THREONINE, ISOLEUCINE, AND ISOLEUCINE-VALINE MUTANTS OF SALMONELLA TYPHIMURIUM[1]

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THIS paper reports an analysis, by transduction techniques, of the genetics of a series of mutants of *Salmonella typhimurium* that are blocked in the synthesis of the amino acids threonine, leucine, isoleucine, and valine. The results were summarized earlier in the Carnegie Institution of Washington Year Book (DEMEREC, LAHR, MIYAKE, GOLDMAN, BALBINDER, BANIČ, HASHIMOTO, GLANVILLE, and GROSS 1958).

The pathway of biosynthesis of these amino acids has been described in several organisms, and summarized by KNOX and BEHRMAN (1959). The diagram of the pathway shown in Figure 1 indicates the probable points in the synthetic sequence at which the different mutant groups described in this paper are deficient. The approximate positions of the blocks were determined primarily on the basis of growth-requirement and feeding tests, and also by analogy with similar mutants, in other organisms, whose enzymatic properties are known. We have not attempted enzymatic analysis of the mutants studied here; and therefore the precise position of the block in each group is not definite, except in the case of the isoleucine-valine mutants. These have been analyzed biochemically by DR. R. P. WAGNER, whose results are reported in the paper following this one (WAGNER and BERGQUIST 1960).

Table 1 lists the mutants in each group, classified according to their approximate spontaneous mutability. Table 2 shows the growth responses of a representative mutant from each group, and Table 3 gives the results of feeding tests between the groups.

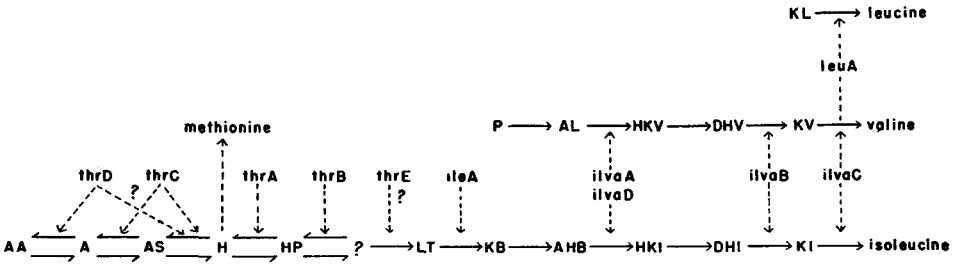

FIGURE 1.—Pathway of biosynthesis of threonine, isoleucine, and valine.

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TABLE 1

Classification of the mutants studied, according to group (locus) and degree of spontaneous mutability

Locus	No. mutants	Mutability		
		Low	Intermediate	High
thrA	14	*thr-2,* 8, 9, 10, 59*	*thr-21, 27, 30, 34, 36, 38, 39, 40, 50*	
thrB	16	*thr-12, 13, 23, 29, 49*	*thr-17, 18, 22, 24, 31, 32, 33, 34, 35, 43*	*thr-28*
thrC	5	*thr-11**		*thr-5, 7, 14, 15*
thrD	1			*thr-16*
thrE	1	*thr-60*		
ileA	12	*ile-3, 6, 11, 13, 14, 16, 18, 23, 24*	*ile-19*	*ile-15, 17*
ilvaA	6	*ilva-8, 12, 14, 19, 22*		*ilva-11*
ilvaB	7	*ilva-2,* 6, 7, 9, 10, 15, 18*		
ilvaC	2	*ilva-13, 16*		
ilvaD	1	*ilva-27*		
leuA	107			
Total	172			

* Indicates high rate of mutation of a suppressor gene, producing slow-growing "revertants".

TABLE 2

Growth responses of mutants

Mutant	L-homoserine	L-threonine	D-threonine	isoleucine	KB	AIIB	AL	valine	DHI	DHV	DHI + DHV	KI	KV	KV + KI	isoleucine + valine	KL
thrD-16	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
thrC-11	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
thrA-10	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
thrB-13	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
thrE-60	—	+	—	+	+	+	—	—	+	—	+	+	—	+	+	—
ileA-14	—	—	±	+	+	+	—	—	+	—	+	+	—	+	+	—
ilvaD-27	—	—	—	—	—	—	—	±	—	±	—	—	±	+	+	—
ilvaA-8	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—
ilvaB-10	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—
ilvaC-13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—
leuA-39	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+

+ = good growth response.
± = slight growth response.

TABLE 3

Feeding relationships

Mutants feeding	*thrD-16*	*thrC-11*	*thrA-10*	*thrB-13*	*thrE-60*	*ileA-14*	*ilvaD-27*	*ilvaA-8*	*ilvaB-10*	*ilvaC-13*	*leuA-39*
thrD-16	—	—	—	—	—	—	—	—	—	—	—
thrC-11	—	—	—	—	—	—	—	—	—	—	—
thrA-10	+	+	—	—	—	—	—	—	—	—	—
thrB-13	—	—	—	—	—	—	—	—	—	—	—
thrE-60	—	—	—	—	—	—	—	—	—	—	—
ileA-14	—	—	—	—	+	—	—	—	—	—	—
ilvaD-27	—	—	—	—	+	+	—	—	—	—	—
ilvaA-8	—	—	—	—	+	+	—	—	—	—	—
ilvaB-10	—	—	—	—	+	+	—	—	—	—	—
ilvaC-13	—	—	—	—	+	+	+	+	+	—	+
leuA-39	—	—	—	—	±	—	+	±	±	—	—
wild type	—	—	—	—	—	—	—	—	—	±	—

+ = good growth response.
± = slight growth response.

MATERIAL AND METHODS

The mutants were from a collection accumulated by several members of this laboratory in work with strains LT-2 and LT-7 of *S. typhimurium* obtained from DR. N. D. ZINDER. Most of them were of spontaneous origin; a few had been isolated from bacteria treated with ultraviolet radiation. Phage stocks were derived from the temperate form (H-1) of PLT-22 (ZINDER and LEDERBERG 1952).

We used DAVIS's minimal medium (LEDERBERG 1950). In transduction experiments it was supplemented with various amino acids, at a concentration of 20 μg/ml. For syntrophism experiments it was enriched with 0.01 percent nutrient broth powder; and in complementation experiments it was unsupplemented.

Transduction was effected by mixing recipient bacteria from a saturated broth culture (2×10^9 cells/ml) with phage, at a multiplicity of infection of five. Adsorption was allowed to proceed at room temperature for five minutes before the cells were plated on selective medium. Plates were incubated for 48 hours at 37°C. When necessary, the genetic constitution of transductant colonies was determined by the replica-plating technique. When a large number of similar mutants was to be tested by transduction, as in the *leu* group, it was done by the spot-testing method. An aliquot of a bacterial culture (about 2×10^8 cells) was spread on a plate containing enriched minimal medium, and drops of different phage suspensions (about 5×10^8 particles) were added at four or more marked spots. Recombination between recipient and donors could be observed on the areas of the spots.

Feeding between strains (syntrophism) was tested by streaking saline suspensions of the two mutants at right angles on enriched minimal agar, and incubating for 48 hours at 37°C.

Growth requirement was determined either by streaking a saline suspension of cells on enriched minimal agar containing the growth factor in a concentration

of 20 μg/ml, or by spreading it on enriched minimal agar and adding crystals or a drop of the growth factor. In the latter procedure, a halo of growth around the spot signified a positive result.

Symbols and abbreviations: The symbols for the kinds of mutants discussed in the paper are as follows: *ara*$^-$, inability to utilize arabinose; *arg*, arginine requirement; *asa*, aspartic acid requirement; *azi-r*, azide resistance; *chl-r*, chloromycetin resistance; *cys*, cystine requirement; *gly*, glycine requirement; *ile*, isoleucine requirement; *ilva*, requirement for isoleucine plus valine; *leu*, leucine requirement; *lys*, lysine requirement; *met*, methionine requirement; *nic*, nicotinic acid requirement; *pan*, pantothenic acid requirement; *phl*, phenylalanine requirement; *pro*, proline requirement; *ser*, requirement for serine or serine-glycine alternative; *str-r*, streptomycin resistance; *thi*, thiamine requirement; *thr*, threonine requirement; *try*, tryptophan requirement.

The complete symbol for an individual mutant marker includes a letter designating its locus and a numeral identifying it as an independently derived mutant. Examples: *serB-80*, *thrA-59*.

Biochemical compounds are represented in Figure 1 and elsewhere by the following abbreviations: A = β-aspartyl phosphate; AA = aspartic acid; AHB = α-aceto-α-hydroxybutyric acid; AL = α-acetolactic acid; AS = aspartic-β-semialdehyde; DHI = α, β-dihydroxy-β-methylvaleric acid; DHV = α, β-dihydroxyisovaleric acid; H = homoserine; HKI = α-keto-β-hydroxy-β-methylvaleric acid; HKV = α-keto-β-hydroxyisovaleric acid; HP = homoserine phosphate; KB = α-ketobutyric acid; KI = α-keto-β-methylvaleric acid (ketoisoleucine); KV = α-ketoisovaleric acid (ketovaline); LT = L-threonine; P = pyruvic acid.

Complementation and abortive transduction: Mutants were divided into groups primarily on the basis of genetic complementation, which was determined by the occurrence of abortive transduction between members of different groups but not within a group (OZEKI 1956; DEMEREC and OZEKI 1959). Abortive transduction gives rise to minute colonies. In stable transductants, which form a large colony, there is permanent recombination between the donor and recipient genomes, but in the progeny of an abortively transduced cell the genetic fragment derived from the donor strain does not multiply and does not recombine with the recipient genome.

Linkage relations and the order of gene loci: Two markers can be simultaneously transduced only if their loci are carried in the same transduction fragment; that is, they must be closely linked. Thus the occurrence of simultaneous transduction is the best criterion of such linkage and was so employed in this work whenever possible. The order of loci within a transducing fragment was determined in two ways:

1. By finding the ratio of the number of joint incorporations of two donor markers to the number of recombinations between them and the recipient markers, which is an indication of the distance between the two loci on a linkage map. Tables 6, 7, 9, 10, and 11 give the results of transduction experiments between one standard marker as donor (or recipient) and a series of other markers.

The ratio of joint to single transductions decreases with increase in the distance between the loci.

2. By application of the three-point test of classical genetics (DEMEREC and HARTMAN 1956). When the double mutant *serB-80 thrA-59* is transduced with phage from the wild type and plated on minimal medium supplemented with serine, which is selective for *thr*$^+$ transductants but not for the *ser* marker, and when the genotypes of the transductants are later determined by replica plating on medium lacking serine, a characteristic proportion of the *thr*$^+$ cells is also found to be *ser*$^+$. If the donor is not the wild type but another *thr* mutant (e.g., *thrB-13*), there is a distortion in the frequency of *ser*$^+$ transductants, depending on the position of *thrB-13* in relation to the *serB-80* and *thrA-59* markers. Table 7 gives the results of these and of reciprocal tests. The possible relative positions of the three markers are shown in Figure 2; the regions where recombination can occur are numbered. Table 4 shows the locations of the crossovers that would be necessary to produce *thr*$^+$ *ser*$^+$ recombinants in the reciprocal crosses, for each of the three possible orders of loci, together with the observed frequencies of *thr*$^+$ *ser*$^+$ transductants. In accordance with these results, Order II can be excluded. We can exclude Order III on the basis of results of two-point tests (Table 5), which show that frequency of recombination between *thrB-13* and *thrA-59* is very much less than that between *thrB-13* and *serB-80*, and thus, that the two *thr* markers are much closer to each other than to *ser*.

From the results of a series of such tests it is possible to construct a linkage map of markers located within one transducing fragment. A comparison of the efficiency of the two methods for determining the order of mutant loci can be made from Tables 6 and 7.

Ghost colonies after transduction: In tests involving transduction of *thrA* mutants with phage from *thrC* or *D* mutants, three sizes of colonies appear on the

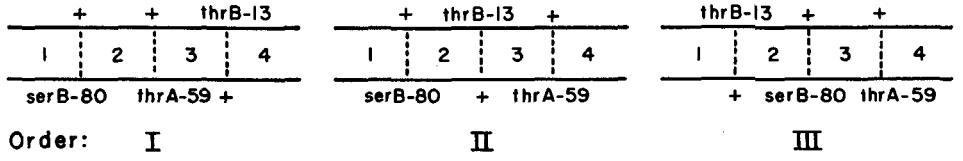

FIGURE 2.—Diagrammatic representation of the three possible orders of the *serB-80, thrA-59,* and *thrB-13* markers. The regions where recombination can occur are numbered.

TABLE 4

Location of the crossovers that would be necessary to produce thr$^+$ ser$^+$ *recombinants in the reciprocal experiments, for each of the three possible orders of loci*

Experiment	Order			Percent observed
	I	II	III	
serB-80 thrA-59 (×) *thrB-13*	1,3	1,2,3,4	2,4	23.2
thrB-13 (×) *serB-80 thrA-59*	3,4	2,3	1,2	96.1
serB-80 thrA-59 (×) wild	1,3 or 1,4	1,4	2,4	7.7

TABLE 5

Frequency of recombination (stable transduction), and presence (+) or absence (—) of complementation (abortive transduction), between threonine mutants. Total number of colonies on three plates in experiments made under standard conditions

	Donor										
		thrA				*thrB*		*thrC*	*thrD*	*thrE*	*serB*	
Recipient	Wild	*-8*	*-9*	*-10*	*-59*	*-12*	*-13*	*-11*	*-16*	*-60*	*-80*	Control
thrA-8	184	0	1	6	15	34	17	38	35	264	146	0
	+	—	—	—	—	+	+	+	+	+	+	
thrA-9	191	0	0	1	4	13	42	27	46	217	206	0
	+	—	—	—	—	+	+	+	+	+	+	
thrA-10	201	0	9	0	3	26	26	29	45	185	177	1
	+	—	—	—	—	+	+	+	+	+	+	
thrA-59	263	4	3	8	0	18	14	45	68	236	269	0
	+	—	—	—	—	+	+	+	+	+	+	
thrB-12	185	6	21	2	8	2	8	28	12	128	129	1
	+	+	+	+	+	—	—	+	+	+	+	
thrB-13	225	23	38	16	6	10	2	66	54	319	204	4
	+	+	+	+	+	—	—	+	+	+	+	
thrC-11	218	46	65	41	35	85	48	1	28	266	211	0
	+	+	+	+	+	+	+	—	+	+	+	
thrD-16	332	210	168	177	89	77	102	58	54	194	186	85
	+	+	+	+	+	+	+	+	—	+	+	
thrE-60	204	171	199	254	142	163	281	254	233	0	181	2
	+	+	+	+	+	+	+	+	+	—	+	
serB-80	241	129	256	194	286	199	204	170	188	225	1	2
	+	+	+	+	+	+	+	+	+	+	—	

plate after 48 hours of incubation: large, minute, and intermediate. Large colonies contain wild-type cells. Minute colonies result from abortive transduction. The intermediate-sized colonies develop from cells of the donor genotype, produced by double transduction of the two markers. They have been called "ghost" colonies because their development is due to feeding by the cells in the background growth of the recipient bacteria. They were first identified in experiments with *try* mutants in 1955 (unpublished observations).

EXPERIMENTAL RESULTS

Leucine mutants: The 107 leucine-requiring mutants analyzed in this study form a single group. They are closely linked genetically, as judged by the low recombination frequencies observed in transduction experiments. From recombination values obtained in reciprocal crosses between different *leu* markers, a linkage map of the *leu* alleles has been constructed, comprising more than 50 sites. Since it is well known that various factors, not yet controllable, may affect frequency of recombination, our present map only indicates the approximate order of the sites within what may be a single locus. All the *leu* markers that we have tested have been found to be closely linked to two loci controlling arabinose

TABLE 6

Frequency of joint transduction of ser *and* thr *markers. Selection was made for* thr$^+$ *on minimal-plus-serine plates; and the transductants (either* thr$^+$ ser *or* thr$^+$ ser$^+$*) were replica plated on minimal medium to identify the two classes. Order:* serB—thrD—C—A—B

Recipient	Donor	Total transductants	No. *ser*$^+$	No. *ser*	Percent *ser*
serB-10	*thrD-16*	320	213	107	33.4
	C-11	325	227	98	30.2
	C-5	346	250	96	27.7
	A-10	280	229	51	18.2
	A-8	387	323	64	16.5
	B-12	167	145	22	13.2
	B-13	328	293	35	11.7
	E-60	256	256	0	0.0
serB-46	*thrD-16*	226	156	70	31.1
	C-11	599	435	164	27.3
	A-10	352	269	83	23.5
	B-12	139	114	25	18.0
	E-60	244	244	0	0.0
serA-11	*thrD-16*	210	210	0	0.0
	C-11	349	349	0	0.0
	B-12	162	162	0	0.0
	A-10	96	96	0	0.0
	E-60	338	338	0	0.0
serB-80 thrA-59	wild	141	27	114	19.2
serB-103 thrC-11	wild	247	71	176	28.7

TABLE 7

Order of threonine loci, determined by three-point tests with serB *and* thr *markers. Selection was made for* thr$^+$ *on minimal-plus-glycine plates (glycine satisfies the requirement in* serB-80 *and* serB-103 *mutants). The transductants (either* thr$^+$ ser *or* thr$^+$ ser$^+$*) were replica plated on minimal medium to identify the two classes. Order:* serB—thrD—C—A—B

Recipient	Donor	Total	Percent *ser*$^+$	Percent *ser*
serB-80 thrA-59	wild	52	7.7	92.3
serB-80 thrA-59	*thrA-10*	58	6.9	93.1
	reciprocal	162	40.7	59.3
serB-80 thrA-59	*thrB-13*	86	23.2	76.8
	reciprocal	103	96.1	3.9
serB-80 thrA-59	*thrC-11*	40	4.7	95.3
	reciprocal	106	37.7	62.3
serB-80 thrA-59	*thrD-16*	68	5.9	94.1
serB-103 thrC-11	wild	130	31.5	68.5
serB-103 thrC-11	*thrA-10*	140	46.4	53.6
	reciprocal	97	94.8	5.2
serB-103 thrC-11	*thrB-13*	66	53.0	47.0
	reciprocal	87	96.5	3.5
serB-103 thrC-11	*thrD-16*	121	15.8	84.2

fermentation (*araA* and *araB*), and so material is now available for making three-point tests to determine exactly the order of the sites. The leucine mutants are being used by DR. PAUL MARGOLIN in studies of the structure of the *leu* region and its response to mutagens (MARGOLIN, VANDERMEULEN-COCITO, and SIMRELL 1959).

Our experiments have shown that *leu* mutants grow on leucine or ketoleucine, but not on ketovaline, their precursor in *Escherichia coli* and probably also in Salmonella (Table 2). In other words, these mutants cannot convert ketovaline to ketoleucine. It has been found that *ilvaC* mutants feed *leu* mutants and presumably accumulate ketoleucine.

In an effort to find out whether any of the *leu* mutants are identical, 52 of them were tested in 572 transduction experiments. In every test recombination was detected, an indication that the mutational change responsible for each one of these 52 mutants had occurred at a different site of the *leu* locus. All 107 mutants investigated are capable of reverting to wild type; therefore none is a multisite mutant.

Threonine mutants: TEAS, HOROWITZ, and FLING (1948) described mutants of *Neurospora crassa* that require for their growth either homoserine or methionine plus threonine. They concluded that both methionine and threonine are derived from homoserine. TEAS (1950) came to the same conclusion in studying mutants of *Bacillus subtilis*.

In *E. coli*, mutants described by COHEN and HIRSCH (1954) have requirements for: (1) L-threonine, (2) L-threonine or L-homoserine, (3) L-threonine plus methionine, (4) L-threonine plus methionine or L-homoserine. The *S. typhimurium* mutants of the present study correspond to the first two of these classes, with the addition of a group growing on either L-threonine or L-isoleucine. Mutants requiring both methionine and threonine have not been detected in Salmonella.

The mutants growing on L-threonine were divided into five groups on the basis of complementation tests. Table 5 records the occurrence of abortive transduction between some of the mutants. Abortive colonies were about ten times more frequent than large colonies. They were clear and distinct in the tests with members of groups *A* and *B* as recipients, less so in the tests of *C*, *D*, and *E* mutants.

The results of growth-requirement tests are summarized in Table 2. Members of groups *C* and *D* grow rapidly on L-threonine and slowly on L-homoserine. Growth on homoserine is greatly stimulated by the addition of isoleucine, but isoleucine alone does not support growth of these mutants. *A*, *B*, and *E* mutants will grow on L-threonine; homoserine does not support their growth. *E* mutants differ from *A* and *B* in being able to utilize L-isoleucine. Neither D-threonine nor D-homoserine is able to support growth of *thr* mutants. Thus *thrA* and *B* mutants are blocked at a later step in the biochemical sequence than *thrC* or *D* mutants; and *thrE* mutants appear to be blocked at a later stage than any of the other *thr* mutants.

The results of syntrophism tests are shown in Table 3. The only feeding ob-

served among the *thr* mutants was by *thrA*, which feeds *C* and *D* but not *B* or *E; thrE* is fed by *ileA* and *ilvaA, B, C,* and *D.*

Two independent lines of evidence indicate that *thrA, B, C,* and *D,* but not *thrE,* are carried in the same donor fragment and, therefore, are closely linked in the bacterial genome.

The yield of prototrophic recombinants in crosses between mutants of *thrA, B, C,* and *D* is always smaller than that in crosses between any of them and wild type (Table 5). The yield of recombinants in crosses between *thrE* and other *thr* mutants approximates that obtained with a wild-type donor, showing that *thrE* is not closely linked to the other *thr* loci.

The four loci *thrA, B, C,* and *D* are closely linked to *serB,* whereas *thrE* is not. Frequencies of joint transduction are shown in Table 6. The results indicate that the order of loci in the transducing fragment is *serB—thrD—thrC—thrA—thrB.* This conclusion was confirmed by three-point tests, made by the method already described, with double mutants carrying both *ser* and *thr* markers (Table 7).

Isoleucine and isoleucine-and-valine mutants: The precursors of valine and leucine differ from those of isoleucine (Figure 1), but the enzymes involved in part of their synthesis are the same, and so it might be expected that a single mutation would result in a double or triple requirement. In fact, mutations affecting the enzyme that controls the final step of synthesis of all three amino acids result in an absolute requirement only for isoleucine and a partial requirement for valine. Presumably, alternative transaminases provide for leucine and partial valine synthesis from their keto acids, as has been shown to occur in *E. coli* (RUDMAN and MEISTER 1953).

All the isoleucine-requiring (*ile*) mutants tested fell into a single group. Complementation tests were difficult to interpret owing to the heavy background growth of all mutants on minimal agar. Growth in the absence of isoleucine may be accounted for by the activity of alternative threonine deaminases (UMBARGER and BROWN 1957). All mutants grow on KB, AHB, DHI, KI, and isoleucine. L-threonine has no effect, but D-threonine supports slow growth. Mutants of this group probably lack the "biosynthetic L-threonine deaminase" (UMBARGER and BROWN 1957). The mutants that require both isoleucine and valine or their precursors for growth fall into four groups on the basis of complementation tests (Table 8) and growth requirements (Table 2). Mutants of group *ilvaC* grow only on the amino acids; the corresponding keto acids support growth of *ilvaA, B,* and *D.* According to the sequence of their biochemical blocks, therefore, the order of the *ilva* mutants is: *ilva(A,D)—ilvaB—ilvaC.*

Some groups can also be distinguished by feeding tests (Table 3). Mutants of the *ilvaC* group feed *ilvaA, B,* and *D,* but there is no feeding between members of the last three groups. All four *ilva* groups feed *ileA* and *thrE* mutants. According to feeding tests the sequence of the loci appears to be *(ileA, thrE)—ilva(A,B, D)—ilvaC.*

The growth requirements of the groups, combined with their feeding properties, allow their relative positions in the biochemical sequence to be determined

TABLE 8

Frequency of recombination (stable tranduction), and presence (+) or absence (—) of complementation (abortive transduction), between ilva *and* ile *mutants. Total number of colonies on three plates*

	Donor												
		ilva									*ile*		*thr*	
Recipient	Wild	*C-13*	*C-16*	*B-10*	*B-9*	*B-7*	*A-8*	*A-14*	*A-19*	*D-27*	*A-14*	*A-18*	*E-60*	Control
ilvaC-13	264	1	4	14	47	59	33	68	104	41	76	109	240	1
	+	—	—	+	+	+	+	+	+	+	+	+	+	
ilvaC-16	199	12	1	67	55	94	76	88	142	74	241	89	189	0
	+	—	—	+	+	+	+	+	+	+	+	+	+	
ilvaB-10	342	66	98	0	39	23	48	51	64	49	59	47	245	2
	+	+	+	—	—	—	+	+	+	+	+	+	+	
ilvaB-9	286	18	61	28	0	3	24	32	41	68	72	39	79	0
	+	+	+	—	—	—	+	+	+	+	+	+	+	
ilvaB-7	241	32	59	26	6	0	56	27	39	55	41	78	268	0
	+	+	+	—	—	—	+	+	+	+	+	+	+	
ilvaA-8	309	89	122	42	84	33	1	12	26	89	57	61	175	4
	+	+	+	+	+	+	—	—	—	+	+	+	+	
ilvaA-14	366	122	109	36	64	28	58	1	26	34	73	89	99	0
	+	+	+	+	+	+	—	—	—	+	+	+	+	
ilvaA-19	287	73	27	22	38	49	10	22	0	18	44	51	143	1
	+	+	+	+	+	+	—	—	—	+	+	+	+	
ilvaD-27	182	128	48	55	62	35	28	5	27	0	38	37	254	3
	+	+	+	+	+	+	+	+	+	—	+	+	+	
ileA-14	332	248	89	77	81	43	42	55	39	41	2	31	171	6
	+	+	+	+	+	+	+	+	+	+	—	—	+	
ileA-18	285	86	108	64	75	96	72	33	64	36	19	3	244	8
	+	+	+	+	+	+	+	+	+	+	—	—	+	
thrE-60	251	194	88	87	127	149	48	94	66	127	143	168	2	2
	+	+	+	+	+	+	+	+	+	+	+	+	—	

—except for *ilvaA* in relation to *ilvaD*. The order is: *thrE—ileA—(ilvaA, ilvaD)—ilvaB—ilvaC*.

Detailed studies of the biochemical aspects of the *ilva* mutants have been made by DR. R. P. WAGNER, and are described in this issue (WAGNER and BERGQUIST 1960).

Table 8 gives the results of two-point tests between *ile* and *ilva* mutants, and shows whether or not abortive transduction occurred. The lower frequencies of transduction to prototrophy between members of the different groups than between them and the wild type indicate that *ileA*, *ilvaA*, *B*, *C*, and *D*, but not *thrE*, are closely linked.

The order of the *ile* and *ilva* loci has been investigated by two-point linkage tests (Tables 9, 10, and 11), determining the frequencies of transductant cells that both lost the requirement of the recipient bacteria and acquired that of the donor (double transduction). The higher the frequency of double, compared to single, transduction of the markers, the closer together the loci are presumed to

TABLE 9

Frequency of joint transduction of ile *and* ilva *markers. Selection was made for* ilva$^+$ *on minimal-plus-isoleucine plates. Transductants (either* ile$^+$ ilva *or* ile$^+$ ilva$^+$*) were replica plated on minimal medium to identify the two classes. Order:* ileA—ilvaA—D—B—C

	Donor							
	ile-14		*ile-19*		*ile-18*		*ile-23*	
Recipient	Total	% Donor	Total	% Donor	Total	% Donor	Total	% Donor
ilvaA-7	606	60.1	129	63.5	99	74.7	400	57.7
ilvaA-8	122	67.2	165	50.9	...	...	...	...
ilvaD-27	374	50.0	224	50.0	131	57.3	693	45.5
ilvaB-9	420	41.2	...	...	...	...	...	...
ilvaB-10	267	41.6	247	42.1	245	39.2	238	41.2
ilvaC-13	151	29.1	194	18.0	...	...	...	...
thrE-60	...	...	241	0.0	...	...	...	...
thrB-13	...	...	304	0.0	...	...	...	...
thrC-11	...	...	191	0.0	...	...	...	...

TABLE 10

Frequency of joint transduction of ilva *markers. Selection was made for the donor type on minimal-plus-KI-plus-KV plates; and transductants were replica plated on minimal medium to identify the two classes. Recipient:* ilvaC-13

Donor	Total	Percent donor type
ilvaA-8	53	34.5
ilvaB-12	88	77.3

TABLE 11

Frequency of joint transduction of ile *and* ilva *markers. Plating on minimal-plus-valine medium. Donor type appeared as small colonies (ghosts) after three days of incubation at 37°C. Donor:* ilvaD-27. *Order of loci:* ileA—ilvaA—D—B—C

Recipient	Total	Percent donor	Recipient	Total	Percent donor
ileA-14	175	24.0	*ilvaC-13*	106	12.5
ilvaA-7	131	30.5	*ilvaC-16*	133	12.0
ilvaA-8	293	42.7	*thrE-60*	179	0
ilvaB-10	82	35.4	*thrB-13*	298	0
ilvaB-9	210	26.2			

be on the linkage map. The absolute frequency of joint transduction varies when different donor strains are used (cf. *ilvaD-27* and *ileA-14*), but all results agree that the order of the loci is *ileA—ilvaA—ilvaD—ilvaB—ilvaC*. The distance between *ileA* and *ilvaA* markers seems to be greater than the distance between other groups of markers in the series.

Although these results are strongly indicative of the order shown above, it cannot be proved conclusively without three-point tests. Attempts to isolate the

double mutant *ile ilva* have failed, and as no other marker is known to be linked to the group, we have not been able to carry out a more rigorous analysis.

DISCUSSION

The most striking result of this study is the finding that gene loci controlling biochemically related functions are clustered together on the chromosome and are arranged within each cluster in such a way that their order corresponds to the sequence of the biochemical reactions they control. The "assembly line" order exemplified by the four threonine and five isoleucine-and-valine loci has previously been demonstrated with respect to four tryptophan loci (DEMEREC and Z. HARTMAN 1956), eight histidine loci (P. E. HARTMAN 1956; HARTMAN, LOPER, and SERMAN 1960), and very likely three proline and three pantothenate loci (MIYAKE and DEMEREC 1960; DEMEREC, LAHR, BALBINDER, MIYAKE, MACK, MACKAY, and ISHIDSU 1959).

The biosynthetic and genetic order of the loci involved in isoleucine synthesis is:

| *thr(D,C)—thrB—thrA* | *thrE* | *ileA—ilva(A,D)—ilvaB—ilvaC* |

These loci are carried in three separate transducing fragments, as indicated above. The significance of the points of breakage is not clear, and the relative positions of the fragments in the total genome of the cell are not yet known. The question is being investigated by the recently developed technique for studying conjugation between *S. typhimurium* and *E. coli* (MIYAKE and DEMEREC 1959), which makes it possible to analyze the order of markers located in different donor fragments.

A linkage map of each of the loci involved in threonine and isoleucine synthesis is shown in Figure 3. The order of the mutational sites is based on the results of two-factor recombination tests and, thus, is only approximate.

A number of *leu*, *ile*, *ilva*, and *thr* mutants were tested for joint transduction (close linkage) with the following markers: *araA-1; arg-3, -4, -5; asa-3; cysA-20, B-18, C-38, D-23, E-2; gly-2; lys-1; metA-15, B-49, C-50, E-47, F-59; nic-1; pan-2; phl-5; proA-39, B-12, C-96, D-102; serA-11, B-80; thi-1; tryD-7; azi-r* (0.5 μg); *chl-r* (10 μg); and *str-r* (100 μg). Joint transduction was detected only

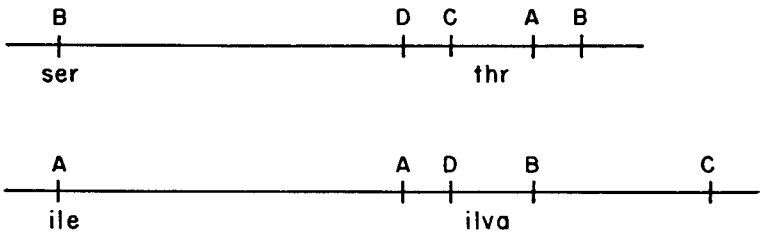

FIGURE 3.—Linkage maps of *ser–thr* and *ile–ilva* fragments. Relative distances between points on the maps correspond approximately to the recombination frequencies. The approximate order of alleles within certain of the loci is as follows: *thrD-7, -5, -11, -15, -14; thrA-10, -8, -9; thrB-17, -12, -13; ileA-19, -11, -14, -3, -6, -2, -16, -18, -17, -15, -13; ilvaA-8, -12, -19, -14, -11; ilvaB-18, -2, -10, -15, -6, -21, -7, -9; ilvaC-13, -16.*

between *leu* and the two arabinose-utilization loci (*araA* and *araB*), and between *serB* and four of the *thr* loci (*thrA*, *B*, *C*, and *D*).

Transduction studies with *E. coli* strains K-12 and B (LENNOX 1955; DEMEREC *et al.* 1958) have shown that phage P1 is able to transfer markers arranged in the following order: *thr—ara—leu—pan*. The results (DEMEREC *et al.* 1958) indicate that this whole section cannot be carried by one phage particle but that some particles carry the *thr–leu* and others the *leu–pan* region. Conjugation studies with Salmonella have revealed that the *thr*, *leu*, and *pan* loci are close together and are arranged in the same order as the functionally similar loci in *E. coli* (DEMEREC *et al.* 1959). Joint transduction of either *thr* and *leu* or *leu* and *pan* markers has not been detected, however, in tests made on a large enough scale to justify the conclusion that any one transducing fragment carried by the Salmonella phage PLT-22 is unable to include two of these three loci.

The difference between the observed behavior in *E. coli* and that in Salmonella might be explained as due to the difference in size of the two transducing phages. Since phage PLT-22 is considerably smaller than phage P1, it seems probable that it can carry less chromosomal material. OZEKI's (1959) results indicate that for any one section of bacterial chromosome transducing fragments carried by phage PLT-22 are the same; that is, the limits of each fragment are predetermined. Thus, it appears probable that the bacterial chromosome has definite weak spots, at which it can break, and that a donor phage has the capacity to transport the fragments located between breaks. In that case it can be supposed that a small phage particle, like that of PLT-22, could carry only a small portion of chromosome—one fragment, resulting from two adjacent breaks—whereas a larger phage, like P1, might carry lager pieces comprising two or more adjoining fragments.

The leucine region, where 107 mutations have been investigated, reveals two interesting characteristics: a lack of sites at which spontaneous changes frequently occur (hot spots), and a lack of structures that would produce multisite changes with the frequency usually observed in Salmonella. As we have mentioned, transduction tests between 52 *leu* mutants showed that every one of the mutations producing them had occurred at a different site of the *leu* locus. If one assumes that the different sites of this gene locus mutate with equal or nearly equal frequency, an estimate can be made of the number of sites present at the *leu* locus. Calculations estimating this number have been made by PROFESSOR HOWARD LEVENE, Department of Mathematical Statistics, Columbia University. His statement regarding "inferences on number of mutational sites" follows:

"Let there be r independently arisen mutations and n mutational sites. If the probability of mutation is the same at each site, then the probability that each of the r mutations is at a different mutational site is $p = \frac{n!}{(n-r)!\, n^r} = \frac{n(n-1) \ldots (n-r+1)}{n^r}$. As an approximation, $\log_e p = -\frac{r(r-1)}{2n}$. (See FELLER, *Probability Theory and Its Applications*, 1st ed. page 29, 2nd ed. page 31, 'birthday problem.')

"Now, to find the minimum number of mutational sites, we find a number of sites, n, for which the probability of all r mutations being different is some small number, α, say $\alpha = .05$ or $\alpha = .01$. We then must solve

$$log_e\alpha = -\frac{r(r-1)}{2n} \text{ for } n,$$

or

$$n = -\frac{r(r-1)}{2\ log_e\alpha}. \tag{1}$$

"It should be noted that the approximate formula

$$p = e^{-\frac{r(r-1)}{2n}} \tag{2}$$

is not valid when p is small (that is, when n is small), and the exact formula should then be used:

$$p = antilog_{10}\ [log_{10}n! - log_{10}(n-r)! - r\ log_{10}n]. \tag{3}$$

The Biometrika Tables, Statistical Tables and Formulas by HALD (Wiley), and volume 1 of PEARSON's tables give $log_{10}n!$ up to $n = 1000$. For $n = 55$ the percentage error in p from formula (2) is about seven percent for $n = 1000$ and 11 percent for $n = 500$, whereas the corresponding error in n from formula (1) is two percent for $p = .217$ and three percent for $p = .05$.

"Table 12 gives n and p (or α) calculated by (3) for n up to 1000 and by (1) for $n > 1000$.

"The important conclusions are: If the number of mutable sites is 230, the probability that all 55 mutations are different is only .001: that is, 230 is the lower 0.1 percent confidence limit of n. Similarly, 335 is the lower one percent confidence limit and 510 the lower five percent confidence limit. The most reasonable number of sites is the 50 percent limit, that is, the number n for which there is a 50-50 chance of all mutants' being different; this value is 2100. Finally, the maximum likelihood estimate of n is ∞ as long as all mutants are different. This last estimate is clearly unreasonable.

TABLE 12

p = probability that all of r = 55 mutants are different when there are n sites of mutation

Exact		Approximate*	
n	p	p	n
200	.00027	.001	230
250	.00162	.01	335
300	.00508	.05	510
400	.0204	.10	650
500	.0457	.25	1070
646	.0938	.50	2100
991	.217	.75	5200
		.90	14000
		.95	26000
		.99	150000

* For $n < 1000$, n obtained by graphic interpolation from exact table; for $n > 1000$, n obtained by formula (1).

"Note that if the mutation rate differs at different sites, all the above values are too small."

SUMMARY

The 107 leucine-requiring mutants studied form a single group, and the *leu* locus is closely linked to the *araA* and *araB* loci. Intercrosses involving 52 of the mutants showed that all the mutations had occurred at different sites of the locus. On the assumption that the probability of mutation is the same at each site, calculations made by PROFESSOR HOWARD LEVENE estimate the number of possible mutational sites as about 300 at the one percent confidence limit, about 500 at the five percent confidence limit, and about 2000 at the 50 percent confidence limit. These values are too small if the mutation rate differs at different sites.

Mutants requiring L-threonine for growth (*thr* mutants) fall into five groups, distinguishable by complementation. All but two of the groups (*C* and *D*) can also be distinguished by their growth requirements and cross-feeding properties. From these properties the order of the groups with regard to their biosynthetic blocks has been determined. The relative order is: *thr(C,D)—thrA—thrB—thrE*. Four of the loci (*thrA, B, C, D*) are carried in the same transducing fragment as *serB*. The order on a linkage map is *serB—thrD—thrC—thrA—thrB*. Thus the genetic sequence of the loci corresponds to the sequence of the biochemical steps they control. The fifth locus, *thrE*, is not linked to the others.

On the basis of complementation, mutants blocked in the synthesis of isoleucine (*ile*) and isoleucine-valine (*ilva*) have been divided into five groups, representing five gene loci. All five groups can be distinguished by their growth requirements or feeding effects, and from these properties the position of the block in the biochemical sequence in each group of mutants has been inferred. Only the relative positions of *ilvaA* and *D* cannot be discovered by this method. For the rest, the biochemical sequence corresponds to the genetic sequence of the loci: *ileA—ilva(A,D)—ilvaB—ilvaC*.

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