GENETIC AND BIOCHEMICAL STUDIES ON ISOLEUCINE AND VALINE REQUIRING MUTANTS OF *ESCHERICHIA COLI*

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IN microorganisms, a single mutation event can result in the growth requirement of two amino acids, isoleucine and valine. This is not considered unusual since the two amino acids share a common biosynthetic pathway (UMBARGER and DAVIS 1962), (Figure 1). In *Salmonella typhimurium* (GLANVILLE and DEMEREC 1960), the genes controlling the synthesis of the enzymes of the isoleucine-valine pathway are clustered in a short region of chromosome. On the other hand, the genes of *Neurospora crassa* (KIRITANI 1962; WAGNER, BERGQUIST, BARBEE and KIRITANI 1964) and *Saccharomyces cerevisiae* (KAKAR and WAGNER 1964) that control the production of similar enzymes are not clustered in any one region of a chromosome. The finding of what may be organizational mutants in *Neurospora* (WAGNER, KIRITANI and BERGQUIST 1962) and *Saccharomyces* (KAKAR and WAGNER 1964) provides a clue with which one can analyze the relationship between the cytoplasmic organization of enzymes of the biosynthetic pathway and the location of the genes controlling these enzymes. A study of the

FIGURE 1.—The biosynthetic pathway leading to isoleucine and valine. AL=α-acetolactate, AHB = α-aceto-α-hydroxybutyrate, DHV = α,β-dihydroxyisovalerate, DHI = α,β-dihydroxy-β-methylvalerate, KV=α-ketoisovalerate, KI=α-keto-β-methylvalerate. (From KAKAR and WAGNER 1964).

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same system in *Escherichia coli* might shed further light on the relationship. Hence, the purpose of this paper is to analyze the genetic structure of isoleucine-valine loci, and to correlate the genetic information with biochemical characteristics that have been obtained with isoleucine-valine requiring mutants of *E. coli*. Recently, two papers have been published along this line (PITTARD, LOUTIT, and ADELBERG 1963; RAMAKRISHNAN and ADELBERG 1964) without referring to the fine structure of isoleucine-valine loci.

MATERIALS AND METHODS

Strains: *E. coli* K-12 and its derivative strain W2252 (Hfr, methionine requiring) were used, and isoleucine (Hfr, *met*⁻, *isol*⁻) and isoleucine-valine (Hfr, *met*⁻, *iv*⁻) requiring mutants were induced in the strain W2252. The marker "Hfr" has been omitted in later descriptions of these strains. The phage used for the transduction experiments was P1kc.

Media: N-B broth: Bacto peptone (Difco) 10g, Bacto yeast extract (Difco) 5g, NaCl 10g, and distilled water 1 liter. N-B soft agar: N-B broth with 0.65% agar. Minimal medium: K₂HPO₄ 7.0 g, KH₂PO₄ 2.0 g, Na₃-citrate 0.5 g, (NH₄)₂SO₄ 1.0 g, MgSO₄·7H₂O 0.1 g, glucose 1.0 g, and distilled water 1 liter. Supplemented media: L-isoleucine, L-valine, L-leucine and/or L-methionine were added to the minimal medium in concentrations of 15 mg, 10 mg, 10 mg, and 15 mg per liter, respectively. Since repression of the reductoisomerase was observed when the molar ratio of isoleucine to valine in a synthetic medium was below one, the amount of isoleucine in a supplemented medium was always greater than that of valine (KIRITANI and IKEDA 1965).

Isolation of mutants: Ultraviolet light (UV, Mazda germicidal lamp, 15 w), X rays (Mazda KXC-19 type X-ray generator, 250 kv, 25 ma), and 2, 6-diaminopurine (Nutritional Biochemical Co.) were used as mutagenic agents. Strain W2252 was treated with one of these mutagenic agents and then subjected to the penicillin screening method (LEDERBERG 1950). Mutants were selected from those clones that required isoleucine and valine, in addition to methionine, for growth. The 2, 6-diaminopurine treatment was carried out according to the method of FREESE (1959).

Chemicals: α -Ketoisovaleric acid (KV) was obtained from Sigma Chemical Company. The other substrates were synthesized by the methods indicated in each reference: α -aceto- α -hydroxybutyric acid (AHB), RADHAKRISHNAN and SNELL 1960; α -acetolactic acid (AL), KRAMPITZ 1948; α,β -dihydroxy- β -methylvaleric acid and α,β -dihydroxyisovaleric acid (DHI and DHV), SJOLANDER, FOLKERS, ADELBERG and TATUM 1954; α -keto- β -methylvaleric acid (KI), MEISTER 1952.

Growth of cells and preparation of cell-free extract for enzymatic analysis: 0.05 ml of an inoculum culture (grown overnight) was added to 300 ml of supplemented medium and grown overnight under forced aeration at 37°C. The cells were collected by centrifugation, washed once with 100 ml of 0.1 M phosphate buffer (pH 7.5), and resuspended in 5 ml of the same buffer. The cell suspension was sonically disrupted for 5 min in a Toyo sonic disruptor (10 kc), then centrifuged at 60,000 $\times$ g. The resulting supernatant fraction was used as a cell-free extract. These procedures were carried out in the cold.

Assay of enzyme activity. Reductoisomerase activity was assayed by the method of RADHAKRISHNAN, WAGNER and SNELL (1960). In the assay mixture, 0.02 M phosphate buffer was used; β -mercaptoethanol and a boiled extract of *E. coli* were omitted. The assay was carried out at 35°C, and the oxidation of NADPH₂ was measured at 340 m μ , using a Beckman recording spectrophotometer. Dehydrase activity was assayed as described by MYERS (1961). α -Keto acids were determined by the indirect method of FRIEDMANN and HAUGEN (1943). The specific activity of these enzymes was measured as μ moles per mg protein per hour. Protein was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

Auxanography: Auxanography was carried out on methionine-supplemented agar plates. Approximately 10⁸ cells in 1 ml saline, which were harvested from an overnight culture of a

mutant strain, were dispersed in 25 ml liquid methionine-agar medium maintained at 40°. The mixture was then poured into a petri dish and allowed to harden. Filter paper discs (Toyo No. 1, 0.3 cm in diameter) were placed on the medium, and then 0.05 ml aliquot of isoleucine or valine or a precursor of these amino acids (1 mg per ml) was pipetted onto the discs. Plates were incubated 24 hours at 37°, then inspected for growth.

Generation time of bacteria. For determining generation time, 0.05 ml aliquot of an overnight inoculum culture was grown in 10 ml medium on a shaker at 37°. The generation time was scored as the amount of time required for the turbidity of the bacterial suspension to double. Turbidity was measured as percent transmission in a Kotaki nephelometer.

Determination of the frequency of reverse mutation. An inoculum containing approximately 100 cells was grown in 10 ml supplemented medium, centrifuged, washed with 10 ml sterile saline, and resuspended in 10 ml sterile saline. A 0.1 ml aliquot of the suspension (diluted 10^{-6}) was plated on medium supplemented with isoleucine, valine, leucine and methionine to determine the number of viable cells of bacteria. One tenth or one ml of the undiluted suspension was dispersed in 75 ml liquid methionine supplemented agar medium at 40°, which was then poured into three petri dishes and allowed to harden. Triplicated plates were incubated 48 hours at 37°, then scored for revertant colonies. The frequency of reverse mutation was calculated by dividing the number of revertants by the number of viable cells.

Preparation of phage P1kc. A P1kc lysate was prepared by the method of LENNOX (1955). A 0.1 ml aliquot of the original phage suspension (supplied by DR. H. OZEKI, National Institute of Health, Tokyo) and 0.5 ml of a young donor culture (ca. 2 to 5×10^8 cells) were mixed in 2.5 ml N-B soft agar, and then layered on a N-B agar plate containing 2.5×10^{-3} M Ca^{++} ion. Several plates were prepared in this manner. After 4 to 7 hours incubation at 37°, 5 ml N-B broth was poured on plates where confluent lysis had occurred. The plates were kept overnight at 4°, and then the lysate was collected. Phage was obtained in the supernatant fraction after centrifugation of the lysate at 10,000 rev/min for 10 min. The phage preparation, with a few drops of chloroform added, was preserved in a refrigerator.

Transduction experiments: A 4.5 ml aliquot of a young recipient culture (ca. 5×10^8 cells per ml) was infected with 0.5 ml phage lysate, prepared from a donor culture, at a multiplicity of infection (M.O.I.) of less than 1. The number of recipient cells was low enough to avoid the interference of spontaneous revertants. The mixture was shaken for 20 min at 37°. Cells were chilled, centrifuged in a refrigerated centrifuge, and suspended in 1 to 2 ml saline. A 0.1 ml aliquot of the suspension was plated on an appropriate medium to detect transductants. Transductant colonies were scored after 48 hours incubation at 37°. Controls were carried out by using sterile water instead of the phage lysate.

RESULTS

Characteristics of mutants: Table 1 lists the 18 mutant strains obtained by the treatment of strain W2252 with UV, X rays and 2,6-diaminopurine. Of these strains, 16 require isoleucine and valine (iv^-) and two require isoleucine ($isol^-$), in addition to methionine, for growth. The growth of the iv^- mutant strains is stimulated by an addition of leucine to medium supplemented with isoleucine and valine. The generation time of iv^- bacteria is 100 min without leucine and 50 to 60 min with it. The frequencies of reverse mutation to iv^+ or $isol^+$ are listed in Table 1.

Grouping of the mutants by auxanography: The iv^- mutant strains were subdivided into three categories; those unable to grow on AHB plus AL (group I, to which mutants 101, 102, 105, 110, 116, 117, 120, and 122 belonged); those unable to grow on combinations of precursors that precede KI and KV (group II, to which mutants 106, 107, 109, 111, 118, and 126 belonged); and a mutant

TABLE 1
Genetic stability of the isolated mutants

Mutant No.	Mutagen used	Requirements	Frequency of reverse mutation
101	ultraviolet	isoleucine plus valine	5.2×10^{-7}
102	X rays	isoleucine plus valine	3.3×10^{-8}
104	X rays	isoleucine	
105	ultraviolet	isoleucine plus valine	5.9×10^{-8}
106	ultraviolet	isoleucine plus valine	5.0×10^{-9}
107	ultraviolet	isoleucine plus valine	$<1.2 \times 10^{-9}$
109	ultraviolet	isoleucine plus valine	2.0×10^{-9}
110	X rays	isoleucine plus valine	$<1.9 \times 10^{-9}$
111	X rays	isoleucine plus valine	$<2.3 \times 10^{-9}$
112	ultraviolet	isoleucine plus valine	3.3×10^{-9}
113	ultraviolet	isoleucine	1.2×10^{-8}
115	ultraviolet	isoleucine plus valine	$<1.8 \times 10^{-9}$
116	ultraviolet	isoleucine plus valine	2.8×10^{-8}
117	ultraviolet	isoleucine plus valine	2.0×10^{-8}
118	ultraviolet	isoleucine plus valine	8.5×10^{-9}
120	ultraviolet	isoleucine plus valine	3.7×10^{-9}
122	ultraviolet	isoleucine plus valine	1.7×10^{-9}
126	DAP*	isoleucine plus valine	1.6×10^{-9}

* DAP: 2,6-Diaminopurine.

strain (iv⁻ 115) able to grow only on isoleucine plus valine. (It was found to be a deletion mutant in subsequent experiments.) Although the experiments were repeated three times, conclusive results on the growth response of strain 112 to DHI plus DHV were not obtained. The isol⁻ mutant strains, 104 and 113, can utilize AHB, DHI, KI, and isoleucine for growth.

Enzyme activities of the mutant strains: Reductoisomerase and dehydrase activities of cell-free extracts of the iv⁻ mutant strains were assayed, and the results are listed in Table 2. The extracts prepared from group I mutant strains exhibit, in general, low reductoisomerase activities when AHB was used as the substrate. When AL was used as the substrate, the enzyme activities of group I mutant strains were not significantly different from that of strain W2252. The mutant strain 101 is an exception because it possesses wild-type levels of both reductoisomerase and dehydrase activities. According to the results of the auxanographic test, the mutant strain 101 should be reductoisomerase-deficient. This strain is referred to as group Ib, whereas the previously described are classified as Ia. Cell-free extracts from group II mutants exhibit essentially no dehydrase activity.

On the basis of these experiments, it may be concluded that in the group I mutants the enzymatic step for the conversion of AHB (AL) to DHI (DHV) is blocked and in group II mutants the step between DHI (DHV) and KI (KV) is blocked (see Figure 1). In mutant strain 115, it appears that the genetic damage involves the two genes which control the production of the reductoisomerase and the dehydrase, since both activities in the cell-free preparation are very low.

Mapping of the iv loci: The linear arrangement of the iv mutational sites was

TABLE 2

Enzyme activities of the mutants

	Group	Mutant No.	Specific activity*			
			Reductoisomerase		Dehydrase	
			AHB†	AL	DHI	DHV
<i>iv</i> ⁻	Ia	102	2.5	1.6	1.7	1.2
		105	1.7	1.0	1.6	1.1
		110	1.4	1.3	1.4	1.4
		116	1.9	1.6	1.4	1.1
		117	1.6	1.8	1.0	0.7
		120	2.9	2.2	1.9	1.5
		122	1.9	2.6	1.0	0.8
	Ib	101	8.0	3.4	1.6	1.1
	II	106	9.8	2.6	0.0	0.0
		107	9.3	4.0	0.0	0.0
		109	14.9	3.8	0.0	0.0
		111	9.1	3.5	0.0	0.0
		112	12.6	4.2	0.0	0.0
		118	14.5	3.7	0.0	0.0
		126	8.7	4.3	0.0	0.0
	Deletion	115	0.8	0.9	0.0	0.0
<i>isol</i> ⁻		104	8.7	2.2	1.1	0.7
		113	7.6	2.5	0.1	0.1
<i>iv</i> ⁺		W2252	6.5	2.3	0.5	0.4

* Micromoles substrate converted per hour per mg protein.

† See Figure 1 for identification of substrates.

determined by transduction studies between the individual *iv* mutant sites. Prior to the transduction experiments, stable *met*⁺ *iv*⁻ strains from one or two representative *met*⁻ *iv*⁻ strains of groups I and II were produced by a transduction with *met*⁺ *iv*⁺ (wild-type) phages. No *met*⁺ *iv*⁺ joint transductants were observed in these experiments, i.e., the *met* locus is not closely linked to *iv* loci. On the other hand, the same phage preparations can transduce the *iv*⁺ trait into any *iv*⁻ strain. Transduction experiments between the *met*⁺ *iv*_x⁻ donor strain (phage) and the *met*⁻ *iv*_y⁻ recipient strain (bacteria) were carried out, and numbers of *iv*⁺ and *met*⁺ recombinants were scored and are presented in Table 3. Relative distance between two *iv* mutational sites, calculated as the recombination index, was obtained using the formula:

$$\text{Recombination index} = \frac{iv^{+}}{met^{+}} \times 1 / \frac{iv^{+} \text{ (wild)}}{met^{+} \text{ (wild)}}$$

In the equation, the terms *iv*⁺ and *met*⁺ represent the number of those recombinants obtained from a transduction cross between *met*⁺ *iv*_x⁻ donor and *met*⁻ *iv*_y⁻ recipient strains, and the *iv*⁺ (wild) and the *met*⁺ (wild) signify the number of recombinants obtained from the cross between *met*⁺ *iv*⁺ (wild type) donor and the *met*⁻ *iv*_y⁻ recipient strains.

TABLE 3

Numbers of transductants produced from crosses $met^+ iv^-_x$ (donor strain) $\times$ $met^- iv^-_y$ (recipient strain)

Recipient type			Donor type							
			(Ia)		(Ib)		(II)			
			110		101		106		111	
			<i>met</i> ⁺	<i>iv</i> ⁺	<i>met</i> ⁺	<i>iv</i> ⁺	<i>met</i> ⁺	<i>iv</i> ⁺	<i>met</i> ⁺	<i>iv</i> ⁺
<i>iv</i> ⁻	Ia	102	2038	152	58	5	119	38	2238	1048
		116	5340	312	2676	163	226	75	3090	3780
		110	1992	0	3886	78	1101	210	3204	1681
		120	2596	39	5920	77	1776	450	5070	2956
		122	241	17	284	11	106	21	217	119
		105	2044	191	56	1	100	27	2415	1058
		117	1650	143	85	0	208	102	2955	1203
	Ib	101	966	45	1681	0	141	32	1075	753
	II	106	566	243	58	29	73	0	1474	57
		118	2472	1404	215	183	257	6	2300	92
		126	1554	557	113	35	179	1	3522	10
		107	3240	2100	3288	3204	223	3	2760	6
		112	1005	840	41	59	103	3	2298	3
		109	1102	1738	58	62	63	5	18.0	0
		111	328	423	52	81	52	3	1043	0
	Deletion	115	720	0	1425	0	1095	0	406	0
<i>isol</i> ⁻			(<i>isol</i> ⁺)		(<i>isol</i> ⁺)		(<i>isol</i> ⁺)		(<i>isol</i> ⁺)	
	113	1725	954	1650	1200	1365	238	393	8	
	104	1692	750	1605	720	1320	850	495	194

FIGURE 2.—Fine structure of *iv* regions in *E. coli*. Arrows indicate the direction of transduction from the donor to the recipient.

As can be seen in Figure 2 in which a tentative order of the mutational sites as determined by the recombination indexes is shown, the mutational sites of group I mutants are closely linked to that of mutant 110 and the sites of group II mutants are close to those of mutant 106 and 111. The mutational site of mutant 101 is found to be in the group I cluster. Since no recombinant has been recovered in crosses of mutant 115 with mutants 101, 110, 106, and 111, the *iv* region of groups I and II of mutant 115 may be deleted. The distance between groups I and II is significantly longer than a distance between mutational sites within the individual two groups. The values of the recombination index between groups I and II fall within a wide range. The order of the mutational sites within individual groups I and II, with exceptions 101 and 118, agrees well with the data. Although the mutational site 101 is placed at the end of the group I region, it can also be placed in a position between the sites 105 and 122.

Crosses of *isol⁻* mutants, 104 and 113, with *iv* mutants indicate that these sites are located outside of the group I or II region. The site of mutant 113 appears to be closely linked with the group II.

DISCUSSION

From the results of the biochemical and the genetic experiments, the isoleucine-valine requiring mutants of *E. coli* can be classified into three categories: those, with the exception of strain 101, having low reductoisomerase activity (group I), those lacking dehydrase activity (group II) and a mutant strain lacking both activities. The mutational sites of strains of groups I and II are clustered in two genetic regions as shown in Figure 2. Since a limited number of mutants was used for this study, the true size of the two regions was not measurable. Although data of crosses between groups fluctuate within a wide range, there is significant gap between the two regions. Generally, when group I mutants were used as donors, crosses between the groups gave high recombination indexes. Strain 115 is a deletion mutant, as shown by mutual transduction experiments. Although the transaminase activity was not assayed in this study, this enzyme might also be lacking in strain 115, as judged by the auxanographic results. If this is true, the locus controlling the transaminase must be linked to regions of groups I and II, because the deficient region of strain 115 can be transferred as a unit by an *iv⁺* phage. Strain 104 (*isol⁻*) has a mutational site located between or outside of the two *iv* regions.

Strain 101 in the group I has an apparent defect in the reductoisomerase as judged by the auxanographic results, yet the enzyme activity in the cell-free extract is as high as wild-type level. These properties of this strain resemble those of the putative organizational *iv⁻* mutants of *Neurospora* (WAGNER, KIRITANI and BERGQUIST 1962). In spite of wild-type levels of *in vitro* reductoisomerase and dehydrase activities in the putative organizational mutants of *Neurospora*, these strains accumulate carbinols and dihydroxy acids in their culture media, as if they had defects in the loci controlling the reductoisomerase and the dehydrase. Genetic sites for these mutants of *Neurospora* (KIRITANI 1962) are

located in a region between the reductoisomerase and dehydrase controlling regions or in the dehydrase controlling region of linkage groups V. On the other hand, the site for strain 101 of *E. coli* is located in the reductoisomerase controlling region. The nature of the genetic and biochemical block of this mutant remains to be studied.

α -acetolactate (AL) is only one third as active as AHB in the reductoisomerase assay carried out on wild-type extracts (Table 3). Although AHB activity is significantly lower in the group I mutants, the AL activity in these mutants is comparable to that in wild type. The so-called residual reductoisomerase activity and the nonspecific reductases, as described by ARMSTRONG and WAGNER (1961; 1962) in their studies on the reductoisomerase of *Salmonella typhimurium*, may account for this apparent inconsistency.

Dehydrase activities when assayed using extracts of strains W2252 and 113 are relatively low as compared to the results of the activity of wild type K-12 reported by MEYERS. According to ARMSTRONG and WAGNER (1963), wild-type *Salmonella* retains the maximal level of dehydrase activity during logarithmic growth. However, the activity declines until none is detectable following prolonged cultivation of bacteria in the stationary phase. In contrast to the dehydrase, reductoisomerase activity is not influenced by the change from logarithmic to stationary growth phases. Since bacteria were harvested in the early stationary phase in the present experiments, low dehydrase activities obtained might be due to the labile nature of the enzyme during the stationary phase.

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SUMMARY

Sixteen isoleucine-valine and two isoleucine-requiring mutants were induced by the treatment of the methionine-requiring mutant strain W2252 of *Escherichia coli* K-12 with ultraviolet light, X rays or 2, 6-diaminopurine. These mutants were partially characterized biochemically and genetically. With regard to the isoleucine-valine requiring mutants, group Ia mutants were reductoisomerase-deficient and group II strains dehydrase-deficient. The mutational sites of each group are clustered in a region; the two regions are distinctly different from one another, but linked on the chromosome. The mutant strain 101 in the group I cluster possesses a wild-type level of reductoisomerase activity in spite of the apparent block at this step. Mutant strain 115 appears to possess an extensive multisite or deletion mutation that includes the groups I and II. The mutational sites of the isoleucine-requiring mutants are located outside of these two regions.

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