLEMAN and ARMSTRONG 1971); and threonine dehydratase (UMBARGER and BROWN 1957).

Determination of α-acetolactate in cell-free extracts: For the determination of the amount of α-acetolactate in cell-free extracts, 2 ml of each extract were used. One ml was treated with 0.1 ml 6 N H_2SO_4 and the other with 0.1 ml 5 N NaOH (to estimate the amount of acetoin present). The acid-treated extract was incubated for 15 min at 60°C to allow for decarboxylation of α-acetolactate to acetoin. The extracts were analyzed for acetoin according to the procedure used for acetohydroxy acid synthetase activity. The base-treated samples served as the blanks.

RESULTS

Enzyme activities: In an attempt to relate the phenomenon of valine sensitivity to the biosynthesis of isoleucine and valine, enzymatic analyses of the five enzymes of the pathway were carried out with cell-free extracts prepared from the four VS strains. The results of this study, as well as those that have been obtained with the wild-type strain of *S. typhimurium* (LT2), are presented in Table 1. All five enzymatic activities are observed in the VS strains. The values for these activities, except that of reductoisomerase, are comparable to (or higher than) those routinely observed in the wild-type strain. The low values observed

TABLE 1

Enzyme analysis of valine-sensitive and wild-type strains

Strain	Supplement to minimal medium*	Specific activity (μmoles/hr/mg)				
		Threonine dehydratase (*ilvA*)	Acetohydroxy acid synthetase (*ilvB*)	Reducto-isomerase (*ilvC*)	Dihydroxyacid dehydratase (*ilvD*)	Transaminase B (*ilvE*)
LT2	none	15.0–30.0	0.3–1.0	6.0–10.0	10.0–20.0	6.0–10.0
LT2	ile,val,leu	4.0–10.0	<0.3	1.0–3.0	2.0–5.0	<3.0
VS-7	none	80.3	2.7	2.5	30.6	20.9
VS-9	none	42.4	2.1	2.5	25.6	7.4
VS-10	none	26.6	2.9	2.5	16.3	11.0
VS-13	none	63.5	1.7	2.1	33.8	33.6

* per ml: ile = 100 μg L-isoleucine; val = 200 μg L-valine; leu = 100 μg L-leucine.

TABLE 2

Cotransduction with ilv *and valine-sensitive strains*

Recipient strains	Donor strains: VS-7 Total*	VS-7 %WT†	VS-9 Total	VS-9 %WT	VS-10 Total	VS-10 %WT	VS-13 Total	VS-13 %WT	ilvC66 Total	ilvC66 %WT	ilvC42 Total	ilvC42 %WT
ilvC42	5,357	29.3	7,362	30.3	6,095	30.4	5,716	28.6	.....		.....	
ilvC66	6,276	23.7	6,138	23.3	6,333	23.1	6,696	23.0	.....		.....	
ilvA118	6,244	24.8	7,224	24.3	6,748	24.7	6,181	24.0	10,429	45.6	.....	
ilvD18	9,654	55.9	10,788	55.3	9,812	49.0	9,186	50.5	16,637	76.8	14,702	83.5

* Total number of recombinants.
† Percentage wild type.

for reductoisomerase activity in the VS strains are representative of "repressed" levels of the enzyme (Table 1: values obtained when LT2 is grown in the presence of isoleucine, valine, and leucine). Variations in the length of time the cells are grown or omission of 1% nutrient broth from the minimal medium does not affect the level of reductoisomerase observed in the VS strains. Thus, with respect to the patterns of enzymatic activities, a low level of reductoisomerase is the single feature that distinguishes the VS strains from wild type.

Genetic mapping: Cotransduction studies were utilized to determine if the mutation sites in the VS strains are located in the *ilv* region of the chromosome and, if so, their position in the *ilv* cluster. The order of the *ilv* loci in the cluster is as follows: *ilvE–ilvD–ilvA–ilvC* (ARMSTRONG and WAGNER 1964); they specify transaminase B, dihydroxyacid dehydratase, threonine dehydratase, and reductoisomerase, respectively. It is apparent from the results listed (Table 2) that the sites in the VS strains are located in the *ilv* cluster; and the four sites map as a group within the cluster (Figure 1). Because each of the four VS strains used in this project exhibits characteristic traits, it is doubtful that any two of the sites are identical; however, this point cannot be considered established.

Growth studies: To obtain information on the nature of the sensitivity to valine, growth studies were carried out with the four VS strains, as well as with

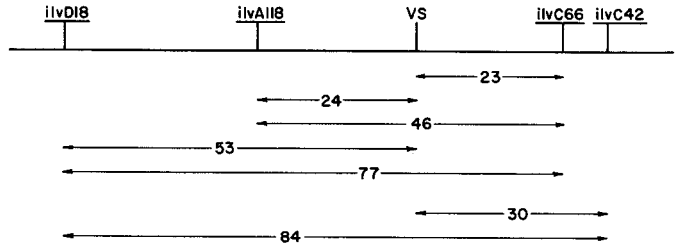

FIGURE 1.—Schematic summation of cotransduction results presented in Table 2. Numerical values refer to frequency (percent) of wild-type recombinants observed in the given cross. Gene–enzyme relationships: *ilvD* = dihydroxyacid dehydratase; *ilvA* = threonine dehydratase; and *ilvC* = reductoisomerase. The *ilvC66* allele was purposely selected for this study because the lesion in this strain maps as one of the *ilvC* sites most proximal to the *ilvA* locus (LESLIE and ARMSTRONG, unpublished).

TABLE 3

Growth studies on valine-sensitive strains in the presence of valine

	Supplement to minimal medium* (μg per ml)							
	1	2	3	4	5	6	7	8
Strain	None	0.1 val	5 val	5 val 10 ile	5 val 10 leu	5 val 10 ile 10 leu 1 pan	5 val 10 ile 10 met	5 val 10 met
LT2	0.55†	0.55	0.55	0.55	0.56	0.56	0.57	0.56
VS-7	0.08	0	0	0.30	0.01	0.34	0.48	0
VS-9	0.10	0	0	0.12	0.01	0.11	0.17	0.01
VS-10	0.12	0	0	0.14	0.02	0.14	0.44	0.01
VS-13	0.12	0	0	0.28	0.01	0.33	0.40	0

* val = L-valine; ile = L-isoleucine; leu = L-leucine; met = L-methionine; pan = D-pantothenate.

† OD units (600 nm) after 22 hr incubation at 37°C.

wild-type *S. typhimurium* LT2. A representative example of the pertinent results derived from the study is presented in Table 3. The figures in column 1 illustrate the slow growth of the VS strains in minimal medium. The results in column 2 depict the inhibition of growth by the presence of 0.1 μg of valine per ml in the medium, demonstrating the extreme sensitivity of these strains to the amino acid. One μg per ml of exogenous glycyl-L-valine or the α-keto acid analogue of valine also results in complete inhibition of growth. Five μg of valine per ml (column 3) were routinely used in the study; this quantity of the amino acid was sufficient for the dual role demanded of valine in the study, i.e., as an agent for the inhibition of growth and as an essential metabolite.

Inhibition of the isoleucine-valine pathway can be expected to affect the synthesis of four essential metabolites (isoleucine, valine, leucine, and pantothenate) because the α-keto acid precursor of valine also serves as a precursor for leucine and pantothenate syntheses (RAMAKRISHNAN and ADELBERG 1965a). When minimal medium is supplemented with both valine and isoleucine (column 4), inhibition is relieved; and growth, although not comparable to that of wild type, is as good as that observed in minimal medium (VS-9 and -10) and is usually better (VS-7 and -13). As seen in column 5, leucine cannot replace isoleucine. A combination of the four end products (column 6) is generally no more effective than valine and isoleucine (VS-9 and -10), although a slight enhancement of growth (0.03–0.05 OD units) is sometimes observed (VS-7 and -13). These results imply that under the conditions of this test, 5 and 10 μg per ml amounts of valine and isoleucine, respectively, are usually sufficient to provide the required amounts of the four end products. It is presumed that because of the reversible nature of the transaminase B reaction, sufficient quantities of the α-keto acid of valine are produced to meet the cell's demand for leucine and pantothenate. Various combinations and varying amounts of the four end products do not result in a growth response better than that observed with an isoleucine + valine combination. Increased amounts of isoleucine (up to 100 μg per ml) do not result in increased growth, and this is interpreted to mean that

isoleucine is serving as an essential metabolite, not as an antagonist of the valine sensitivity *per se*.

When the results are compared with growth responses obtained with wild type, it is apparent that other factors besides the isoleucine-valine pathway are involved in this phenomenon of valine sensitivity. Because of the metabolic interrelationships among the syntheses of valine, isoleucine, threonine, methionine, and lysine (DATTA 1969; UMBARGER 1969a), it is plausible that additional effects may involve the synthesis of the last three amino acids. As seen in column 7 of Table 2, the addition of methionine to an isoleucine + valine supplement results in a growth response better than that obtained without methionine (column 4). The results with VS-7, -10, and -13 are typical of the response obtained; the VS-9 result represents a minimal response that is occasionally observed. The figures in column 8 reveal that methionine alone is not effective in overcoming the sensitivity to valine. The results in columns 8 and 5 demonstrate that a decrease in the synthesis of isoleucine and valine is the critical feature in the inhibition of growth by valine. The enhancement of growth by the addition of methionine, however, suggests an involvement of the synthesis of this amino acid in the phenomenon. The methionine effect is not mimicked when cysteine, reduced glutathione, or homoserine is substituted for methionine. Repeated attempts to show an involvement of threonine and/or lysine in this system have not been successful. Thus, to date, the maximum growth response observed with VS strains grown in minimal medium has been obtained with a supplement containing valine, isoleucine, and methionine; and, as the results in column 7 depict, the wild-type growth rate has yet to be attained.

Reversal of endogenous valine inhibition: The results presented in Table 3 are those obtained when, by the addition of valine, the sensitivity to this amino acid is imposed on the bacteria. A second series of growth studies was carried out, and these assays were designed to study the effects of supplementation (other than with valine) on the slow rate of growth in minimal medium. Isolates of VS-10 and -13 were utilized, and a summary of the results is presented in Table 4. As seen in columns 2 and 3, when either methionine or isoleucine serves as the supplement, there is an enhancement of growth; and methionine exerts the

TABLE 4

Growth studies on valine-sensitive strains in the absence of valine

	Supplement to minimal medium* (μg per ml)							
	1	2	3	4	5	6	7	8
Strain	None	20 met	20 ile	20 met 20 ile	10 cys	10 cys 20 met	10 cys 20 ile	10 cys 20 met 20 ile
LT2	0.52†	0.51	0.53	0.52	0.51	0.53	0.53	0.55
VS-10	0.10	0.23	0.14	0.38	0.05	0.05	0.05	0.05
VS-13	0.12	0.26	0.19	0.38	0.08	0.09	0.08	0.08

* met = L-methionine; ile = L-isoleucine; cys = L-cysteine.
† OD units (600 nm) after 22 hr incubation at 37°C.

greater effect. These results suggest that the inhibition by endogenous valine is exerting a more critical effect on the synthesis of methionine than on that of isoleucine and valine. However, a combination of the two amino acids (column 5) is more effective than either alone. Studies on *Escherichia coli* K12 (DATTA 1967) provide evidence that both cysteine and threonine exert a feedback inhibition control on the synthesis of methionine by inhibiting the activity of homoserine dehydrogenase. To determine if this form of control mechanism is operating in the VS strains, cysteine was furnished to the cells (assuming that the restricted synthesis of isoleucine in the VS strains results in a high endogenous level of its precursor, threonine). As seen in column 5, levels of 10 μg of cysteine per ml result in a decreased rate of growth. If the control mechanism cited above is indeed responsible for this inhibition, it would be expected that a supplement of methionine (the end product) would overcome it. However, as depicted in column 6, this is not the case. Supplements of isoleucine or an isoleucine + methionine combination (columns 7 and 8) are also unable to effect a reversal of the inhibition. Addition of 20 μg of valine per ml (not shown) to the isoleucine + methionine supplement does not exert any influence. Thus, these results suggest that cysteine is either enhancing an inhibition already present (other than the synthesis of methionine) or imposing a new one.

The recently reported evidence for substrate induction of the reductoisomerase of *S. typhimurium* (ARFIN, RATZKIN and UMBARGER 1969) allows for the following considerations concerning the nature of the mutation in the VS strains. If the valine sensitivity that is observed is the result of an inhibition by this amino acid of the acetohydroxy acid synthetase (the enzyme that catalyzes the synthesis of the substrates required for the induction of the reductoisomerase), then the low levels of reductoisomerase that are characteristic of these strains may be explained as a secondary manifestation of the mutation, i.e., little or no induction of the enzyme. To test this rationale, *in vitro* inhibition studies on the acetohydroxy acid synthetase, reductoisomerase, and threonine dehydratase of VS strains were carried out. The last two activities are not inhibited when the assay mixture contains 10^{-3} M L-valine or when the cell-free extract is preincubated 30 min with 10^{-2} M of the amino acid. The results obtained with the acetohydroxy acid synthetase are presented in Table 5. This enzyme is known to be sensitive

TABLE 5

In vitro *inhibition of acetohydroxy acid synthetase activity in cell-free extracts*

Strain	Percentage inhibition		
	10^{-3}M val*	10^{-4} M val	10^{-2} M ile
LT2	30	0	28
VS-7	74	22	64
VS-9	77	20	61
VS-10	72	15	50
VS-13	75	21	60

* Abbreviations used: val = L-valine; ile = L-isoleucine.

TABLE 6

Acetohydroxy acid synthetase activity and α-acetolactate present in cell-free extracts of various S. typhimurium *strains*

	Cell-free extract							
	LT2	*ilvC8*	*ilvD47*	*ilvD6*	VS-7	VS-9	VS-10	VS-13
Specific activity*	2.1	2.9	11.0	7.6	36.4	31.6	12.0	10.5
μg α-acetolactate per mg protein	0.12	2.7	1.2	1.2	0	(0.05)	(0.05)	(0.05)

* μmoles/hr/mg.

to feedback control (UMBARGER 1969b); and, as seen in Table 5, the wild-type activity is inhibited 30% in the presence of 10^{-3} M L-valine. However, the inhibition observed with the VS strains is markedly enhanced (72 to 77%). A concentration of 10^{-2} M valine does not increase the amount of inhibition observed. At levels of 10^{-4} M L-valine, inhibition (15–22%) of the activity of the VS strains is noted, whereas the wild-type activity is not affected. The enzyme in the VS strains also shows an increased sensitivity to L-isoleucine.

A similar inhibition of acetohydroxy acid synthetase by endogenous valine during growth can be indirectly evaluated by an examination of cell-free extracts for the presence of one of the products of the reaction, α-acetolactate. Extracts prepared from VS and other *S. typhimurium* strains were analyzed. For this study, extracts of the *ilv* strains were prepared from cells grown under derepressed conditions in an attempt to obtain acetohydroxy acid synthetase activities comparable to the high levels routinely observed in VS strains. Wild-type extracts were prepared from cultures that were harvested during midlog phase of growth. As seen in Table 6, each of the extracts prepared from the *ilv* strains (*C8*, *D6*, and *D47*) contains appreciable amounts of enzyme activity, as well as α-acetolactate. In the case of LT2, although the enzyme is readily detectable, the amount of α-acetolactate is low. Because these cells were still in an active growth period when harvested, rapid utilization of the α-acetolactate being produced could account for this observation. The results presented for the VS strain include analysis of cell-free extracts prepared from cells that were harvested at four different time intervals of growth. In each case, the level of enzyme activity is high; but the level of α-acetolactate is below the valid limits of detection. To date, the highest value that has been obtained for α-acetolactate in an extract of a VS strain is 0.12 μg per mg of protein. The high specific activities recorded in Table 6 for the acetohydroxy acid synthetase of VS strains are typical of the derepressed levels frequently observed. Specific activities ranging from 1.5 (Table 1) to 50 have been obtained with these strains.

The induction of the reductoisomerase by its substrate, α-aceto-α-hydroxybutyrate, in growing culture of *S. typhimurium* has been demonstrated by ARFIN, RATZKIN and UMBARGER (1969). Following the general procedure described by these authors, cell-free extracts of VS strains, grown either in the absence or in the presence of α-aceto-α-hydroxybutyrate (AHB), were assayed for three of the

TABLE 7

Specific activities in extracts of valine-sensitive strains

	Acetohydroxy acid synthetase (*ilvB*)		Reductoisomerase (*ilvC*)		Threonine dehydratase (*ilvA*)	
	Minimal	Minimal + AHB*	Minimal	Minimal + AHB	Minimal	Minimal + AHB
VS-7	30.0	28.2	2.3	6.8	84.0	79.5
VS-9	39.1	30.0	2.3	7.6	101	71.5
VS-10	40.5	36.7	2.1	9.6	65.0	52.0
VS-13	18.7	23.3	2.4	6.0	53.1	51.0

* AHB = 10 mM α-aceto-α-hydroxybutyrate.

enzyme activities of the pathway. The results are presented in Table 7. The activities for the acetohydroxy acid synthetase and threonine dehydratase in each of the strains are present in derepressed amounts; and both growth conditions utilized result in similar levels of activity for each of these enzymes. However, when the reductoisomerase activity is considered, it is apparent that supplementation of the medium with AHB results in a 2.5- to 4.0-fold increase in activity. These data support the contention that under these conditions of AHB supplementation, an induction of reductoisomerase occurs. It is of interest to note that in growth studies, AHB and isoleucine relieve valine inhibition to the same extent (AHB → ile); and acetolactate (AL), the valine precursor, inhibits growth (AL → val). These results point out that the level of reductoisomerase in these mutants is not the critical feature of the mutation. The VS strains cannot be considered reductoisomerase-deficient mutants; the latter possess no detectable activity in cell-free extracts (ARMSTRONG and WAGNER 1962).

DISCUSSION

The *S. typhimurium* mutant strains described in this report are phenotypically characterized by slow growth on minimal medium and by inhibition of growth when the medium is supplemented with small quantities of valine. The enzymatic, genetic, and growth studies carried out on four of these valine-sensitive strains have yielded the following information and considerations about the nature of the mutation.

A low level of reductoisomerase activity, one of the enzymes of the isoleucine-valine biosynthetic pathway, is a distinguishing feature of these strains (Table 1). This observation, when coupled with the mapping data (Figure 1) that places the mutation sites between the *ilvA* (threonine dehydratase) and *ilvC* (reductoisomerase) alleles tested, suggests that the mutation is involved in regulation of the synthesis of the reductoisomerase. At the time of these findings, available evidence on the control of the *ilvC* locus implied that this locus was contained in its own operon and was independently repressible (UMBARGER 1969b). Thus the data on the valine-sensitive strains could be explained as the results of a mutation involving the operator region that controls the production of the reducto-

isomerase. However, the report by ARFIN, RATZKIN and UMBARGER (1969) which furnished evidence for the synthesis of the reductoisomerase by an inducible mechanism rather than the previously supposed repressible mechanism, called for a reinterpretation of the results obtained with the valine-sensitive strains. These new considerations focused on the acetohydroxy acid synthetase, rather than the reductoisomerase, as the enzyme more directly associated with valine sensitivity. The results presented in Tables 5 through 7 provide evidence that in these mutant strains, the acetohydroxy acid synthetase is the valine-sensitive enzyme and that the low levels of reductoisomerase routinely observed are the indirect result of an insufficient production of α-aceto-α-hydroxybutyrate and α-acetolactate (substrate inducers required for the synthesis of reductoisomerase). These data also support the evidence for the inducibility of the reductoisomerase (ARFIN, RATZKIN and UMBARGER 1969).

Whereas the enzymatic studies were designed to elucidate the biochemical block, the growth studies were carried out to examine more closely the observed phenotypes of these mutant strains, i.e., slow growth on minimal medium and the sensitivity to valine. The results presented in Tables 3 and 4 demonstrate that in the presence of valine the primary site of inhibition is the synthesis of isoleucine and valine; however, the syntheses of other essential metabolites are also affected. The growth studies do not allow for any definite conclusions concerning the pleiotropic effects encountered, but the results do demonstrate that sensitivity to valine brings about what can generally be described as an imbalance in intermediary metabolism. The slow growth on minimal medium (Table 4) is probably the result of such an imbalance, i.e., a continuous inhibition of the acetohydroxy acid synthetase activity by endogenously produced valine, the results of which affect other biosynthetic pathways. The result depicting the inhibition of growth by cysteine (Table 4) is most likely a phenomenon that can be produced by a number of metabolic compounds which when added to the medium would serve to enhance the imbalance in metabolism already present. It should be noted that the valine-sensitive strains display a high frequency of reversion. Because of the multiple factors that appear to be involved in the mutant phenotype of slow growth, it is not surprising that this characteristic is not more stable.

Valine-sensitive strains possess characteristics very different from the *ilv* mutants that have previously been studied in this laboratory. They represent a distinct class of mutations within the *ilv* cluster. In a recent report by MARINUS and LOUTIT (1969) on the characterization of *ilv* mutants of *Pseudomonas aeruginosa*, one strain is described that resembles the valine-sensitive strains. The strain OT517 (*ilv-17*) produces large amounts of acetohydroxy acid synthetase but no reductoisomerase. The enzymatic and genetic data on this strain imply that the strain possesses a mutation in the operator region of the *ilvC* (reductoisomerase) locus. Although this comparison is being made about mutations in two distinctly different microorganisms, it would be interesting to determine the sensitivity of strain OT517 to valine.

The available evidence on the valine-sensitive strains leads to the conclusion

that acetohydroxy acid synthetase, the product of the *ilvB* gene, is the enzyme that is sensitive to valine. If these mutations represent alterations in the structural gene for the enzyme, then the mapping data presented in Figure 1 place the *ilvB* locus between the *ilvC* and *ilvA* loci in the *ilv* cluster. When this assignis correlated with previous data (ARMSTRONG and WAGNER 1964), the following order for the *ilv* loci of *S. typhimurium* is derived: *ilvE–ilvD–ilvA–ilvB–ilvC*. This order differs from that derived for *E. coli*, which is *ilvE–ilvD–ilvA–ilvC–ilvB* (RAMAKRISHNAN and ADELBERG 1965b). Although this difference in gene order is a distinct possibility, the evidence in this report does not clearly establish that it is the structural gene for the acetohydroxy acid synthetase that is involved in the mutations under study. The isolation of strains that lack the enzyme activity would offer a more direct means of assessing the present data. Attempts to isolate strains deficient in acetohydroxy acid synthetase have, to date, been unsuccessful.

One of the intriguing observations in this study is the sensitivity of these mutants to valine. Although *in vitro* studies demonstrate that the acetohydroxy acid synthetase of wild-type *S. typhimurium* is sensitive to valine (feedback control), this sensitivity has not been shown to be physiologically important (UMBARGER 1969b). Whereas there is no significant inhibition of growth when wild-type cells are grown on minimal medium supplemented with valine (up to 1 mg per ml), the growth of these valine-sensitive strains is completely inhibited by the presence of 0.1 μg of valine per ml (Table 3) and even by levels as low as 0.01 μg per ml, i.e., 10^{-6} to 10^{-7} M. Thus, even taking into account the accumulation of valine by the cells, inhibition of growth of these mutants is effectively accomplished by a low concentration of valine. One explanation, based on the results of the growth studies, is that only a partial inhibition of the acetohydroxy acid synthetase activity is sufficient to result in a build-up of intermediates which either directly or indirectly (by conversion into other compounds) interfere with metabolic processses to the extent that growth is not possible. Finally, there is the possibility that there are factors involved that have not been considered as yet. For example, CRONENWETT and WAGNER (1965) reported that in *S. typhimurium*, the enzymes of the isoleucine-valine pathway exist in the cell as a complex associated with the membrane. The possibility of mutations that result in altering the effective complexing of the enzymes *in vivo* should then also be considered. It is conceivable that the mutation in the valine-sensitive strains not only results in an increased sensitivity of the acetohydroxy acid synthetase to valine but also in an inability of this enzyme to complex properly. The effectiveness of low concentrations of valine in inhibiting growth may be a reflection of both features of the mutant enzyme.

Thus although much information has been obtained about the valine-sensitive strains, there are aspects of the mutation that will require further study before a definitive description of the phenomena observed in this study can be presented.

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SUMMARY

A study of strains of *S. typhimurium* that display an extreme sensitivity to

valine provides evidence that the acetohydroxy acid synthetase, first enzyme in the common pathway for the synthesis of isoleucine and valine, possesses an enhanced sensitivity to the amino acid. Results of enzymatic and growth studies reveal that inhibition of this enzyme by valine not only affects synthesis of isoleucine and valine but also the induction of the reductoisomerase, second enzyme of the pathway. Growth studies indicate that the syntheses of other essential metabolites, e.g., methionine, are also affected by the mutation. It is plausible that *in vivo* inhibition of acetohydroxy acid synthetase by valine results in an accumulation of intermediates of related pathways which directly or indirectly (by conversion to other compounds) affect the syntheses of other metabolites.—The phenomenon of valine sensitivity is currently thought to represent a mutation in the *ilvB* locus, structural gene for acetohydroxy acid synthetase. If so, the results of cotransduction tests place the *ilvB* locus between the *ilvA* and *C* loci and furnish the following order for the *ilv* genes of *S. typhimurium*: *ilvE–ilvD–ilvA–ilvB–ilvC*. This order differs from that reported for *E. coli*, which is *ilvE–ilvD–ilvA–ilvC–ilvB*.

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