OPERATOR CONSTITUTIVE MUTATIONS IN THE LEUCINE OPERON OF *SALMONELLA TYPHIMURIUM*

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IN a previous communication (CALVO, FREUNDLICH and UMBARGER 1969), a group of *Salmonella typhimurium* mutants was described as having the following properties. They were resistant to an analog of leucine, 5′,5′,5′-trifluoro-DL-leucine (RENNERT and ANKER 1963), excreted leucine, had constitutive and derepressed levels of enzymes involved in leucine biosynthesis, and they had mutant sites linked to the leucine operon. This paper describes in more detail the properties of these mutants and the map position of their mutant sites.

MATERIALS AND METHODS

Bacterial strains: All strains discussed here are derivatives of *Salmonella typhimurium* strain LT2 *ara*-9 or *ara*-9 *gal*-205. The isolation and characterization of fluoroleucine-resistant mutants were described in a previous report (CALVO, FREUNDLICH and UMBARGER 1969). The nomenclature in this paper follows the standards proposed by DEMEREC *et al.* (1966). For the purpose of describing genetic crosses, the locus affected in the fluoroleucine-resistant strains is temporarily defined here by the symbol *exc*, referring to leucine excretion. All cultures were incubated at 37°C. Liquid cultures were aerated by bubbling filtered air through a tube containing 10 ml of medium or by placing cultures in Erlenmeyer flasks on a rotary shaker.

Media: Nutrient broth and nutrient agar were used as complete media. A minimal salts solution (SSA) contained per liter of distilled water: K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate dihydrate, 0.97 g; $MgSO_4$, 0.05 g. SSA supplemented with 0.2% glucose or 0.2% glucose and 1.5% agar served as liquid and solid minimal media, respectively. Enriched minimal agar contained 0.01% (w/v) dehydrated nutrient broth. When growth on arabinose was desired, citrate was omitted from the medium and 0.2% arabinose was substituted for glucose (minimal arabinose).

Transduction techniques: Transduction was mediated by PLT22 type H1 phage using media and methods reported by MARGOLIN (1963). With the exception of strain *leuA*517, recipients used in crosses did not revert and controls consisted of separate platings of the donor phage lysate and the recipient culture. When strain *leuA*517 was recipient, control experiments employing phage grown on strain *leuA*517 (homologous phage) were also performed. Leucine excretion was scored by an auxanographic test described previously (CALVO, FREUNDLICH and UMBARGER 1969).

Enzyme assays: Bacteria in the middle log phase of growth were harvested by centrifugation, washed with 0.05 M potassium phosphate buffer, pH 6.8, and were resuspended in the same buffer (10 ml per gram wet weight of bacteria). Extracts were prepared by ultrasonic oscillation of 11 ml of suspension for 60 sec at 4 amps with a Branson S110 Sonifier. α-Isopropylmalate (α-IPM) synthetase was assayed by a fluorometric assay (CALVO, BARTHOLOMEW and STIEGLITZ

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1969) and β-isopropylmalate (β-IPM) dehydrogenase by the procedure of BURNS *et al.* (1963). Protein was determined by the method of LOWRY *et al.* (1951) using bovine serum albumin as standard.

Construction of leu⁻ exc *double mutants:* An overnight broth culture of an *ara*-9 *exc* strain was mixed with an equal volume of phage suspension prepared from strain *leuA*124 or *leuA*517, diluted, and a sample was plated on enriched minimal agar containing 0.2% arabinose as carbon source and 50 μg of L-leucine per ml. After incubation for 48 hrs, *ara*+ recombinants were replica-plated on to minimal arabinose and to minimal arabinose plus leucine plates to distinguish leucine auxotrophs from prototrophs. Tubes containing 0.5 ml of minimal medium supplemented with 50 μg of L-leucine per ml were inoculated with individual *ara*+ *leu*⁻ transductants and incubated until the cultures were fully grown. One drop of toluene was added to each tube and after standing 20 min with intermittent shaking, the following components of the β-IPM dehydrogenase assay were added in a 0.2 ml volume: tris(hydroxymethyl)amino methane-HCl, pH 8.0, 100 μmoles; KCl, 100 μmoles; $MnCl_2$, 0.25 μmole; β-IPM, 0.25 μmole; oxidized nicotine-adenine dinucleotide, 0.5 μmole. After incubation for 10 min at 37°C, 0.75 ml of 10^{-3}M 2,4-dinitrophenylhydrazine in 0.5N HCl was added to each tube followed by 0.25 ml of 40% KOH 15 min later. When this test was carried out with a strain having a high, constitutive level of β-IPM dehydrogenase, a dark brown color resulted; under the same conditions, the wild-type strain gave a light amber color.

RESULTS

The identity, origin and enzyme levels of the pertinent strains are shown in Table 1. In comparison with the wild type, the leucine forming enzyme levels in these strains (grown in a minimal medium) were greatly elevated. When these same strains were grown in a minimal medium supplemented with 50 μg of L-leucine per ml, these enzyme levels were lowered by about 30%. Note that all of these strains were derepressed to about the same level and that the derepression of the synthetase and dehydrogenase was essentially coordinate.

The results of two-factor crosses from earlier experiments suggested that the mutant sites of these strains were located at the left end of the leucine operon (the end distal to the *ara* cluster). This conclusion was confirmed by the results of

TABLE 1

Properties of some fluoroleucine-resistant mutants

Strain	Origin	Specific activity‡ of α-IPM synthetase	Specific activity‡ of β-IPM dehydrogenase
ara-9 *gal*-205		0.25	5.6
CV6	spontaneous	3.46 (13.8)†	65.6 (11.7)†
CV112	2-AP*	3.50 (14.0)	58.4 (10.4)
CV165	2-AP	3.56 (14.2)	72.0 (12.8)
CV173	2-AP	3.00 (12.0)	64.6 (11.5)
CV177	2-AP	2.60 (10.4)	61.3 (11.0)
CV186	2-AP	2.25 (9.0)	66.0 (11.8)
CV200	2-AP	3.46 (13.8)	71.3 (12.7)

* Isolated after mutagenesis with 2-aminopurine.

† Values in parentheses represent the factor by which the specific activity is increased relative to the wild type.

‡ μmoles product former per mg of protein per hr. The strains were grown in minimal medium.

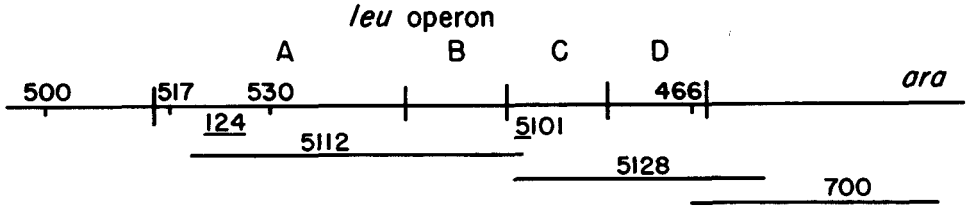

FIGURE 1.—Map position of some of the mutant sites studied in this work. The left boundary of cistron *A* is not known with certainty and it may lie to the left of *leu*-500.

deletion mapping experiments (Figure 1, Table 2). Phage grown on fluoroleucine-resistant mutants was used to transduce *leu* operon deletions to prototrophy and the transductants were scored for leucine excretion by an auxanographic test. Because fluoroleucine was not readily available, leucine excretion rather than fluoroleucine-resistance was chosen as a suitable phenotype for scoring. In crosses of this type involving deletion mutants, the existence of non-excreters among the *leu*$^+$ recombinants indicates that the mutation lies outside of the deletion. The three deletions chosen for this study cover by overlap a region extending from *ara* to a point within *leuA* (Figure 1, MARGOLIN and CALVO, unpublished data). The fact that non-excreters were found among *leu*$^+$ transductants (Table 2) plus the fact that these mutations were much more closely linked to *leuA*124 than to either *leuC*5101 or *leuD*466 (CALVO, FREUNDLICH and UMBARGER 1969), is a clear indication that these sites lie to the left of deletion *leuABC*5112.

An estimate of the proximity of these fluoroleucine-resistance sites to the left end of the *A* cistron was made by two-factor crosses. Strains *leuA*124 and *leuA517*, known from other studies to bear lesions at the left end of cistron *A*

TABLE 2

Deletion mapping analysis of some fluoroleucine-resistant mutants

Cross			Number of *leu*$^+$ transductants analyzed	Number of non-excreters
Recipient	×	Donor		
*leuABC*5112		CV6	400	2
		CV112	400	2
		CV165	400	4
		CV173	400	3
		CV177	400	4
*leuCD*5128		CV6	100	21
		CV112	100	15
		CV165	100	17
		CV173	100	16
		CV177	100	13
leu-700		CV6	100	31
		CV112	100	26
		CV165	100	27
		CV173	100	28
		CV177	100	29

TABLE 3

Linkage of some fluoroleucine-resistance sites to leuA*124 and* leuA*517*

Cross			Number of *leu*+ transductants analyzed	Number of non-excreters	Percent
Recipient	×	Donor			
*leuA*124		CV6	576	8	1.4
		CV112	260	3	1.2
		CV165	312	10	3.2
		CV173	260	3	1.2
		CV177	260	5	1.9
*leuA*517		CV112	416	1	.24
		CV165	312	1	.32
		CV173	266	1	.38
		CV177	260	0	0

were employed as recipients in crosses with phage grown on fluoroleucine-resistant mutants and the *leu*+ recombinants were scored for the excretion phenotype (Table 3). The data indicate that these mutant sites are very close to the left end of cistron *A* (less than one half cistron away) and that they are closer to *leuA*517 than to *leuA*124.

To further characterize the order of fluoroleucine-resistance sites relative to other sites in the operon, three-factor crosses were carried out. Double mutants containing a *leuA* site and an "excreter" marker (temporarily defined as *exc*) were constructed by transductions of the type illustrated in Figure 2. In the cross between an *exc ara*-9 recipient and a *leuA* donor, selection was made for *ara*+ and those recombinants that were leucine auxotrophs were identified by replica plating. The identification of the *exc* marker among *leuA ara*+ types was complicated by the fact that leucine auxotrophs cannot excrete leucine. However, another phenotype of *exc* is constitutive, derepressed enzyme levels and it was possible to screen *leuA ara*+ types for *exc* by developing a rapid enzyme assay for one of the enzymes in the pathway (cistron *B* product, β-IPM dehydrogenase). Two *leu* sites were chosen for this study; *leuA*124 and *leuA*517. The proportion of *leuA exc* among *leuA* types can be estimated from Table 3 as 1:58 and 1:418 for *leuA124* and *leuA517*, respectively. In total, 9 doubly mutant strains were constructed. Six *exc* mutations of independent origin were combined with the *leuA*124 mutation and 3 *exc* mutations of independent origin with the *leuA*517 mutation.

The types of three-factor crosses performed are illustrated in Figure 3. Crosses

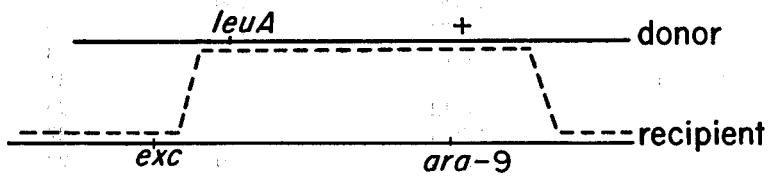

FIGURE 2.—Representation of cross by which *leuA exc* double mutants were constructed.

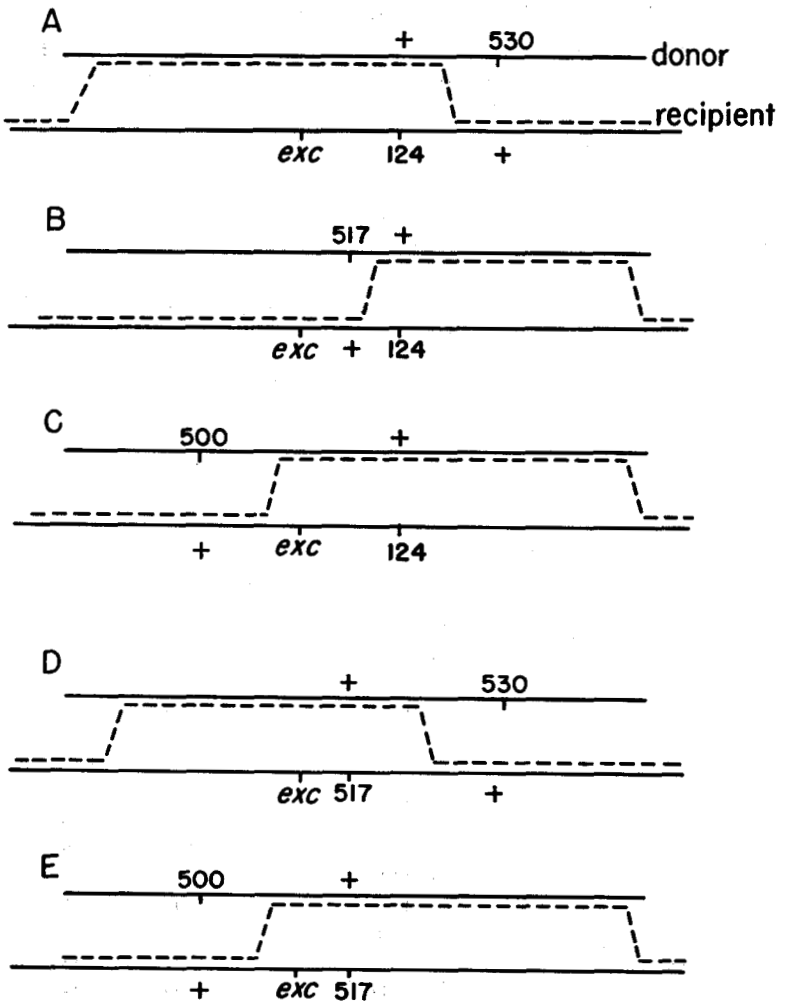

FIGURE 3.—Schematic representation of three-factor crosses that were designed to determine the position of an *exc* site relative to *leu*-500, *leuA*517, *leuA*124 and *leuA*530. See text for explanation.

A, B and C involved *leuA*124 *exc* as recipient against a donor phage carrying a *leu* mutant site either to the right (*leuA*530) or to the left (*leuA*517, *leuA*500) of *leuA*124. In cross A, the formation of a *leu*$^+$ recombinant requires a crossover between *leuA*124 and *leuA*530. If *exc* were to the right of and close to *leuA*530, almost all *leu*$^+$ recombinants would be excreters. For the first 6 crosses in Table 4 involving *leuA*530 as donor, only 8–12% of the *leu*$^+$ recombinants were excreters suggesting that *exc* does not lie to the right of *leuA*530. In crosses of type B (Figure 3), crossovers between *leuA*517 and *leuA*124 are selected. If *exc* were to the right of *leuA*124, *leu*$^+$ recombinants would be almost all non-excreters because the distance between *leuA*124 and *exc* is small relative to the distance between

*leuA*124 and the right end of the donor fragment. In fact, about 96% of the *leu*+ recombinants from these crosses were excreters (Table 4, first 6 crosses involving *leuA*517 as donor) indicating that *exc* is not to the right of *leuA*124. This result is consistent with *exc* being to the left of *leuA*517 or to the right of but very close to *leuA*517. The former possibility is considered more likely. *leu*+ recombinants formed in type C crosses require a crossover between *leu*-500 and *leuA*124. If *exc* were to the left of *leu*-500, almost all *leu*+ recombinants should be excreters because 4 crossovers are required for the recombinants to be non-excreters. The results of these crosses, namely, that about 50% of the *leu*+ recombinants were excreters (Table 4, first 6 crosses involving strain *leu*-500 as donor) indicate that *exc* is not to the left of *leu*-500. To summarize, *exc* lies between *leu*-500 and *leuA*124 and probably to the left of *leuA*517. This is consistent with the results

TABLE 4

Analysis of fluoroleucine-resistance sites by three-factor crosses

Recipient		Donor	Number of plates†	Total colonies analyzed	Number of excreters	Percent excreters
Strain*	Derived from cross					
CV16	*leuA*124 × CV6	*leuA*530	10	100	8	8.0
		*leuA*517	89	366	349	95.4
		leu-500	35	972	478	49.2
CV229	*leuA*124 × CV112	*leuA*530	40	666	83	12.5
		*leuA*517	80	201	193	96.3
		leu-500	40	1176	552	46.9
CV235	*leuA*124 × CV173	*leuA*530	50	720	83	11.5
		*leuA*517	90	301	294	97.7
		leu-500	65	1424	656	46.0
CV236	*leuA*124 × CV177	*leuA*530	40	788	85	10.8
		*leuA*517	80	207	201	97.3
		leu-500	40	1022	500	48.8
CV238	*leuA*124 × CV186	*leuA*530	120	2011	239	11.9
		*leuA*517	80	265	252	95.0
		leu-500	60	1288	644	50.0
CV239	*leuA*124 × CV200	*leuA*530	40	686	73	10.6
		*leuA*517	80	184	179	97.4
		leu-500	40	899	420	46.7
CV231	*leuA*517‡× CV120	*leuA*530	40	303	11	3.6
		leu-500	99	1264	383	30.3
CV234	*leuA*517‡× CV165	*leuA*530	40	593	45	7.7
		leu-500	120	1778	537	30.2
CV237	*leuA*517‡× CV177	*leuA*530	40	290	36	12.4
		leu-500	60	449	146	32.5

* Each recipient strain harbors the *leuA* mutation of one parent and the *exc* mutation of the other parent in the cross that is shown.

† Number of enriched minimal plates spread with 0.2 ml of a transduction mixture containing equal volumes of phage suspension (3×10^{10}/ml) and an overnight broth culture of the recipient.

‡ *leuA*517 reverts at a low frequency and the data for these crosses have been corrected for these reversions. Control crosses (data not shown) employed homologous phage and the number of plates spread were equal to the number used in experimental crosses.

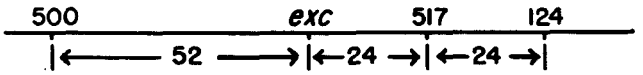

FIGURE 4.—Relative map distances between *leu*-500, *exc*, *leuA*517, and *leuA*124. The *leu*-500-*leuA*124 distance was arbitrarily taken as 100.

of the deletion mapping study which indicated that *exc* must lie to the left of *leuA*124.

The results of the last 3 sets of crosses in Table 4, in which *leuA*517 *exc* strains served as recipient, support this conclusion. The crosses involving strain *leu*-500 as donor are particularly important. The fact that about 30% of the *leu*$^+$ recombinants from these crosses were excreters provides convincing evidence that *exc* is to the left of *leuA*517. If *exc* mutations were to the right of *leuA*517, the expected proportion of excreters should have been 10% or less.

The relative distances between *leu*-500, *exc*, *leuA*517 and *leuA*124 can be estimated from the data in Table 4 from crosses involving strain *leu*-500 as donor. If the *leu*-500–*leuA*124 distance is assigned 100 arbitrary units, then *exc* is 52, 24 and 48 units removed from *leu*-500, *leuA*517, and *leuA*124, respectively (Figure 4).

It is apparent from Table 4 that the *exc* sites analyzed in this study have the same general map location. It was of interest to determine whether some of these sites could be separated by recombination. If a pair of *exc* sites were non-identical and non-overlapping, then *leu*$^+$ recombinants from crosses of the type illustrated in Figure 5 should contain a small proportion of non-excreters. The order of *exc* sites (assuming that the sites are separable) is not known, therefore, reciprocal crosses (Figure 5) were made to ensure that non-excreters could arise by 2 crossovers in at least one of the two crosses. Data from two pairs of crosses are shown in Table 5. The mutant sites of strains CV200 and CV186 are either overlapping or very near one another because no non-excreters were found among 4400 *leu*$^+$ recombinants. The mutant sites of strains CV177 and CV173 were separable by recombination but the very low frequency (about 0.03%) indicates that these sites are very close to each other. The data also suggest the following order of mu-

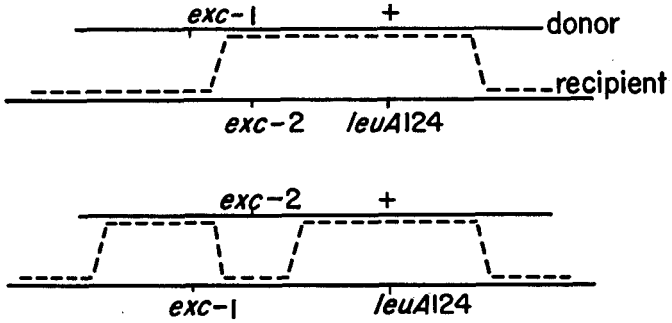

FIGURE 5.—Representation of reciprocal crosses that were performed in an attempt to separate *exc* sites by recombination.

TABLE 5

Analysis of crosses between fluoroleucine-resistant mutants

Recipient		Donor	Number of *leu*+ transductants analyzed	Number of non-excreters
Strain	Derived from cross			
CV238	*leuA*124 × CV186	CV200	7800	0
CV239	*leuA*124 × CV200	CV186	4400	0
CV235	*leuA*124 × CV173	CV177	6480	2
CV236	*leuA*124 × CV177	CV173	9090	0

tant sites: *leu*-500–mutant site of strain CV177–mutant site of strain CV173–*leuA*517.

DISCUSSION

The fluoroleucine-resistant mutants that were studied have the following properties: they have normal growth rates (CALVO, FREUNDLICH and UMBARGER 1969); they excrete leucine but not isoleucine or valine (CALVO, FREUNDLICH and UMBARGER 1969); when grown in a minimal medium, the levels of their leucine forming enzymes are some twelve times higher than the corresponding levels in the wild type (coordinate derepression; BURNS, CALVO, MARGOLIN and UMBARGER 1966); supplementation of the medium with L-leucine results in a 30% reduction in the levels of these enzymes; their mutant sites are located at the extreme left end of the map shown in Figure 1. On the basis of the location of these sites and their effect upon the expression of adjacent genes, they are tentatively considered to be operator constitutive mutations and together with *leu*-500, they define an operator locus, *leuO*. The sites of these eight strains are assigned the following allele designations: *leuO*2001 (CV6), *leuO*2004 (CV112), *leuO*2005 (CV120), *leuO*2006 (CV165), *leuO*2007 (CV173), *leuO*2008 (CV177), *leuO*2009 (CV186), and *leuO*2011 (CV200). Confirmation that these are indeed operator sites will require dominance tests.

Strain *leu*-500, studied extensively by MARGOLIN (1963) and MUKAI and MARGOLIN (1963), has barely detectable levels of enzymes that are specified by *leu* cistrons *A,B,C* and *D*. Multisite mutations in a region of the genome between *trp* and *cysB* (termed *supX*; referred to as *su leu*-500 in earlier papers) restore these enzymes to a level at which the strain can grow slowly in a minimal medium. Thus, *leu*-500 does not seem to be a strong polar mutation of the type described by others (NEWTON 1966; MARTIN *et al.* 1966). MUKAI and MARGOLIN (1963) suggested that *leu*-500 is an alteration in the leucine operator region and that the altered region recognizes and binds a foreign repressor molecule, the latter specified by *supX*. FRIEDMAN and MARGOLIN (1968) subsequently suggested that *supX* codes for a specific catabolite repressor. No other strain with the properties of strain *leu*-500 has been identified among several thousand leucine auxotrophs that have been screened (MARGOLIN and CALVO, unpublished data). The assignment of *leu*-500 and *leuO*2001, *leuO*2004, etc. as alleles of a common locus, *leuO*, is tentative and arbitrary. DEMEREC *et al.* (1966) defined the term

"locus" in a broad sense to include, "nucleotide sequences which themselves may not be transcribed, but which govern the punctuation or regulation of transcription." The nucleotide sequence containing *leuO*2001 is assumed to have a control function. Whether *leu*-500 is located in this sequence or in some other concerned with, for example, punctuation or initiation of transcription or translation, is not known. In some respects, *leu*-500 resembles sites on the genome of *Escherichia coli* that have been described as promoter mutations (MILLER *et al.* 1968).

The sites of these eight strains are all located in the same general region, between *leuO*500 and *leuA*517. Because of the method of isolation (CALVO, FREUNDLICH and UMBARGER 1969), they are all of independent origin, however, there is no strong evidence that they are all unique sites. No distinction can be made between them on the basis of their enzyme levels or on the basis of three-factor crosses (Table 4). In crosses in which the donor and recipient both carried a *leuO* site (Table 5), wild-type colonies were observed in one case at a very low frequency (2/6480). However, it is not certain that these colonies are true recombinants. In similar crosses reported by ROTH *et al.* (1966), two *hisO* sites could not be separated by recombination but were shown to be different by other means. A third *hisO* site could be separated from the other two by recombination. In summary, if these eight sites are unique they must be located very near one another.

How close are these sites to other *leu* sites? *leuA*517 is thought to be a mutation in cistron *A* on the basis of complementation data (MARGOLIN 1963) and because crude extracts lack α-IPM synthetase activity but have normal amounts of β-IPM dehydrogenase activity (*A* and *B* cistron products, respectively). The recombination frequency between *leuA*517 and *leuO*2001, *leuO*2004, etc. is about 0.25% (Table 3). Estimates are available for the relationship between the number of nucleotide base pairs between two markers and the recombination frequency between the same two markers in transduction experiments. YANOFSKY (1963) reported a recombination frequency of 0.003% per base pair for P1 mediated transductions of *trpA* sites in *Escherichia coli*. The histidine operon of *S. typhimurium* was reported to contain 11,000 base pairs (AMES *et al.* 1967) and the recombination frequency between markers at opposite ends of the operon was estimated to be 70–90% (LOPER *et al.* 1964). This leads to an estimate of 0.0065–0.0082% recombination per base pair for P22 mediated transduction. Using these latter estimates, *leuA*517 is about 30 to 38 base pairs removed from *leuO*2001. This is probably a maximum estimate because (1) the estimate of the recombination frequency between markers in the histidine operon did not consider multiple crossovers and (2) the possibility of localized negative interference in the cross between *leuA*517 and *leuO*2001 (HAYES 1964). The above speculations should be considered with caution since several laboratories have reported that the nature of specific mutations can affect recombination frequencies (BALBINDER 1962).

In an extensive analysis of some 1300 leucine auxotrophs (CALVO, unpublished data), only two have sites that lie to the left of *leuA*517. This suggests that the latter is located at the far left end of cistron *A* and that *leuO*2001 is either at the beginning of the *A* cistron or just outside of it. Current experiments should determine whether *leuO* specifies any information for the amino acid sequence of

α-IPM synthetase (*A* cistron product). From the results of three-factor crosses (Table 4) an estimate was made of the relative distances between *leuO*2001, *leuA*517 and *leuO*500 (Figure 4), namely, that the *leuO*500—*leuO*2001 distance is about twice the *leuO*2001—*leuA*517 distance. Thus, *leuO*2001 is at most, 60 to 80 base pairs removed from *leuO*500.

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SUMMARY

The mutant sites of eight leucine operator constitutive mutants are located at one end of a cluster of *leu* cistrons, between *leu*-500 (operator negative phenotype) and *leuA*517. The eight sites are very close to one another (they may be identical) and relatively close to *leuA*517 and *leu*-500 (separation of about 30 and 60 base pairs, respectively).

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