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Regulatory Mechanisms in the Biosynthesis of Isoleucine and Valine

II. Identification of Two Operator Genes

T. RAMAKRISHNAN[1] AND EDWARD A. ADELBERG

Department of Microbiology, Yale University, New Haven, Connecticut

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ABSTRACT

RAMAKRISHNAN, T. (Yale University, New Haven, Conn.), AND EDWARD A. ADELBERG. Regulatory mechanisms in the biosynthesis of isoleucine and valine. II. Identification of two operator genes. J. Bacteriol. 89:654-660. 1965.—A tightly clustered set of five structural genes governs the synthesis of the five enzymes of isoleucine and valine biosynthesis in *Escherichia coli*. Three of the genes governing transaminase B, dehydrase, and threonine deaminase, are controlled by a single operator locus, designated *oprA*. The structural gene governing the condensing enzyme is controlled by a second operator locus, designated *oprB*. Both *oprA* and *oprB* have been shown to regulate structural genes which are *cis*, but not *trans*, to their own operator. No mutations have yet been found which affect the level of reductoisomerase, but the existence of a third operator controlling the synthesis of this enzyme can be inferred. Enzyme derepression resulting from mutations in *oprA* confers resistance to high levels of valine. Derepression of the condensing enzyme resulting from mutations in *oprB* confers resistance to low levels of valine, and to α-aminobutyric acid. The significance of these findings with respect to the valine sensitivity of *E. coli* strain K-12 is discussed.

In the first paper of this series (Ramakrishnan and Adelberg, 1964), we described the isolation of mutants of *Escherichia coli* K-12 which were coordinately derepressed for three of the five enzymes of the isoleucine-valine biosynthetic pathway. The five enzymes, listed in their biosynthetic order, are: threonine deaminase, which converts threonine to the isoleucine precursor α-ketobutyrate; condensing enzyme, which condenses pyruvate with α-ketobutyrate to form α-aceto-α-hydroxybutyrate, or with another pyruvate to form the valine precursor, α-acetolactate; reductoisomerase, which reduces and rearranges α-aceto-α-hydroxybutyrate and α-acetolactate to form α-β-dihydroxy-β-methylvalerate and α,β-dihydroxyisovalerate, respectively; dehydrase, which dehydrates the dihydroxy acids to the corresponding keto acids, α-keto-β-methylvalerate and α-ketoisovalerate; and transaminase B, which transaminates the keto acids with α-ketoglutarate to form isoleucine and valine, respectively. The structures of the above-named intermediates are shown in Fig. 1.

The three genetically derepressed enzymes are threonine deaminase, dehydrase, and transaminase B. The derepressed mutants were selected as valine-resistant strains; in the present paper, we will show that these strains owe their valine resistance entirely to their increased levels of threonine deaminase. We will also present complete evidence for designating the locus within which the mutations occurred as an operator locus (*oprA*).

The fact that derepression of threonine deaminase, dehydrase, and transaminase B leads to an increased production of isoleucine but not of valine tells us that either condensing enzyme or reductoisomerase must catalyze the rate-limiting reaction in the valine biosynthetic pathway. Thus, it should be possible to isolate a strain derepressed in one or both of these enzymes by selecting for mutants resistant to an antimetabolite of valine. α-Aminobutyric acid (ABA) is such an antimetabolite (Gladstone, 1939); in the present paper, we will also show that certain mutants resistant to ABA are derepressed for the condensing enzyme, and that their mutations have occurred in an operator locus (*oprB*) controlling the synthesis of condensing enzyme only.

None of the mutants we have studied thus far is significantly different from the parental strain in its level of reductoisomerase; this enzyme is subject to end-product repression,

[1] Present address: Pharmacology Laboratory, Indian Institute of Science, Bangalore, India.

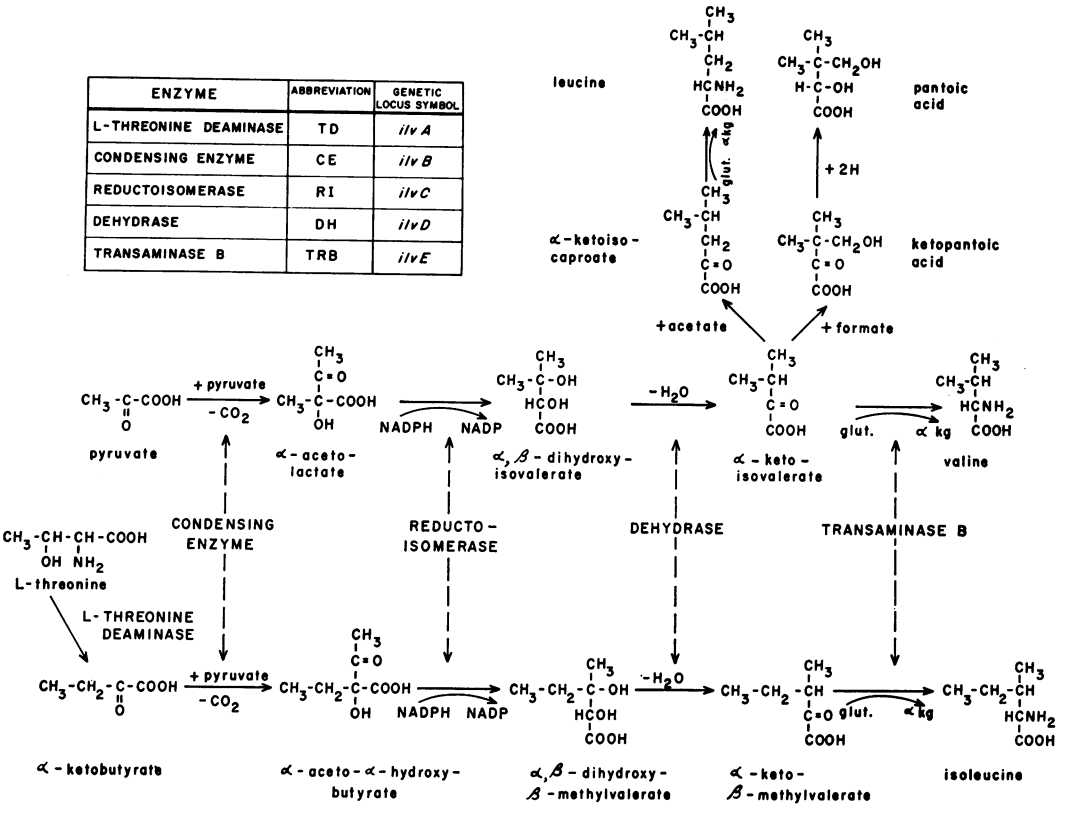

FIG. 1. *Intermediates and enzymes in the biosynthesis of isoleucine and valine*

however, implying that a third repressor-binding site (operator locus) exists which is responsible for the regulation of reductoisomerase formation.

The criteria by which an operator locus can be recognized are the following: (i) mutations occurring in such a locus can bring about derepression of the operon which it controls; (ii) an operator locus regulates structural genes if they are on the same deoxyribonucleic acid (DNA) structure (that is, genes which are *cis*, but not *trans*, to the operator in a diploid cell); and (iii) an operator locus is immediately adjacent to the structural gene or genes which it controls (Jacob et al., 1960). In the present paper, we will furnish the evidence which satisfies the first two criteria for both *oprA* and *oprB*. In the following paper (Ramakrishnan and Adelberg, 1965), we will furnish the evidence satisfying the third criterion, in the form of genetic mapping data.

MATERIALS AND METHODS

Organisms. A summary of strains used in this work is presented in Tables 1 and 2.

Media and culture methods. The media and culture methods used in this work were described previously (Adelberg and Burns, 1960).

Mating conditions. The conditions employed for measuring the kinetics of zygote formation and for carrying out genetic crosses were those described by Pittard, Loutit, and Adelberg (1963). Single recombinant clones were picked from the selective plate and purified twice on the same medium on which they were originally isolated.

Transduction technique. The procedure used for transduction was that described by Lennox (1955).

Enzyme assays and analytical procedures. The procedures used were described earlier (Ramakrishnan and Adelberg, 1964). *N*-methyl-*N*-nitroso-*N'*-guanidine was obtained from K & K Laboratories, Plainview, N.Y.

RESULTS

Isolation of structural gene mutants for threonine deaminase, dehydrase, and transaminase B. The valine-resistant strain AB1005 was grown overnight in nutrient broth, diluted 1:10 in the same broth, and aerated for 2 hr to bring the cells into early log phase. An aqueous solution of *N*-methyl-*N*-nitroso-*N'*-nitroguanidine was added to a final concentration of 100 μg/ml, and the cells were incubated for 1 hr at 37 C with shaking. The cells were then centrifuged, washed twice

TABLE 1. *Genotypes of strains*

Strain no.[a]	Genetic locus[b]							Sex
	ilvA	*ilvB*	*ilvC*	*ilvD*	*ilvE*	*oprA*	*oprB*	
AB1005[c]	+	+	+	+	+	1	+	♂ F_{14}
AB1025[c]	+	+	+	+	193	1	+	♂ F_{14}
AB1026[c]	194	+	+	+	+	1	+	♂ F_{14}
AB1027[c]	+	+	+	195	+	1	+	♂ F_{14}
AB1048[c]	+	+	+	+	+	+	1	♂ F_{14}
AB1206[c]	+	+	+	+	+	+	+	♂ F_{14}
AB1051[d]	+	+	+	+	+	+	1	F^-
AB1052[d]	+	196	+	+	+	+	1	F^-
AB1055[d]	+	+	+	+	+	+	1	F^-
AB1254[d]	+	+	+	+	+	+	+	F^-
AB1255[e]	201	+	+	+	+	+	+	F^-
AB2140[f]	+	+	+	16	+	+	+	F^-

[a] Numbers refer to allele numbers allotted to mutant loci in these laboratories.

[b] Phenotypes associated with mutant alleles: *ilvA194*, *ilvA201*, loss of threonine deaminase activity; *ilvB196*, loss of *p*H 8 condensing enzyme activity; *ilvD195*, loss of dehydrase and transaminase activity (polarity effect); *ilvD16*, loss of dehydrase activity; *ilvE193*, loss of transaminase B activity; *oprA1*, derepression of *ilvA*, *ilvD*, and *ilvE* when in *cis* position; *oprB1*, derepression of *ilvB* when in *cis* position.

[c] Also carries *thi-1*, *pro-2*, *his-4*, *gal-2*, *lac-1*, *str-8*.

[d] Also carries *arg-1*, *met-1*, *thi-1*, *try-14*, *his-1*, *mal-1*, *xyl-4*, *T6r-6*, *str-8*.

[e] Also carries *thi-1*, *arg-1*, *met-1*, *his-1*, *xyl-1*, *mal-1*

[f] Also carries *thi-1*, *arg-1*, *met-1*, *mal-1*, *str-8*.

TABLE 2. *Locus designations*

Locus	Genetic function
ilvA	Structural gene for threonine deaminase
ilvB	Structural gene for condensing enzyme
ilvC	Structural gene for reductoisomerase
ilvD	Structural gene for dehydrase
ilvE	Structural gene for transaminase B
oprA	Operator locus regulating *ilvA*, *ilvD*, and *ilvE*
oprB	Operator locus regulating *ilvB*

with buffer, and incubated overnight in broth with aeration. They were again grown to log phase, centrifuged, and washed twice with buffer, after which they were suspended in minimal medium containing 1% citrate and aerated for 2 hr. Penicillin was added at 1,000 units per ml to dilutions of this suspension and the dilutions were incubated for 24 hr; 0.1 ml of each of the dilutions was spread on nutrient agar. When colonies appeared, they were replicated onto minimal medium and onto minimal medium with isoleucine and valine, and with isoleucine, valine, and leucine. The colonies which required isoleucine alone were found to have a mutated threonine deaminase, those which required isoleucine and valine were found to have a mutated dehydrase or reductoisomerase, and those which required all three amino acids were found to have a mutated transaminase B.

The structural genes governing the synthesis of the five enzymes in the biosynthetic pathway will be designated as follows: *ilvA*, (L-threonine deaminase); *ilvB*, (condensing enzyme); *ilvC*, (reductoisomerase); *ilvD*, (dehydrase); and *ilvE*, (transaminase B).

Cis-trans tests of oprA activity. The *oprA* mutations producing the valine-resistance phenotype were isolated in F′ strain AB1206, in which the isoleucine-valine operons are carried on the sex factor, F_{14} (Pittard et al., 1963). Strain AB1206 was recently shown to have a chromosomal deletion corresponding to the entire F_{14} merogenote (Pittard and Ramakrishnan, 1964); it is thus haploid for the genetic region in which *oprA* lies. When AB1206 transfers F_{14} to an F^- recipient, however, a new F′ strain is formed which is a partial diploid, suitable for *cis-trans* tests.

Accordingly, F_{14} carrying the mutant locus *oprA1* was transferred to a series of F^- strains, each bearing a different structural gene mutation. In some cases, the donor strain carried a structural gene mutation on F_{14} as well. The diploids formed in this manner were verified for their ability to transfer F_{14} at high frequency, were tested for valine resistance, and were assayed for their levels of enzyme activity. The results (Table 3) show that *oprA1* derepresses the structural genes *ilvE* (governing transaminase B), *ilvD* (governing dehydrase), and *ilvA* (governing threonine deaminase), when the structural gene is in the *cis* position but not in the *trans* position relative to *oprA1*.

Table 3 also reveals the highly significant fact that the diploids are resistant to 10^{-2} M valine only when threonine deaminase is derepressed; strains having derepressed levels of transaminase B and dehydrase, but normal levels of threonine deaminase, are valine-sensitive. Thus, threonine deaminase is the rate-limiting enzyme for isoleucine biosynthesis in *E. coli* K-12, when valine is acting as a corepressor of enzyme synthesis. Only by derepressing threonine deaminase formation can sufficient isoleucine be made to overcome the inhibition of strain K-12 produced by high levels of valine in the medium.

Isolation of mutants derepressed for condensing enzyme synthesis. ABA has been reported to be

TABLE 3. *Cis-trans tests of oprA activity*

Diploid strain	Parent strains	Partial genotype	Enzyme activity*				
			TRB (*ilvE*)	DH (*ilvD*)	TD (*ilvA*)	RI	CE
AB1019 (valine-sensitive)	AB1206 × AB1254	*ilvE*$^+$ *ilvD*$^+$ *ilvA*$^+$ *oprA*$^+$ / *ilvE*$^+$ *ilvD*$^+$ *ilvA*$^+$ *oprA*$^+$	2.40	2.00	3.50	0.39	0.81
AB1020 (valine-resistant)	AB1005 × AB1254	*ilvE*$^+$ *ilvD*$^+$ *ilvA*$^+$ *oprA1* / *ilvE*$^+$ *ilvD*$^+$ *ilvA*$^+$ *oprA*$^+$	35.80	38.60	46.80	0.39	0.81
AB1031 (valine-resistant)	AB1027 × AB1255	*ilvE*$^+$ *ilvD195 ilvA*$^+$ *oprA1* / *ilvE*$^+$ *ilvD*$^+$ *ilvA201 oprA*$^+$	1.80†	1.00	47.00	0.40	0.80
AB1032 (valine-sensitive)	AB1026 × AB2140	*ilvE*$^+$ *ilvD*$^+$ *ilvA194 oprA1* / *ilvE*$^+$ *ilvD16 ilvA*$^+$ *oprA*$^+$	35.30	38.00	1.70	0.38	0.78
AB1033 (valine-resistant)	AB1025 × AB2140	*ilvE193 ilvD*$^+$ *ilvA*$^+$ *oprA1* / *ilvE*$^+$ *ilvD16 ilvA*$^+$ *oprA*$^+$	2.38	38.00	46.70	0.42	0.82

* Activities are expressed as micromoles of substrate converted or product formed per hour per milligram of protein. TRB = transaminase B; DH = dehydrase; TD = threonine deaminase; RI = reductoisomerase; CE = condensing enzyme.

† The low level of transaminase B appears to be due to a polarity mutation in the dehydrase locus (Ames and Hartman, 1963).

an antagonist of valine (Gladstone, 1939) and to compete with valine for the valine-activating enzyme of *E. coli* (Bergmann, Berg, and Dieckmann, 1961). We have found that ABA inhibits the growth of strain AB1206 when incorporated into minimal agar at a concentration of 10^{-3} M, and that the inhibition can be overcome by an equimolar concentration of valine, isoleucine, or leucine. Accordingly, about 2×10^8 washed cells from each of 20 subcultures of strain AB1206 were spread on plates containing 10^{-3} M ABA, and one ABA-resistant (ABA^r) colony from each culture was isolated and purified by restreaking on nutrient agar. The pure cultures were found to be resistant to 10^{-3} M ABA and to 2×10^{-4} M valine. Three of the ABA^r mutants were found to carry their mutant locus on F_{14}, as shown by their ability to transfer it at high frequency. One of the mutants was analyzed for the time at which it transferred the resistance locus to a sensitive female strain; the results (Fig. 2) show that the locus enters at about 23 min. The mutant locus is thus closely linked to the loci governing the isoleucine-valine biosynthetic enzymes, which loci are transferred by F_{14} males at about 20 min (Pittard et al., 1963).

Derepression of the condensing enzyme in ABA^r strains. Strain AB1048, one of the ABA^r mutants, was grown both in minimal medium and in medium to which isoleucine, leucine, and pantothenate were added to effect multivalent repression (Freundlich and Umbarger, 1963). Cell-free extracts were prepared and assayed for condensing enzyme, reductoisomerase, and transaminase B activity; the extracts were incubated both in the presence and in the absence of valine, a feed-back inhibitor of condensing enzyme activity (Leavitt and Umbarger, 1961). The data are presented in Table 4 along with similar data for AB1206, the parental ABA-sensitive (ABA^s) strain. The results show that the ABA^r strain has a 20-fold increase in condensing enzyme activity, and that this activity is inhibited by valine in the assay system to the same extent as the activity in AB1206. In contrast, the reductoisomerase and transaminase B activities are approximately equal in the ABA^r and ABA^s strains.

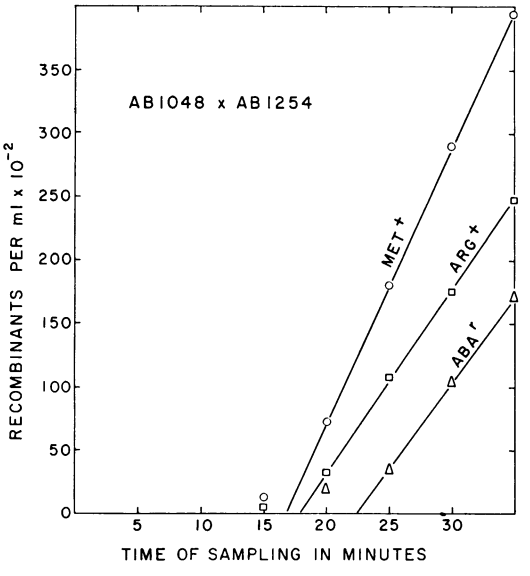

FIG. 2. *Kinetics of zygote formation for the locus controlling resistance to α-aminobutyric acid (ABA), compared with the kinetics for markers known to be carried on F_{14}. The female strain, AB1254, is methionineless, argininеless, and ABA-sensitive (see Table 1 for alleles).*

TABLE 4. *Derepression of the condensing enzyme*

Strain no.	Supplements to growth medium	Enzyme activity*			
		Condensing enzyme		RI	TRB
		Valine absent	Valine, 10^{-3} M		
AB1206	None	0.50	0.30	0.21	1.35
	All four end products†	0.28	—	0.15	1.10
AB1048	None	9.80	5.80	0.20	1.30
	All four end products†	10.20	—	0.23	1.28

* Activities are expressed as micromoles of substrate converted or product formed per hour per milligram of protein. RI = reductoisomerase; TRB = transaminase B.

† Isoleucine, leucine, valine, and pantothenate (Freundlich and Umbarger, 1963).

Thus, resistance to ABA reflects the genetic derepression of the condensing enzyme. We have designated the locus in which the mutation occurred as *oprB*, because the *cis-trans* tests reported below indicate that it is a true operator locus. The mapping experiments reported in the following paper (Ramakrishnan and Adelberg, 1965) confirm the close proximity of *oprB* and *ilvB*, the condensing enzyme structural gene.

Isolation of structural gene mutants lacking condensing enzyme. To perform the *cis-trans* test of *oprB* activity, it was necessary to isolate strains having a mutation in the *ilvB* locus. Although many mutants have been isolated which are defective for transaminase B, dehydrase, reductoisomerase, or threonine deaminase, none has ever been found which was defective for condensing enzyme. This failure is understandable because *E. coli* possesses two condensing enzymes, one with an optimum at *p*H 8.0 and the other with an optimum at *p*H 6.0 (Radhakrishnan and Snell, 1960). Assuming two different structural genes for the two enzymes, a mutation in either one would still leave the other functional and the pathway open.

Following a suggestion by Sidney Brenner, mutants lacking the *p*H 8.0 enzyme were isolated by selecting for ABA-sensitive derivatives of an ABA-resistant strain. It will be recalled that a mutant such as AB1048 owes its resistance to the fact that it is genetically derepressed for the *p*H 8.0 condensing enzyme; hence, any derivative of this strain which loses the *p*H 8.0 enzyme will have only normal levels of the *p*H 6.0 enzyme and will be ABA-sensitive.

The ABA-resistant strain chosen for this experiment was F^- strain AB1051, which had received the *oprB1* allele from AB1048 by phage transduction. AB1051 was treated with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine and plated on nutrient agar. The colonies which formed were tested for resistance to ABA by replica plating. Several ABA-sensitive mutants were isolated, and their cell-free extracts were assayed for condensing enzyme activity. One of the ABA-sensitive mutants, F^- strain AB1052, was found to lack the *p*H 8.0 enzyme, but to have sufficient *p*H 6.0 enzyme to enable it to grow slowly without added isoleucine or valine. The residual

TABLE 5. *Condensing enzymes at pH 8.0 and pH 6.0*

Strain no.	Condensing enzyme activity*			
	Before acid treatment		After acid treatment	
	*p*H 8.0	*p*H 6.0	*p*H 8.0	*p*H 6.0
AB1051	9.80	7.50	0.14	0.29
AB1052	0.15	0.30	0.15	0.30
AB1055	9.65	7.50	0.15	0.29

* Activities are expressed as micromoles of substrate converted or product formed per hour per milligram of protein.

TABLE 6. *Cis-trans tests of oprB activity*

Diploid strain	Parent strains	Partial genotype	Enzyme activity*		
			CE	RI	TRB
AB1053	AB1206 × AB1052	F *ilvB*$^+$ *oprB*$^+$ / *ilvB196 oprB1*	0.49	0.38	2.60
AB1054	AB1048 × AB1254	F *ilvB*$^+$ *oprB1* / *ilvB*$^+$ *oprB*$^+$	10.20	0.43	2.40

* Activities are expressed as micromoles of substrate converted or product formed per hour per milligram of protein. CE = condensing enzyme; RI = reductoisomerase; TRB = transaminase B.

enzyme activity was shown to be the *p*H 6.0 enzyme by virtue of its sensitivity to acid (Radhakrishnan and Snell, 1960). The relevant data are presented in Table 5.

The mutant lacking the *p*H 8.0 condensing enzyme has the genotype *ilvB196 oprB1*. This strain, AB1052, was used to carry out *cis-trans* tests of *oprB* activity, as described in the following section.

That strain AB1052 still carries the *oprB1* allele was proved by transducing in *ilvB*$^+$ from strain AB1206, and showing that the transductant (AB1055) has derepressed levels of condensing enzyme (Table 5).

Cis-trans tests of oprB activity. A diploid strain (AB1053) having the *trans* configuration *F ilvB*$^+$ *oprB*$^+$/*ilvB196 oprB1* was constructed by the transfer of F_{14} from strain AB1206 to the F$^-$ strain AB1052. Strain AB1054, having the genotype *F ilvB*$^+$ *oprB1*/*ilvB*$^+$ *oprB*$^+$, was prepared as the *cis* control. Cell-free extracts were prepared from both strains, and their condensing enzyme activities were compared with that of the repressed strain AB1206 and the derepressed strain AB1048. The results (Table 6) show that the *ilvB*$^+$ locus is derepressed when it is *cis*, but not *trans*, to *oprB1*.

DISCUSSION

The data presented above, together with the mapping data given in the accompanying paper (Ramakrishnan and Adelberg, 1965), show that three of the five closely linked loci governing the isoleucine-valine biosynthetic enzymes, *ilvE*, *ilvD*, and *ilvA*, form a single operon under the control of the operator locus, *oprA*. Another structural gene, *ilvB*, is under the control of a second operator, *oprB*. No mutations have yet been observed to alter the levels of reductoisomerase activity; the *ilvC* locus, however, is under end-product regulation and is thus inferred to be controlled by still a third operator locus.

When *oprA* mutates to the derepressed state, there is an increase in the rate of synthesis of isoleucine but not of valine. This increase, which is correlated with resistance to 10^{-2} M valine, has been shown to be a function of threonine deaminase derepression. When the *ilvB* locus is derepressed by a mutation in *oprB*, so that the level of condensing enzyme is raised 20-fold, the synthesis of both isoleucine and valine is increased. This increase is correlated with resistance to 10^{-3} M ABA, and to 2×10^{-4} M valine.

Thus, isoleucine biosynthesis can be increased either by derepressing threonine deaminase or by derepressing the condensing enzyme; the former confers resistance to high levels of valine, and the latter confers resistance only to low levels of valine. These observations suggest that the extreme sensitivity of *E. coli* K-12 to valine results from the efficiency with which valine represses the condensing enzyme and threonine deaminase in this strain. Still another factor is the ability of valine to inhibit the activity of the condensing enzyme (Leavitt and Umbarger, 1961). Pittard et al. (1963) described a mutant in which the condensing enzyme is resistant to valine inhibition; this change confers on the strain the ability to grow at high levels (10^{-2} M) of valine.

Work is now in progress on a large number of valine-resistance mutations which map outside of the region of the structural genes. One such mutation has been found to bring about derepression of the condensing enzyme but not of the other four enzymes. This mutation has not yet been mapped, but indicates the existence of

at least one regulator locus, the product of which specifically acts on *oprB*. A search for other regulator loci is in progress.

ACKNOWLEDGMENT

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