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Valine-resistant mutants of *Escherichia coli* K-12

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INTRODUCTION

One unusual feature of K-12 strains of *Escherichia coli* is their sensitivity to the amino-acid valine. The growth of wild-type K-12 can be inhibited by as little as 0·4 μg./ml. valine, while the growth of *E. coli* strain B remains unaffected by 10,000 μg./ml. Mutants resistant to valine occur spontaneously with a frequency of about 5×10^{-7}. One such valine-resistant mutant was shown to be closely linked to the *leu* locus in K-12 by conjugation experiments (Manten & Rowley, 1953). Rowley (1953) demonstrated that inhibition by valine could be prevented by leucine and came to the conclusion that the close proximity of *leu* and *val-r* was not fortuitous. Inhibition by norleucine can be prevented by methionine (Rowley, 1953), but conjugation experiments failed to demonstrate linkage between the loci for resistance to norleucine and the ability to synthesise methionine (Clowes & Rowley, 1954).

In the work to be described, a large number of spontaneous valine-resistant mutants were isolated and their genetic location determined by transduction with the temperate phage P1 and by conjugation with Hfr strains.

MATERIALS AND METHODS

Media

(*a*) Difco agar for the preparation and assay of P1 lysates:
Oxoid tryptone, 10 g.; NaCl, 8 g.; glucose, 1·0 g.; Difco Bacto agar, 10 g.; distilled water, 1 litre.

(*b*) Soft agar: as above, with agar concentration reduced to 0·6%.

(*c*) Phage buffer:
Na_2HPO_4, 10·5 g.; KH_2PO_4, 4·5 g.; NaCl, 7·5 g.; ·01 M $MgSO_4$, 15·0 ml.; 0·01 M $CaCl_2$, 15·0 ml.; 1% gelatin, 1·5 ml.; water, 1·47 litres.

(*d*) Buffer:
KH_2PO_4, 3·0 g.; Na_2HPO_4, 7·0 g.; NaCl, 4·0 g.; $MgSO_4 7H_2O$, 0·2 g.; water, 1 litre.

(*e*) Minimal medium:
NH_4Cl, 20 g.; NH_4NO_3, 4·0 g.; Na_2SO_4, 8·0 g.; K_2HPO_4, 12·0 g.; KH_2PO_4, 4·0 g. $MgSO_4 7H_2O$, 0·4 g.; water, 1 litre. Solidified with 3 volumes of 2% Davis agar, and supplemented with 0·2% glucose.

(*f*) Nutrient broth:

Oxoid No. 2 nutrient broth, 25 g.; water, 1 litre.

(*g*) Nutrient agar:

Oxoid No. 2 nutrient broth, 25 g.; Davis agar, 12·5 g.; water, 1 litre.

Amino-acid supplements at 20 μg./ml. unless stated otherwise.

The preparation of phage lysates

The temperate phage P1*kc* (Lennox, 1955) designated hereafter P1 was used for transduction. Transducing phage lysates were prepared by confluent lysis of donor bacteria on Difco agar plates supplemented with 0·5% yeast extract and 1×10^{-3} M $CaCl_2$. About 1×10^8 donor bacteria grown in nutrient broth and 1×10^6 P1 particles were mixed together and plated in 3 ml. of soft agar overlay. After 16 hours' incubation, the surface layer was collected and shaken with 2 ml. of phage buffer to elute the phage, then centrifuged at low speed. The supernatant was treated with chloroform and centrifuged again at low speed. Lysates prepared in this way were sterile, had titres of about 5×10^{10} phages/ml. and remained stable for several months at 4°. *Shigella dysenteriae* strain Sh was used as the standard indicator for P1 assay.

Transduction procedure

Recipient bacteria were grown in nutrient broth at 37° to a titre of 2×10^8 bacteria/ml., centrifuged and taken up in one-fifth the volume of buffer supplemented with 1×10^{-3} M $CaCl_2$. Equal parts, usually 1·0 ml. of this suspension and a P1 lysate from the donor bacteria were mixed and kept at room temperature (*c.* 20°) for 90 min. This adsorption mixture was centrifuged and the pellet resuspended in buffer supplemented with 0·5% sodium citrate. After appropriate dilution, aliquots of 0·1 ml. were spread on selective solid media for the detection of stable transduced clones. In a typical experiment about 80% of the P1 is adsorbed and about 50% of the recipient cells survive infection at a multiplicity of approximately 10. Low temperature (20°), which blocks the lytic cycle (Bertani & Nice, 1954), and the addition of 0·5% sodium citrate, which prevents reinfection on the plates, did not markedly affect the frequency of transduction but ensure that the majority of transduced clones are P1 sensitive. Under these conditions the frequency of transduction per P1 particle varies depending on the marker selected, but is usually of the order of 1×10^{-5} (Lennox, 1955).

Conjugation procedures

(a) *Hfr crosses* (Wollman, Jacob & Hayes, 1956). The Hfr donor and F^- recipient parent cultures were grown in nutrient broth to mid-log phase, approximately 1×10^8 bacteria/ml., and mixed in the ratio 1 Hfr:10 F^- cells. Conjugation was allowed to proceed at 37° in a waterbath and where necessary the mating was interrupted by shaking on a Microid flask shaker. Suitable dilutions of the zygotes were spread on selective minimal medium plates and the recombinants scored after 48 hours' incubation at 37°.

(b) *Vhf crosses* (Taylor & Adelberg, 1960). The Vhf (very high frequency) donor and F^- recipient parent cultures were grown in nutrient broth to mid-log phase, approximately 1×10^8 bacteria/ml., and mixed in the ratio 1 Vhf: 10 F^- cells. Conjugation was allowed to proceed at 37° in a waterbath. To interrupt the mating at suitable time intervals 0·1 ml. samples of the mixture were removed and added to 0·5 ml. of a suspension of phage T6 (5×10^{10} particles/ml.) previously warmed to 37° and shaken vigorously by hand. Twenty minutes at 37° were allowed for adsorption of T6 after which the zygotes were diluted and aliquots spread on selective minimal medium plates, and the recombinants scored after 48 hours' incubation at 37°.

RESULTS

Isolation of val-r *mutants*

One hundred small tubes each containing 1 ml. of nutrient broth were inoculated with approximately 1×10^3 cells of a *thr leu thi* strain of *E. coli* K-12. The tubes were incubated at 37° for 18 hours without aeration and then the cultures were centrifuged, washed once, and resuspended in buffer. Aliquots of 0·1 ml. were plated from each tube on to minimal medium supplemented with threonine, leucine and thiamin and 40 μg./ml. valine. After 48 hours' incubation 62 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 62 valine-resistant mutants (*val-r1, 2, . . .* etc.) of independent spontaneous origin were obtained.

Joint transduction of valine resistance and other markers

Since Manten & Rowley (1953) had demonstrated linkage between valine resistance and the *leu* locus by conjugation, attention was first directed to the *thr-leu* region. In preliminary experiments a P1 transducing lysate was prepared on a prototrophic, azide resistant (*azi-r*) valine resistant (*val-r*) donor which probably carries the same valine resistance marker as that used by Manten and Rowley. This lysate was used to transduce a *thr . leu . thi val-s, azi-s* recipient, and selection made for each of the characters in turn and some combinations, and the joint transduction of the other markers scored by streaking or replicating on to suitable supplemented media. Table 1 shows the results of these experiments which are in good agreement with the similar data of Lennox (1955). They indicate that *leu* lies between *thr* and *azi-r*, and is more closely linked to *azi-r* than to *thr*. The absence of joint transduction between the markers *thr* and *azi-r* indicates that the distance between these markers is greater than that part of the bacterial genome normally carried by a transducing phage particle.

The fall in the joint transduction frequency of *val-r* and the selected markers from 96·5 when selection is made for leu^+ to 52·0 when selection is made for thr^+leu^+ confirms the close linkage of leu^+ and *val-r* and indicates that it lies between leu^+ and *azi-r*.

Table 1. *Joint transduction frequencies in the* thr-leu *region of* E. coli *K*-12

Selected marker	Unselected markers				
	thr+	*leu*+	*val-r*	*azi-r*	*thr*+*leu*+
thr+	—	6·6	4·2	0	—
leu+	4·2	—	96·5	56·0	—
val-r	3·2	94·0	—	54·0	3·0
azi-r	0	55·0	63·0	—	—
thr+*leu*+	—	—	52·0	—	—

The joint transduction frequency is expressed as that percentage of the number of transduced clones carrying the selected marker that also carry the non-selected marker.

A P1 lysate was prepared on a prototrophic *val-s* donor and used to transduce each of the *thr leu thi val-r* mutants in turn. In the first series of experiments selection was made for *thr*+*leu*+ on minimal medium supplemented with thiamin. The transduced clones were scored for valine sensitivity by streaking or replicating on to medium supplemented with 40 μg./ml. valine. From Table 2, which summarizes the results of these experiments, it is evident that the *val-r* mutants can be divided into three classes. One, comprising 26 mutants, in which *val-s* and *thr*+*leu*+ are not jointly transduced; another, comprising 26 mutants, in which the joint transduction frequency of *val-s* and *thr*+*leu*+ is approximately 45% (groups B and C); and a third consisting of 1 mutant only, *val-r-57*, in which the joint transduction frequency is 30%.

Table 2. *Joint transduction of* val-s *and* thr+leu+ *from a* val-s *prototroph to* thr.leu.thi val-r *mutants*

Thr.leu.thi recipient mutant	Group	Joint transduction frequency %
val-r-1, -2, -4, -6, -7, -8, -9, -10, -12, -23, -32, -34, -36, -38, -47, -48, -49, -50, -51, -52, -53, -54, -55, -56, -61, -62		0
val-r-13, -33, -39, -42, -59	B	Range 40–54; mean 48·5
val-r-3, -5, -11, -14, -15, -20, -21, -22, -24, -25, -27, -30, -31, -35, -37, -43, -44, -45, -46, -58, -60	C	Range 40–54; mean 44·6
val-r-57	D	30·0

In the second series of experiments, selection was made for *leu*+ transduced clones on minimal medium supplemented with threonine and thiamin. As before, the colonies were scored for valine sensitivity by streaking or replicating on to medium supplemented with 40 μg./ml. valine. The results in Table 3 show that the same group of *val-r* mutants which could not be jointly transduced to *val-s* and *thr*+*leu*+ cannot be jointly transduced to *val-s* and *leu*+. It is clear that the class of mutants which gave a joint transduction frequency for *val-s* and *thr*+*leu*+ of 45% can now

be divided into two. One group (B) shows only 3% joint transduction of *val-s* and *leu*$^+$, while the other (C) is about 86%. *Val-r-57* is clearly differentiated from the others, yielding 19·0% joint transduction of *val-s* and *leu*$^+$.

Table 3. *Joint transduction of* val-s *and* leu$^+$ *from a* val-s *prototroph to* thr.leu.thi val-r *mutants*

Thr.leu.thi recipient	Group	Joint transduction frequency %
val-r-1, -2, -4, -6, -7, -8, -9, -10, -12, -23, -32, -34, -36, -38, -47, -48, -49, -50, -51, -52, -53, -54, -55, -56, -61, -62		0
val-r-13, -33, -39, -42, -59	B	Range 2–5; mean 3·0
val-r-3, -5, -11, -14, -15, -20, -21, -22, -24, -25, -27, -30, -31, -35, -37, -43, -44, -45, -46, -58, -60	C	Range 82–97; mean 86·6
val-r-57	D	19·0

The third series of experiments involved selection for *thr*$^+$ transduced clones on minimal medium supplemented with leucine and thiamin, from *thr leu thi val-s* mutants treated with P1 lysates of a prototrophic *val-s* donor followed by scoring for valine sensitivity on minimal medium supplemented with leucine, thiamin and 40 μg./ml. valine. No threonine independent *val-s* colonies were detected. Rowley (1953) reported that valine sensitivity could be antagonized by either leucine or *iso*leucine and Manten & Rowley (1953) showed that a *leu* mutant of *E. coli* K-12 was phenotypically valine resistant when tested on media supplemented with leucine and valine, but behaved as if it were valine sensitive in conjugation experiments. It was concluded therefore that the presence of leucine in the medium made the scoring of valine sensitivity impossible, although it had not prevented the successful isolation of *val-r* mutants from a *thr leu thi val-s* strain. To overcome this difficulty, leucine independent derivatives were obtained from each of the *thr leu thi val-r* mutants by P1 transduction from an appropriate donor, and the isolation of P1 sensitive *thr leu*$^+$ *thi val-r* colonies. These were treated with the same P1 lysate as in the previous experiments, and selection for *thr*$^+$ was exercised on media supplemented with thiamin. The frequency of valine sensitivity among the transduced clones was scored as before, and a summary of the results is presented in Table 4. It is clear that the mutants *val-r-1, -2, -4, -6, -7, -8, -9, -10,*

Table 4. *Joint transduction of* val-s *and* thr$^+$ *from a* val-s *prototroph into* thr.leu$^+$.thi val-r *mutants*

Thr.leu$^+$*.thi* recipient	Group	Joint transduction frequency %
val-r-1, -2, -4, -6, -7, -8, -9, -10, -12, -23, -32, -34, -36, -38, -47, -48, -49, -50, -51, -52, -53, -54, -55, -56, -61, -62		0
val-r-13, -33, -39, -42, -59	B	Range 89–99; mean 95
val-r-3, -5, -11, -14, -15, -20, -21, -22, -24, -25, -27, -30, -31, -35, -37, -43, -44, -45, -46, -58, -60	C	Range 0·5–5; mean 1·0
val-r-57	D	70·0

-12, *-23*, *-32*, *-34*, *-36*, *-38*, *-47*, *-48*, *-49*, *-50*, *-51*, *-52*, *-53*, *-54*, *-55*, *-56*, *-61* and *-62* do not lie in the *thr-leu* region. The mutants in group C are closely linked to *leu*$^+$ and group B mutants are closely linked to *thr*$^+$.

Ozeki (1959) working with *Salmonella* and phage PLT-22 obtained evidence which indicates that the chromosomes of the donor bacteria are broken up into fragments of predeterminate composition prior to transduction. If this occurred in the *E. coli* phage P1 system, then clearly any fragment which bore the markers *leu*$^+$ could be expected to carry also the markers *thr*$^+$ and *azi-r* linked to *leu*$^+$, and should be able to transduce *thr*$^+$ and *azi-r* jointly. The absence of such joint transductions (see Table 1) indicates that the fragments involved in P1 transduction are not of predeterminate composition. If, on the other hand, the chromosome of *E. coli* K-12 is broken at random prior to transduction and P1 can transduce any of the resulting fragments up to a limiting size determined presumably by the protein head membrane of the P1 particle, the absence of *thr*$^+$ *azi-r* joint transductions can be understood. It is now possible to explain the drop from 86% to 45% in the joint transduction frequency of valine sensitivity and the selected markers *leu*$^+$ and *thr*$^+$*leu*$^+$ when *val-r* C mutants are transduced by a P1 lysate of a prototrophic *val-s* donor. If *val-r* C lies on the opposite side of *leu*$^+$ to the marker *thr*$^+$, then when selection is made for *leu*$^+$ transduction the joint transduction frequency of *leu*$^+$ and *val-s* is a measure of the linkage between them. When selection is made for *thr*$^+$ in addition to *leu*$^+$, only a fraction of the fragments that carry *leu*$^+$ will also bear *thr*$^+$ and among this fraction the number that also bear *val-s* will be less than among those that carry *leu*$^+$ alone, because the distance between *thr*$^+$ and *leu*$^+$ is large relative to the maximum size of a fragment participating in a transduction. Consequently the number of *thr*$^+$*leu*$^+$ *val-s* transductions should be less than the number of *leu*$^+$ *val-s* transductions. Similarly *val-r- B* mutants show a drop from 95% to 48% in the joint transduction frequency of *val-s* when selection is switched from *thr*$^+$ to *thr*$^+$*leu*$^+$ and can therefore be mapped close to *thr*$^+$ but on the opposite side to *leu*$^+$. The mutant *val-r-D57* most probably lies between *thr*$^+$ and *leu*$^+$, giving transduction frequencies with these markers of 70% and 20% respectively. The low figure of 30% joint transduction frequency for this marker when the selection is for *thr*$^+$*leu*$^+$ is most likely due to double exchanges between the fragment carrying *thr*$^+$*val-s leu*$^+$ and the *thr val-r-57 leu* recipient chromosome, which result in integration of *thr*$^+$ and *leu*$^+$ and the elimination of *val-s*.

Crosses between Hfr val-s *and F*$^-$ val-r *mutants*

Conjugation experiments were carried out between HfrH *met val-s* and each of the F$^-$ *thr leu thi val-r* mutants following the procedure detailed in the section on Materials and Methods. Mating was allowed to proceed at 37° for 25 min., after which time the zygotes were diluted and plated on minimal medium supplemented with thiamin for the selection of *thr*$^+$*leu*$^+$ recombinants. After 48 hours' incubation the recombinants were scored by streaking or replica plating for valine sensitivity. It was found that when the F$^-$ recipient was *val-r-1*, *-2*, *-4*, *-6*, *-7*, *-8*, *-9*, *-10*, *-12*, *-23*, *-32*, *-34*, *-36*, *-38*, *-47*, *-48*, *-49*, *-50*, *-51*, *-52*, *-53*, *-54*, *-55*, *-56*, *-61* and *-62*, no

val-s thr$^+$*leu*$^+$ recombinants were produced. This means that none of these mutants is located in that part of the Hfr H chromosome transferred during the first 25 min. of conjugation that is, in the region *thr leu lac gal* (Wollman, Jacob & Hayes, 1956). However, when the F$^-$ recipient was *val-r-B*, *val-r-C* or *val-r-D*, most of the *thr*$^+$*leu*$^+$ recombinants observed were *val-s*.

Crosses between Vhf val-r *and F*$^-$ val-s

Each of the *val-r* mutations *val-r-1*, *-2*, *-4*, *-6*, *-7*, *-8*, *-9*, *-10*, *-12*, *-23*, *-32*, *-34*, *-36*, *-38*, *-47*, *-48*, *-49*, *-50*, *-51*, *-52*, *-53*, *-54*, *-55*, *-56*, *-61* and *-62* was transferred to Vhf strains AB 311, AB 312 and AB 313 (Taylor & Adelberg, 1960). Conjugation experiments were then carried out between each of these Vhf *val-r* donors and an F$^-$ recipient *try*, *his*, *pro T6-r*, *val-s*, and the kinetics of transfer of *val-r* followed. The crosses were performed following the procedure described in the section on Materials and Methods, using a high titre stock of phage T6 to stop chromosome transfer and kill the T6-s Vhf parent. Appropriate dilutions of the zygotes were plated onto minimal medium supplemented with proline, histidine, tryptophan and 40 μg./ml. valine, and the number of *val-r* recombinants scored after 48 hours' incubation. Aliquots of the zygotes were plated onto minimal medium supplemented with proline and tryptophan from crosses involving Vhf AB 311 and Vhf AB 313, and onto minimal medium supplemented with histidine and tryptophan from crosses involving Vhf AB 312. The number of recombinants on these plates is a measure of the transfer of *his*$^+$ from Vhf AB 311 and Vhf AB 313, and *pro*$^+$ transfer from Vhf AB 312, and also provides an adequate control on the kinetics of *val-r* transfer.

Table 5. *The times, in minutes, required for the transfer of* val-r *from Vhf* val-r *donors to an F*$^-$ val-s *recipient*

	Group	Vhf AB313	Vhf AB312	Vhf AB311
val-r-4, *-6*, *-7*, *-8*, *-9*, *-61*	A	> 80	38–40	> 60
val-r-10, *-12*, *-23*, *-34*, *-48*, *-49*, *-50*, *-51*, *-56*	E	9–10	33–34	> 60
val-r-1, *-2*, *-32*, *-36*, *-38*, *-47*, *-52*, *-53*, *-54*, *-55*, *-62*	F	13–14	33–34	> 60

The times at which *val-r* begins to occur among the recombinants are given in Table 5, from which it is clear that the *val-r* mutants listed fall primarily into two groups. One, comprising *val-r-4*, *-6*, *-7*, *-8*, *-9*, and *-61*, which is not transferred by Vhf AB 313, and the remainder which can be transferred by Vhf AB 313. The latter may be further sub-divided on the basis of the times of entry of *val-r* from Vhf AB 313 into two groups, one of which is transferred after about 9 min. (*val-r-10*, *-12*, *-23*, *-34*, *-48*, *-49*, *-50*, *-51* and *-56*), and the other, which is transferred after about 14 min. (*val-r-1*, *-2*, *-32*, *-36*, *-38*, *-47*, *-52*, *-53*, *-54*, *-55* and *-62*). But this second subdivision is not reflected in the times of transfer from Vhf AB 312. The reason for this is not known.

Joint transduction of val-r *and* ilva$^+$ *by P1*

Comparison of the circular chromosome map of *E. coli* K-12 (Jacob & Wollman, 1958) and the location of the *val-r* mutants described in the previous section revealed that *ilva* had been mapped in the same region. Recently isolated *iso*leucine (*ile*) and *iso*leucine plus valine (*ilva*) requiring mutants were treated with P1 lysates from the *val-r* strains and plated on minimal medium to select protrophic transduced clones. After incubation the resulting colonies were scored for valine resistance. It was found that three of the markers, *val-r-1*, *-2* and *-12*, were very closely linked to *ile* and *ilva*, i.e. all of the transduced prototrophs were valine resistant. We may therefore infer that our *ile* and *ilva* mutants are linked together presumably in the same manner as the analogous mutants studied by Glanville & Demerec (1960) in *Salmonella*. One of the *ile* and one of the *ilva* mutants were mapped by kinetics of transfer experiments with Vhf AB312 and Vhf AB313, and both gave the same times for transfer as did *val-r-1*.

A recently isolated mutant with growth requirements for methionine, *iso*leucine and valine (*met*.*ilva*) was found to carry a mutation extending over several cistrons. A P1 lysate from a prototrophic strain could transduce *met*$^+$ into the mutant, making it *met*$^+$*ilva*, or could transduce *ilva*$^+$, making it *met*.*ilva*$^+$, but in a single transduction no *met*$^+$*ilva*$^+$ colonies were observed. Either of the two types *met*$^+$*ilva* or *met*.*ilva*$^+$ obtained as a result of the first transduction could be made *met*$^+$*ilva*$^+$ as a result of a second transduction. Thus it appears that the mutation in this strain extends over a region larger than the fragment normally transduced by a P1 lysate. This extensive mutation was mapped by kinetics of transfer experiments, using Vhf AB313 and Vhf AB312 as donors and the *met*.*ilva* F$^-$ mutant as recipient. No prototrophic recombinants were recovered from the cross involving Vhf AB313, but they began to appear after 38 min. in the cross with Vhf AB312. This gave reason to suppose that the *val-r* mutants which behaved in crosses in a like manner to the *met*.*ilva* mutant might be linked to it close enough for the detection of joint transductions. P1 lysates from each of these *val-r* mutants, *val-r-4*, *-6*, *-7*, *-8*, *-9* and *-61* were prepared and used in transduction experiments with *met*.*ilva*. No linked transduction of *val-r* and either *met* or *ilva* was observed.

Levels of valine resistance

Minimal agar containing threonine, leucine and thiamin was supplemented with valine to give final concentrations from 20 to 10,000 μg./ml. Suspensions, in buffer, of each of the *val-r* mutants were streaked on to the surface to find the level of valine to which each was resistant. The plates were examined after 24 and 48 hours' incubation at 37° and the results after 48 hours are presented in Table 6. For comparison, the results obtained with *E. coli B.* and *Shigella dysenteriae* (*Sh*) are also shown. It is clear that none of the *val-r* mutants show the same high level of resistance characteristic of *E. coli* B. Two of the *val-r* groups, *val-r-B* and *val-r-D*, are resistant up to 80 μg./ml. *Val-r-C* mutants show an intermediate level of resistance, 1000 μg./ml., while the remainder are resistant up to 5000 μg./ml. In general, mutants which were assigned to a particular group by the genetic tests

described earlier showed the same level of resistance. There were, however, some exceptions. The mutants *val-r-C3*, *-C11* and *val-r-A7*, *-A23* showed a lower level of resistance than did the other members of the group to which on genetic criteria they belong.

Table 6. *Levels of valine resistance among mutants of* E. coli *K*-12

	Valine concentration in μg./ml.					
	80	100	1000	2000	5000	10,000
val-r-B	+	−	−	−	−	−
val-r-D	+	−	−	−	−	−
val-r-C	+	+	+	−	−	−
val-r-A	+	+	+	+	+	−
val-r-E	+	+	+	+	+	−
val-r-F	+	+	+	+	+	−
E. coli B	+	+	+	+	+	+
Shigella	+	+	+	+	+	±
E. coli K-12 *val-s*	−	−	−	−	−	−

(+) indicates growth; (−) indicates no growth; after 48 hours' incubation.

Valine antagonists

Reference was made earlier to the fact that Rowley (1953) reported that either leucine or *iso*leucine would prevent inhibition by valine. It was discovered early in this work during the isolation of *val-r* mutants from a *thr . leu . thi val-s* strain that the presence of 20 μg./ml. of leucine in the medium did not prevent inhibition of the background of *val-s* cells, and hence the recovery of *val-r* colonies. Since so little as 0·05 μg./ml. *iso*leucine is sufficient to permit the growth of a *val-s* strain otherwise inhibited by 1·0 μg./ml. valine, the presence of quite small amounts of contaminating *iso*leucine or other antagonist in commercial samples of leucine may lead to the conclusion that leucine itself will antagonize inhibition by valine.

A series of plates were prepared containing 0, 1, 5, 15, 45 and 90 μg./ml. valine, and to each series of plates increasing concentrations of *iso*leucine were added. A *val-s* strain was streaked onto each plate to determine what concentration of *iso*-leucine was sufficient to antagonize the inhibition caused by each of the levels of valine. Table 7, which summarizes these results shows that over the range tested *iso*leucine antagonism of valine inhibition is competitive. It was found that the presence of 20 μg./ml. of leucine in the medium made no difference to these results. Essentially the same results were also obtained when a leucine-requiring *val-s* strain was used and 20 μg./ml. leucine was added to the plates.

The quantitative nature of the antagonism by *iso*leucine of valine inhibition was confirmed by experiments in which plates containing 5 μg./ml. valine were spread with a lawn of *val-s* cells and one drop of increasing concentrations of *iso*leucine added to small holes bored in the centre of each plate. After incubation, the zones of growth around each well of *iso*leucine were measured. It was found that the size of the zone was directly proportional to the logarithm of the concentration.

In addition, experiments similar to those just described with *iso*leucine were carried out using precursors of *iso*leucine and valine. These experiments showed that α,β-dihydroxy-β-methyl-valeric acid (DHI) and α-aceto-α-hydroxy-butyric acid (AHB) will antagonize inhibition by valine while α,β-dihydroxy-*iso*valeric acid

Table 7. Iso*leucine-valine antagonism*

Antagonism by *iso*leucine μg./ml.	Inhibition caused by valine μg./ml.					
	0	1·0	5·0	15·0	45·0	90·0
0	+	−	−	−	−	−
0·05	+	+	−	−	−	−
0·15	+	+	+	−	−	−
0·5	+	+	+	+	−	−
1·5	+	+	+	+	+	−
3·0	+	+	+	+	+	+

(DHV), α-keto *iso*valeric acid (KV) and α-aceto-lactic acid (AL) will not. α-keto-β-methylvaleric acid (KI) was not available, and so could not be tested. The effective antagonists, DHI and AHB are intermediates in *iso*leucine synthesis, while those compounds not effective, AL, DHV and KV, are the immediate precursors of valine (Wagner & Bergquist, 1960).

Syntrophism

During the course of this work it has been frequently observed that on plates containing valine the *val-r* colonies were surrounded by a halo of growth of the *val-s* cells which formed the background lawn. This halo was not always present, and when it did occur it was usually on old plates. The most probable explanation appears to be that the *val-r* cells were accumulating and excreting in the vicinity of the colony something which antagonized inhibition of the sensitive cells by valine. Attempts to test this possibility and to identify the antagonist excreted were made.

Supernatants from minimal medium grown cultures of the *val-r* mutants were added to holes bored in plates containing 5 μg./ml. valine and which had been previously seeded with *val-s* cells. After incubation the plates were examined for zones of growth around the wells. It was found that with one exception, *val-r-9*, all the supernatants contained something which was able to antagonize the inhibition caused by 5 μg./ml. valine. Neither a supernatant from a minimal medium grown culture of the *val-s* strain, nor minimal medium itself produced a zone of growth of *val-s* cells. The results in the previous section showed that *iso*leucine, DHI and AHB were effective valine antagonists; and Glanville & Demerec (1960) showed that *Salmonella* mutants *ilva C* would grow on *iso*leucine plus valine, but not on DHI or AHB. So that if the supernatants from the growth of the *val-r* mutants in minimal medium contained the valine antagonist *iso*leucine, they should be able to support the growth of *ilva C* in the presence of valine. This was tested by

adding the supernatant to holes bored in minimal medium plates containing 20 μg./ml. valine and previously seeded with the *Salmonella* mutant *ilva C-16*. After incubation, the plates were examined for zones of growth around the wells. With a few exceptions, all of the supernatants were able to support the growth of *ilva C-16*. No *iso*leucine could be detected in this way in the supernatants from *val-r-6*, *-7*, *-15*, *-22*, *-24*, *-44*, *-46*, *-50*, *-54* or *-60*.

DISCUSSION

When any collection of mutants of like phenotype is studied it is pertinent to ask whether they are genetically different, or represent re-isolations of the same mutation. Among the mutants reported on here, six groups can be distinguished. Three, *val-r-B*, *val-r-C* and *val-r-D*, by their different linkage relationships to the markers *thr* and *leu*, revealed by transduction; two more, *val-r-A* and *val-r-E*, and possibly a third, *val-r-F*, by their different times of entry in interrupted mating experiments. It is not possible to say with assurance that all of the mutants in any one group are genetically distinct because it is not possible to select for *val-s* in a cross between two *val-r* mutants.

It was shown that among the *val-r* mutants not linked to either *thr* or *leu*, one group, *val-r-A*, was transferred by Vhf AB312 at about the same time as an extensive *met.ilva* mutation, though it was not linked closely enough to this marker for the occurrence of linked transduction. Two other groups, *val-r-E* and *val-r-F*, are transferred by Vhf AB312 at about the same time as *ile* and *ilva* but only three, *val-r-E12*, *val-r-F1* and *val-r-F2* could be shown to be closely linked to these markers by transduction. If the distinction between *val-r-E* and *val-r-F* based on a difference in the time of transfer from Vhf AB313 is a valid one, these results indicate that *ile* and *ilva* lie between *val-r-E* and *val-r-F*.

It is well known that the pathways for the synthesis of threonine, leucine, *iso*-leucine and valine are interrelated (Adelberg & Rabinovitz, 1956; Kamin & Handler, 1957; Coon & Robinson, 1958; Knox & Behrman, 1959 (see Fig. 1)). In *Salmonella* it has been shown by Glanville & Demerec (1960) that four out of five threonine loci are linked in what is probably the same sequence as the biochemical steps they control, and one *iso*leucine locus together with four *iso*leucine-valine loci are also linked in the same sequence as the biochemical steps they control. In addition, conjugation experiments with *Salmonella* have revealed that the *thr* and *leu* loci are closely linked and arranged in the same order as the analogous loci in *E. coli* (Demerec *et al.*, 1959). The present study has revealed that there is at least one *val-r* locus linked to each of the markers *leu*, *thr*, *ile* and *ilva*. It is tempting, therefore, to suppose that this linkage is not fortuitous.

The model proposed by Jacob & Monod (1961) to explain the regulation of protein synthesis in the case of the enzyme β-galactosidase provides a basis from which one can attempt an extrapolation to cover the situation encountered here. We can suppose that a repressor-controlled operon exists for each of the gene sequences referred to. However, whether the *val-r* mutations represent mutations

of a normally valine sensitive operator gene which render it no longer sensitive to valine, or whether they represent mutations in a repressor locus which prevent the repressor from coupling with valine and so inhibit its normal function, such mutations of either kind would leave the other gene sequences sensitive to valine. It may be that one of the groups of *val-r* mutants functions in this manner.

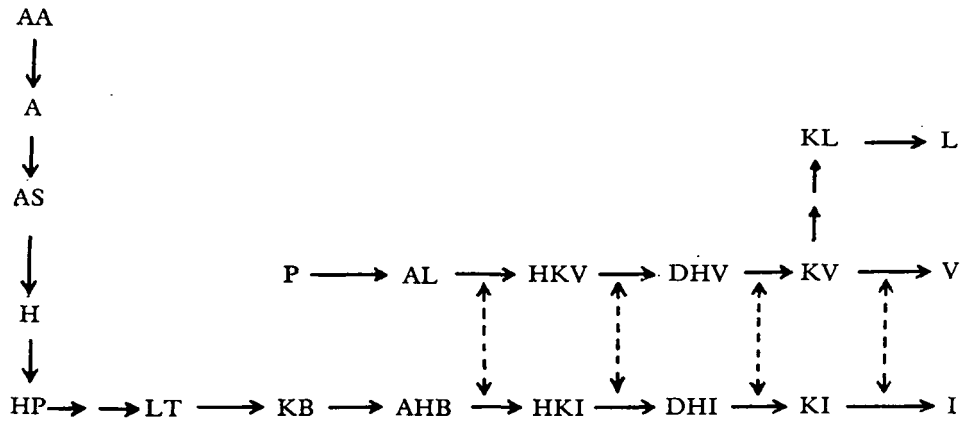

Fig. 1. Pathways of synthesis of threonine, *iso*leucine, valine and leucine. AA = aspartic acid; A = β-aspartyl phosphate; AS = aspartic-β-semialdehyde; H = homoserine; HP = homoserine phosphate; LT = L-threonine; KB = α-ketobutyric acid; AHB = α-aceto-α-hydroxybutyric acid; HKI = α-keto-β-hydroxy-β-methyl valeric acid; DHI = α,β-dihydroxy-β-methylvaleric acid; KI = α-keto-β-methylvaleric acid; I = isoleucine; P = pyruvic acid; AL = α-acetolactic acid; HKV = α-keto-β-hydroxyisovaleric acid; DHV = α,β-dihydroxyisovaleric acid; KV = α-ketoisovaleric acid; V = valine; KL = ketoleucine; L = leucine.

Another may represent permease negative mutations which prevent the uptake of inhibitory concentrations of valine. Any mutation which opened up an alternate pathway for the synthesis of *iso*leucine or facilitated the excess production of isoleucine or its precursors which antagonize valine inhibition, would lead to the development of valine resistance. The phenotypic differences so far demonstrated between the different groups of *val-r* mutants do not provide an adequate basis upon which to decide between these alternatives.

SUMMARY

Sixty-two mutants of *E. coli* K-12 resistant to 40 μg./ml. valine were isolated from a sensitive strain. Transduction experiments using phage P1 showed that one group of these mutants, *val-r-C*, is closely linked to *leu*, another group, *val-r-B* is closely linked to *thr*, and a third mutant, *val-r-D57*, lies between *leu* and *thr*. Conjugation experiments showed that the remainder of the mutants could be divided into three groups, *val-r-A*, *val-r-E* and *val-r-F* on the basis of their different times of transfer from Vhf donors to *val-s* F^- recipients. All the mutants are sensitive to 10,000 μg./ml. valine; *val-r-B* and *val-r-D* mutants are resistant up to 80 μg./ml.

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valine; *val-r-C* mutants are resistant up to 1000 μg./ml. valine, and *val-r-A*, *val-r-E* and *val-r-F* mutants up to 5000 μg./ml. The functional significance of the genetic locations of these groups is discussed.

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