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# REGULATORY MECHANISMS IN THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE

## I. GENETIC DEREPRESSION OF ENZYME FORMATION

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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. I. Genetic derepression of enzyme formation. J. Bacteriol. 87:566-573. 1964. —A total of 60 mutants of *Escherichia coli* K-12 resistant to $10^{-2}$ M valine were isolated from the valine-sensitive F′ strain AB1206. Conjugation experiments showed that in five of these mutants the valine-resistance locus is closely linked to the structural genes governing isoleucine-valine biosynthesis. In these five valine-resistant mutants, three enzymes of the isoleucine-valine pathway were found to be coordinately derepressed: L-threonine deaminase, dihydroxy acid dehydrase, and transaminase B. Two other enzymes of this pathway, the condensing enzyme and the reductoisomerase, were unaffected. The mutation from valine-sensitivity to valine-resistance appears to have altered an operator locus, because the derepressed state is dominant over the repressed state in diploids heterozygous for the valine-resistance locus. The valine-resistant mutants excrete isoleucine into the medium. The significance of these findings with respect to the valine-sensitivity of *E. coli* K-12 and the regulation of the biosynthesis of isoleucine and valine by this organism are discussed.

Isoleucine and valine are synthesized by two parallel series of reactions catalyzed by a set of common enzymes (*see* Umbarger and Davis, 1962). The biosynthetic pathways are diagrammed in Fig. 1. The structural genes governing the formation of these enzymes are clustered in a small region of the chromosome in *Salmonella typhimurium* (Glanville and Demerec, 1960) as well as in *Escherichia coli* K-12 (Pittard, Loutit, and Adelberg, 1963). Both the isoleucine pathway and the valine pathway appear to be under end-product regulation; e.g., isoleucine and valine repress the formation of dihydroxy acid dehydrase (Freundlich, Burns, and Umbarger, 1962), and isoleucine represses the formation of L-threonine deaminase (Umbarger and Brown, 1957). Valine also inhibits the activity of the condensing enzyme (Leavitt and Umbarger, 1961).

The keto acid precursor of valine, α-ketoisovalerate, is the branch point for the pathways leading to pantothenate and leucine. Freundlich et al. (1962) and Freundlich and Umbarger (1963) showed that in *E. coli* W, which is not sensitive to valine, all four end products (isoleucine, valine, pantothenate, and leucine) are necessary for maximal repression of the enzymes catalyzing the common reactions of isoleucine and valine synthesis; the authors have termed this phenomenon "multivalent repression."

The presence of a repression mechanism and the tight clustering of the structural genes suggest that the latter form one or more operons under the control of regulator and operator loci, by analogy with the β-galactosidase system (Jacob et al., 1960). In the present paper, we describe experiments designed to test for the presence of an operator locus. Such a locus should possess the following attributes. (i) It should be able to mutate to a state of lowered affinity for repressor, causing derepression of enzyme formation, or to a state in which the structural genes are totally inactivated. (ii) It should be immediately adjacent to the genes which it controls. (iii) It should be dominant in heterozygotes, and should affect genes which are in the position *cis* but not *trans*.

Adelberg (1958) devised a method for obtaining derepressed mutants by selecting for strains resistant to analogues of the end product. Analogues may inhibit growth by acting as corepressors of biosynthetic enzymes (Moyed,

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1960) or by competing with metabolites for incorporation into macromolecules (Munier and Cohen, 1956). In either case, derepression of the biosynthetic enzymes should lead to analogue resistance.

Valine inhibits the growth of *E. coli* K-12, and this inhibition is reversed by its analogue, isoleucine (Tatum, 1946). Valine-resistant mutants are easily selected on valine-containing medium; some of these may be expected to be derepressed mutants, including regulator gene mutants which fail to produce active repressor, or operator gene mutants which have a lowered affinity for repressor. Of these, only operator mutations would be dominant in heterozygotes.

We have selected our valine-resistant mutants in strain AB1206, an F′ strain in which the structural genes for the isoleucine-valine enzymes are attached to the sex factor (Pittard et al., 1963). The F-merogenote, designated $F_{14}$, is transferred at high frequency, the zygotes so formed giving rise to colonies of cells diploid for the merogenote region. Mutants in which the valine-resistance loci were found to be on $F_{14}$ and to be dominant were then further analyzed genetically and enzymatically.

The following sections describe a series of valine-resistant mutants in which three of the enzymes of isoleucine-valine synthesis are coordinately derepressed as the result of mutations within a locus closely linked to the structural genes. The alleles conferring resistance are dominant in heterozygotes, suggesting that the locus is an operator.

## MATERIALS AND METHODS

*Organisms.* The valine-sensitive strain used in this work was AB1206. AB1206 is an F′ derivative of *E. coli* K-12; it carries the F-merogenote, $F_{14}$. The markers on $F_{14}$ are transferred in the order *O-(arg3-met1-met27)-arg1-met/$B_{12}$1(val-r12-ilv16, 17, 12-ile1)-sex factor*. The valine-resistant mutants and their isolation are described in Results. A summary of the strains used in this work is presented in Table I.

*Media and culture methods.* The media and culture methods used in this work were described previously (Adelberg and Burns, 1960). When the organisms were grown under conditions of "multivalent repression," the medium of Freundlich et al. (1962) as modified by Freundlich and Umbarger (1963) was used; pantothenic acid was added to the medium at 20 μg/ml.

*Mating conditions.* The conditions employed for measuring the kinetics of zygote formation were those described by Pittard and Adelberg (1963). In all the kinetic experiments, the re-

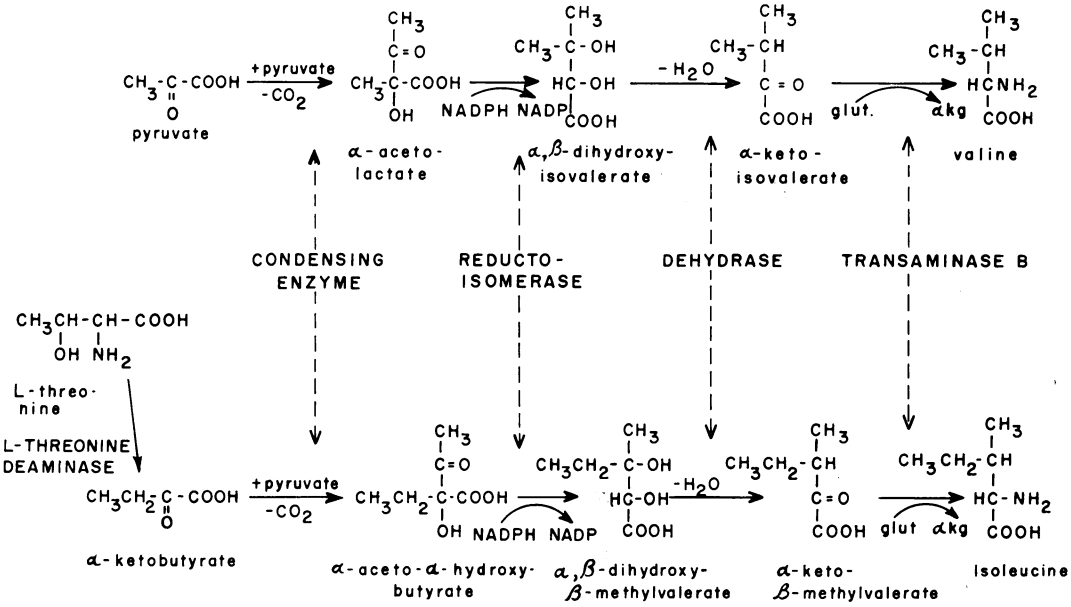


FIG. 1. *Pathways of isoleucine and valine biosynthesis.*

TABLE 1. *List of strains**

| Strain no. | Auxotrophic characters | | | | | | Energy source utilization | | | | Response to | | | Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *arg* | *met* | *thi* | *pro* | *try* | *his* | *gal* | *lac* | *mal* | *xyl* | *val* | *T6* | *str* | |
| AB 1254 | 1 | 1 | 2 | + | 14 | 1 | + | + | 1 | 4 | s | r | r | ♀ |
| AB 1276 | + | + | 1 | + | + | 4 | 2 | + | + | + | s | s | s | ♀ |
| AB 1206 | + | + | 1 | 2 | + | 4 | 2 | 1 | + | + | s | s | r | ♂ $F_{14}$ |
| AB 1001 | + | + | 1 | 2 | + | 4 | 2 | 1 | + | + | r101 | s | r | ♂ $F_{14}$ |
| AB 1005 | + | + | 1 | 2 | + | 4 | 2 | 1 | + | + | r105 | s | r | ♂ $F_{14}$ |
| AB 1008 | + | + | 1 | 2 | + | 4 | 2 | 1 | + | + | r108 | s | r | ♂ $F_{14}$ |
| AB 1009 | + | + | 1 | 2 | + | 4 | 2 | 1 | + | + | r109 | s | r | ♂ $F_{14}$ |
| AB 1013 | + | + | 1 | 2 | + | 4 | 2 | 1 | + | + | r113 | s | r | ♂ $F_{14}$ |
| AB 1020 | +/1 | +/1 | 2 | + | 14 | 1 | + | + | 1 | 4 | r120/s | s | r | ♂ $F_{14}$ |

* The following abbreviations are used in the table and the text: *ile*, isoleucine; *ilv*, isoleucine and valine; *val*, valine; *arg*, arginine; *met*, methionine; *thi*, thiamine; *thr*, threonine; *leu*, leucine; *pro*, proline; *try*, tryptophan; *his*, histidine; *ser*, serine or glycine; *xyl*, xylose; *mtl*, mannitol; *mal*, maltose; *lac*, lactose; *gal*, galactose; *T6*, bacteriophage T6; *str*, streptomycin; r, resistant; s, sensitive; *met*/$B_{12}$, methionine or vitamin $B_{12}$. Numbers refer to allele numbers allotted to mutant loci in these laboratories.

cipient strain used was AB1254, which carries the mutant alleles *met1* and *arg1*.

*Replica plating.* Recombinant colonies were scored for segregants by employing the replica plating technique of Lederberg and Lederberg (1952). The same procedure was used to test the ability of valine-resistant mutants to grow on concentrations of valine higher than those at which they were isolated.

*Preparation of cell-free extracts.* Cells harvested at 14 to 15 hr, while still in the logarithmic phase, were suspended in 5 volumes of 0.03 M phosphate buffer (pH 7.0) and subjected to ultrasonic oscillation in the cold in an ultrasonic disintegrator (MSE Ltd., London, England) for 2 min. The solution was centrifuged at 0 C for 20 min at 11,000 × *g* in a Servall superspeed centrifuge. The supernatant fluid was used for enzyme assays on the same day that the extract was prepared.

*Enzyme assays.* The assay for condensing enzyme was performed according to the method described by Radhakrishnan and Snell (1960). The substrate used was sodium pyruvate, and the reaction was carried out at pH 8.0. Reductoisomerase was assayed according to the method of Armstrong and Wagner (1961). Dihydroxy acid dehydrase was assayed according to the method of Myers (1961). Transaminase B (Rudman and Meister, 1953) was assayed according to the method of Umbarger (*personal communication*): α-ketobutyrate and L-valine were used as substrates, and the formation of α-ketoisovalerate was measured by dinitrophenylhydrazine after extraction with toluene to separate it from α-ketobutyrate. L-Threonine deaminase was assayed according to the method of Umbarger and Brown (1957).

The substrates for enzyme assays were prepared afresh every week. Enzymatic activities are recorded as micromoles of substrate converted, or product formed, per hour per milligram of protein.

*Analytical procedures.* Protein was estimated according to the method described by Lowry et al. (1951). Crystalline bovine serum albumin was used as the standard.

Amino acids excreted by the organisms into the medium were detected chromatographically after evaporating the medium to dryness in a water bath. The amino acids were then extracted by 0.18% (w/v) HCl in ethanol (Baliga et al., 1955), and subjected to descending chromatography on Whatman no. 1 filter paper with isoamyl alcohol-pyridine-water-diethylamine as solvent (Block and Weiss, 1956).

*Chemicals.* α,β-Dihydroxy-β-methylvalerate and α,β-dihydroxyisovalerate were kindly furnished by J. W. Myers. Acetolactate was prepared according to the method of Krampitz (1948). All other chemicals were obtained from Calbiochem.

## RESULTS

*Isolation of valine-resistant mutants.* A total of 60 tubes, each containing 1 ml of broth, were inoculated with approximately $10^3$ cells of *E. coli* AB1206. The tubes were incubated at 37 C for 15 hr with shaking, after which the cultures were centrifuged, washed once, and resuspended in minimal medium. A total of 20 tubes were treated with 2-aminopurine at a final concentration of 200 $\mu$g/ml and incubated at 37 C overnight with shaking. Another 20 tubes were treated with ethylmethanesulfonate at a final concentration of 50 $\mu$moles/ml and incubated at 37 C for 1 hr, after which the cells were harvested, washed with buffer, resuspended in buffer, and "heat shocked" for 1 hr at 48 C (Strauss, 1962). Samples (0.1 ml) were plated from each of the 60 tubes onto minimal medium supplemented with $2 \times 10^{-4}$ M valine. After 48 hr of incubation, each of the plates contained at least one valine-resistant colony. One colony from each plate was picked and purified by single-colony isolation on the same medium. In this way, 60 valine-resistant mutants of independent origin were obtained.

*Crosses between F' val-r mutants and an F⁻ val-s strain.* Conjugation experiments were carried out between each of the valine-resistant mutants and F⁻ strain AB1254. Mating was interrupted after 45 min by the addition of T6 phage and blending, after which the zygotes were diluted and plated on minimal medium containing $2 \times 10^{-4}$ M valine for the selection of valine-resistant recombinants, and on appropriately supplemented media for the selection of other markers on $F_{14}$. After 48 hr of incubation at 37 C, the recombinants were counted. Five of the valine-resistant mutants obtained by spontaneous mutation, but none of the induced mutants, were found to transfer the valine-resistance marker on $F_{14}$. The kinetics of zygote formation by these five strains was measured. The data for one of these strains are given in Fig. 2, from which it can be seen that the valine-resistance locus is transferred at about 23 min. It is, therefore, closely linked to the *ilv* loci mapped by Pittard and Adelberg (1963). The other four strains gave similar kinetics. These five valine-resistant mutants were used throughout the rest of this work.

*Levels of valine-resistance.* The five valine-resistant mutants were replicated onto minimal medium containing $10^{-3}$ and $10^{-2}$ M valine and incubated at 37 C. All the strains were able to grow on the highest concentration of valine.

*Enzymes of isoleucine-valine biosynthesis in the valine-sensitive and valine-resistant strains.* The concentration of enzymes in the valine-sensitive parent strain AB1206 was used as a point of reference in these studies. This strain, as well as the valine-resistant mutants, was grown in minimal medium and in medium to which isoleucine, valine, leucine, and pantothenic acid were added to bring about "multivalent" repression. The cells were harvested and cell-free extracts were prepared as described in Materials and Methods. The extracts were then assayed for the enzymes of isoleucine-valine biosynthesis, with the following results. (i) There was no difference in the levels of condensing enzyme and reductoisomerase between the parent and the mutants. (ii) The condensing enzyme of the valine-resistant mutants was as sensitive as that of the parent strain to inhibition by valine at a level of $10^{-4}$ M. (iii) High concentrations of L-threonine deaminase, dihydroxy acid dehydrase, and transaminase B (about 10 to 15 times those present in AB1206) were found in the valine-resistant strains. The relative increases in enzyme activity were the same whether the substrates used were isoleucine precursors or valine precursors. The ratios of the specific activity of the dehydrase and transaminase to the specific activity of threonine deaminase are plotted in Fig. 3 for each extract. This figure shows that the ratio of the activity of any one

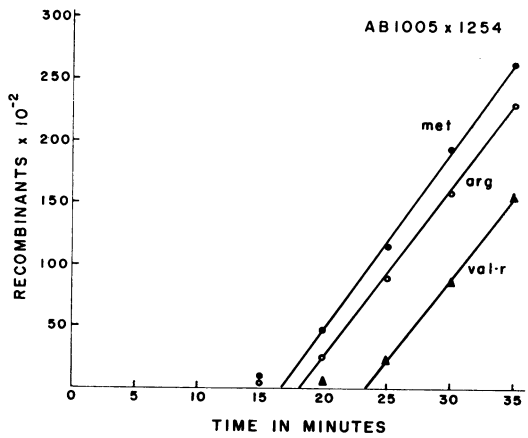


FIG. 2. *Kinetics of zygote formation for markers arg1, met1, and val-r when F⁻ strain AB1254 is mated with F' valine-resistant strain AB1005.*

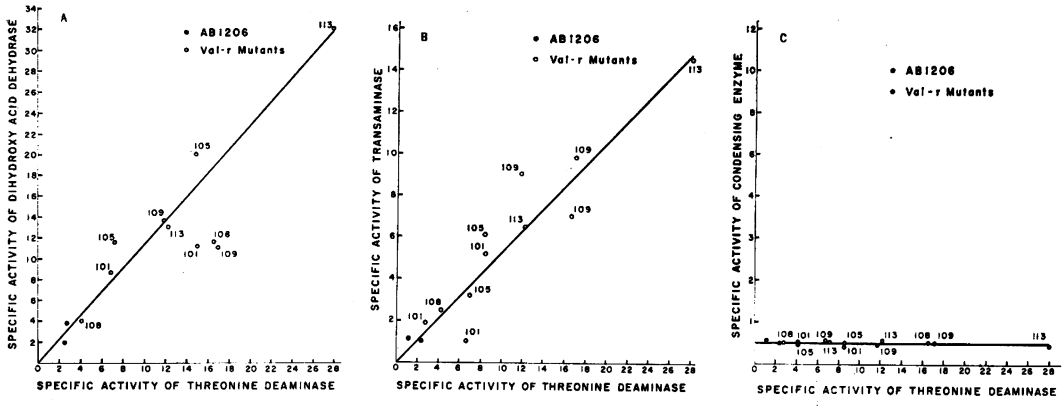


FIG. 3. *Coordinate derepression by valine of the formation of three enzymes concerned with isoleucine-valine biosynthesis. Specific activity of* L*-threonine deaminase plotted against (A) specific activity of dihydroxy acid dehydrase; (B) specific activity of transaminase B; (C) specific activity of condensing enzyme. The numbers refer to the val-r allele numbers of the mutants. The specific activity of each enzyme was measured under two conditions of repression. The reaction mixtures for enzyme assay contained the following.* L*-Threonine deaminase contained in 1 ml (μmoles): phosphate, 100; cysteine, 250;* L*-threonine, 400; cell-free extract, 0.8 mg of protein. Dihydroxy acid dehydrase contained in 1 ml (μmoles): tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.8), 200; cysteine, 250; ferrous sulfate, 50;* DL*-α,β-dihydroxyisovalerate, 50; cell-free extract, 0.8 mg of protein. Transaminase B contained in 1 ml (μmoles): tris buffer (pH 7.8), 200; pyridoxal phosphate, 100; α-ketoglutarate, 125;* L*-valine, 250; cell-free extract, 0.8 mg of protein. Condensing enzyme contained in 1 ml (μmoles): phosphate buffer (pH 8), 200; thiamine pyrophosphate, 4; manganous sulfate, 0.5; sodium pyruvate, 300; cell-free extract, 0.8 mg of protein.*

of these enzymes to the other is constant no matter what the mutant or the growth conditions.

In contrast, the specific activities of the condensing enzyme and the reductoisomerase in the valine-resistant mutants are constant and independent of the activities of the three derepressed enzymes. Data for the condensing enzyme are plotted in Fig. 3C; identical results were obtained for the reductoisomerase.

*Excretion of isoleucine by valine-resistant mutants.* The greatly increased activity of threonine deaminase in the valine-resistant mutants suggested that they might be excreting isoleucine. Since high concentrations of dihydroxy acid dehydrase and transaminase were also present in the mutants, it was possible that they were excreting some valine also.

To test these possibilities, AB1206 and the valine-resistant mutants were grown in minimal medium for 15 hr and centrifuged. The supernatant fluid was subjected to paper chromatography as described in Materials and Methods. Only the valine-resistant mutants were found to excrete detectable amounts of isoleucine; valine was excreted both by the parent strain and by the valine-resistant mutants, in amounts which did not appear significantly different as estimated visually.

*Inhibition of threonine deaminase by isoleucine.* It is known that in *E. coli* K-12 the activity of L-threonine deaminase is inhibited by isoleucine (Umbarger, 1956). It was of interest to determine whether this mechanism operates in the valine-resistant mutants which excrete isoleucine. The threonine deaminase of valine-resistant mutants was found to be inhibited to the same extent (40%) by $10^{-4}$ M L-isoleucine as was that of the valine-sensitive strain AB1206.

*Segregational instability of first-generation valine-resistant strains.* When F′ strain AB1206 or one of its valine-resistant derivatives transfers $F_{14}$ to a suitable $F^-$ recipient, a new F′ strain is formed which has been referred to as a "first-generation male" (Pittard and Adelberg, 1963) Such males are unstable merodiploids, and segregate haploid $F^-$ cells at high frequency. If the valine-resistance mutations which have occurred on $F_{14}$ are truly dominant, then first-generation males formed by the transfer of $F_{14}$ from valine-resistant mutants of AB1206 should

also be valine-resistant and should segregate valine-sensitive clones.

To test this possibility, one colony each of the first-generation males formed in crosses of $F^-$ strain AB1254 with the valine-resistant F′ strains AB1001, AB1005, AB1009, and AB1013 was purified by restreaking on minimal medium and was grown overnight in broth. The broth cultures were streaked on nutrient agar, and 80 colonies from each were transferred to plates of nutrient agar. They were then replicated onto minimal medium and onto minimal medium supplemented with valine at $2 \times 10^{-4}$ M. Each of the four first-generation valine-resistant strains was found to have produced valine-sensitive segregants, the latter ranging in frequency from 10 to 70% of the subclones tested. Thus, the first-generation valine-resistant strains are heterozygous, valine-resistance being dominant.

## DISCUSSION

The results reported above demonstrate the existence of a locus which controls the formation of three of the enzymes of isoleucine-valine biosynthesis. This locus can mutate to a state which allows the derepression of the enzymes L-threonine deaminase, dihydroxy acid dehydrase, and transaminase B, but not of the condensing enzyme or the reductoisomerase. The affected enzymes are derepressed in a coordinate manner (Fig. 3) just as in the case of the enzymes for histidine biosynthesis (Ames and Garry, 1959). The sensitivity of L-threonine deaminase to feedback inhibition by isoleucine and of the condensing enzyme to feedback inhibition by valine is not affected in such mutants. Changeux (1961) also observed that in an *E. coli* strain derepressed for L-threonine deaminase the enzyme activity remains sensitive to inhibition by isoleucine, again illustrating the independence of the repression and feedback-inhibition mechanisms.

The locus described is closely linked to the structural genes which it controls, and the allele conferring derepression is dominant over the wild-type allele. This locus is tentatively designated as the operator locus for an operon comprising three structural genes. Final confirmation of this designation must await *cis-trans* tests of the derepression effect; the strains necessary for such tests are now being prepared.

Valine is known to inhibit isoleucine metabolism at several points. It represses the formation of many of the enzymes needed for isoleucine synthesis, it inhibits the activity of the condensing enzyme, and it competes with isoleucine for a common permease (Cohen and Monod, 1957) as well as for a common activating enzyme (Bergmann, Berg, and Dieckmann, 1961). The latter effect may be the cause of the alteration by valine of protein synthesis observed by Cohen (1958). It is not certain which of these activities is the primary cause of valine sensitivity in strain K-12, but the fact that genetic derepression of threonine deaminase, dihydroxy acid dehydrase, and transaminase B confers high-level resistance to valine eliminates the condensing enzyme and the reductoisomerase as primary sites of valine inhibition.

The excretion of excess isoleucine, but not of excess valine, suggests that one of the three derepressed enzymes is rate-limiting for the synthesis of isoleucine, but not of valine, in wild-type *E. coli* K-12. If so, valine synthesis may be limited by the activity of the condensing enzyme or the reductoisomerase, and a mutant derepressed for either of these enzymes might excrete valine but not isoleucine. Such mutants are now being sought. The failure of the condensing enzyme and the reductoisomerase to be altered by the operator mutation is surprising. Experiments are now in progress to map the operator locus with respect to the five structural genes; presumably, the genes for the condensing enzyme and the reductoisomerase lie outside the operon, and are governed by a separate operator not revealed by our present selection methods.

Many of the valine-resistant mutants isolated from strain AB1206 proved to have mutated at one or more chromosomal loci, rather than at a locus on $F_{14}$. These loci presumably correspond to the valine-resistance sites reported by Glover (1962). It is unlikely that they lie in the chromosomal segment corresponding to $F_{14}$, since AB1206 appears to have a major chromosomal deletion in this region. The evidence for this conclusion will be presented in a separate communication. It is interesting that the mutagens 2-aminopurine and ethylmethanesulfonate induce mutations in various loci conferring valine resistance, but not in the operator locus. This would be understandable if the operator locus comprises only a very limited number of nucleotide pairs.

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