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Antipolarity in the *ilv* Operon of *Escherichia coli* K-12

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The genes governing three of the enzymes of the isoleucine-valine biosynthetic pathway form the operon: *operator-ilvA-ilvD-ilvE*. The enzymes are: *ilvA*, L-threonine deaminase; *ilvD*, dihydroxy acid dehydrase; and *ilvE*, transaminase B. A nonsense mutation in the *ilvD* gene (*D-ochre*) and a nonsense mutation in the *ilvE* gene (*E-amber*) affect the properties of the proximal gene product, L-threonine deaminase (TD), in addition to inactivating the enzymes produced by the genes in which the mutations have occurred. The *D-ochre* mutation causes TD to move in diffusion and gel filtration experiments as though it were 30% smaller than the wild-type enzyme. The *E-amber* mutation causes TD to move in similar experiments as though it were much larger than the wild-type enzyme. Both mutations completely abolish the sensitivity of TD to L-isoleucine, the normal feedback inhibitor of the wild-type enzyme. The effects of the nonsense mutations on TD can be reversed in three ways: by genetic reversion of the *D-ochre* mutation; by treatment of the altered enzymes with 3.0 M urea; and by forming a heterozygous diploid, containing the wild-type allele as well as the mutant allele of *ilvD* or *ilvE*. The results suggest that the subunits of TD undergo abnormal aggregation in the presence of the partial polypeptides produced by the mutant alleles of *ilvD* or *ilvE*; multi-enzyme aggregates in extracts of wild type, however, could not be detected.

In *Escherichia coli* K-12, five enzymes are involved in the biosynthesis of isoleucine and valine (Fig. 1). These enzymes are determined by five *ilv* genes which are tightly clustered on the *E. coli* chromosome (31); the sequence of the genes, and the enzymes which they determine, are shown in Fig. 2.

Two operator loci concerned with the regulation of the *ilv* genes were discovered by Ramakrishnan and Adelberg (30). Originally designated *oprA* and *oprB*, they have been redesignated *ilvO* and *ilvP* (32) to conform to the nomenclature recommended by Demerec et al. (8). Mutations in *ilvO* derepress three adjacent loci: *ilvA*, governing L-threonine deaminase (TD); *ilvD*, governing dihydroxy acid dehydrase (DH); and *ilvE*, governing transaminase B (TRB; 29). These three loci thus constitute an operon, which has been designated "operon A" (30).

Mutations in *ilvP*, the second operator, derepress *ilvB*, governing the condensing enzyme. The existence of a third operator, controlling *ilvC* [the gene determining reductoisomerase (RI)] has been inferred from the fact that RI is regulated by end-product repression just as are the other four structural genes.

The present paper will be concerned only with "operon A," which will henceforth be referred to simply as "the *ilv* operon." The sequence of genes in this operon is *operator-ilvA-ilvD-ilvE*, where the proximal gene, *ilvA*, determines TD. TD is the first enzyme in the biosynthetic pathway; it is an allosteric enzyme, being feedback-inhibited by L-isoleucine and activated by L-valine (5, 6).

The purpose of this paper is to describe and discuss certain pleiotropic effects of two nonsense mutations in the distal genes of the operon, *ilvD* and *ilvE*. Nonsense mutations are chain-terminating and thus cause the complete inactivation of the enzymes determined by the genes in which they lie. They also show varying degrees of *polarity*: the amount of enzyme formed by any gene which is *distal* to the mutation in the operon is usually lower than normal (26). The two nonsense mutations reported here are unusual in that, in addition to inactivating completely the enzymes governed by the genes in which they lie, they have drastically altered the properties of TD, which is determined by the *proximal* gene, *ilvA*.

The pleiotropic effect of one of these nonsense mutations, *ilv-188*, was discovered in the course of a study of external suppressor genes (10). The

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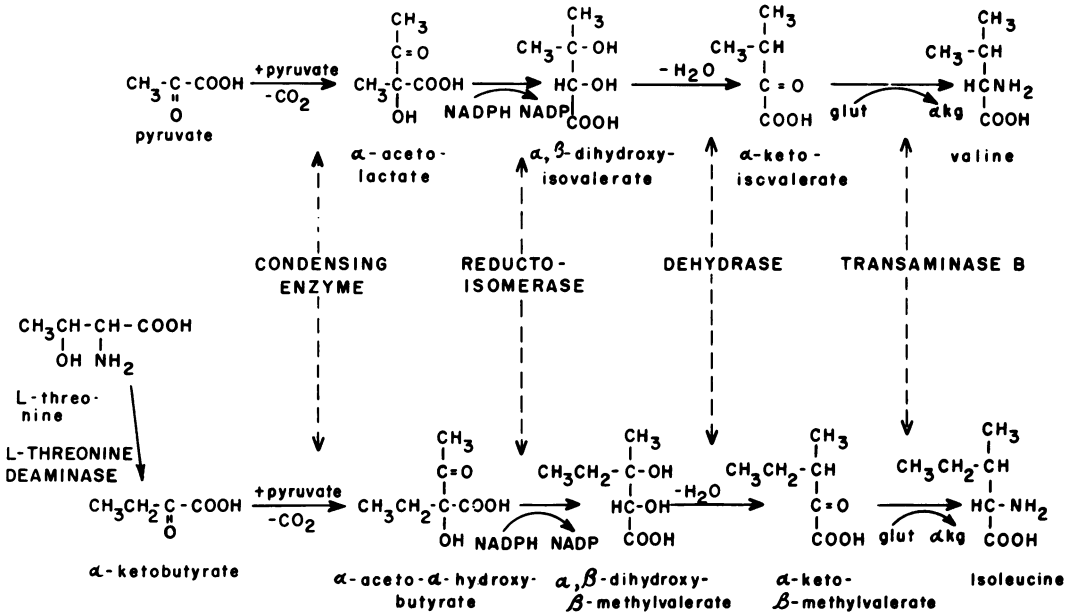

FIG. 1. *Isoleucine-valine biosynthetic pathway.*

ilv-188 mutation was shown to be an *ochre* mutation; it was tentatively assigned to the *ilvD* locus, on the basis of the fact that DH was completely inactivated in two different genetic backgrounds, other enzyme levels being variably and incompletely reduced: We have confirmed this conclusion by genetic complementation and mapping experiments, and will thus designate the mutation as *ilvD188*.

The pleiotropic effect of the second nonsense mutation, *ilv-316*, was discovered in the course of the present work; *ilv-316* is an *amber* mutation (D. Duggan, *personal communication*). On the basis of the genetic and enzymatic analyses to be described in this paper, this mutation appears to lie in the *ilvE* gene, governing TRB. It will thus be designated as *ilvE316*.

Some effects of *ilvD188* and *ilvE316* on the enzymes of the *ilv* operon are summarized in Table 1. Although it is a nonsense mutation, *ilvD188* does not exert a polarity effect on the distal gene product, TRB. On the other hand, the proximal gene product, TD, is reduced to 40% of the wild-type level. Even more striking is the effect of *ilvD188* on an allosteric property of TD: the sensitivity of this enzyme to inhibition by L-isoleucine is completely abolished. The pleiotropic effect of *ilvD188*, which is exerted on the proximal gene product rather than on the distal gene product, will be designated as an *antipolarity effect*.

The nonsense mutation, *ilvE316*, also exerts a pronounced antipolarity effect. When extracts are prepared from cells carrying the *ilvE316* mutation, both proximal genes are found to have produced altered products. DH, the product of the *ilvD* gene, is inactive in fresh extracts, but regains partial activity on standing at 4 C. TD, the prodduct of the *ilvA* gene, is present at the wild-type level, but its sensitivity to inhibiton by L-isoleucine is totally abolished.

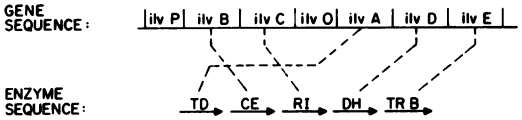

FIG. 2. *Genes and enzymes of the isoleucine-valine biosynthetic pathway. TD, L-threonine deaminase (L-threonine hydrolyase, deaminating, EC 4.2.1.16); CE, condensing enzyme; RI, reducto-isomerase; DH, dihydroxy acid dehydrase (2,3-dihydroxy acid hydrolyase, EC 4.2.1.9); TRB, transaminase B (L-glutamine: 2 oxoacid aminotransferase, EC 2.6.1.15); ilvO, ilvP, operator genes.*

The present paper reports the results of experiments which define the nature of the antipolarity effect of *ilvD188* (*ochre*) and *ilvE316* (*amber*). For the sake of clarity, strains carrying these mutations will always be identified by the notations (*D-ochre*) and (*E-amber*), respectively, following the strain numbers.

MATERIALS AND METHODS

Media and culture methods. Culture media and methods previously described by Adelberg and Burns (1) were routinely employed. Merodiploid strains were maintained as suspensions in minimal buffer at 4 C.

TABLE 1. *Effects of nonsense mutations on enzymes of the ilv operon*

Strain	Genotype	TRB specific activity	DH specific activity	TD specific activity	TD sensitivity to isoleucine[a]
AB1976[b]	Wild type	1.0	1.0	1.0	1.0
AB3511	*ilvD188 (ochre)*	1.25	0.0	0.4	0.0
AB2200[b]	Wild type	1.0	1.0	1.0	1.0
AB3516	*ilvE316 (amber)*	0.0	0.3[c]	1.05	0.0

[a] The sensitivity of the wild-type enzymes corresponds to 83 to 93% inhibition by 10^{-2} M L-isoleucine.

[b] AB1976 is the wild-type parent of strain AB3511. AB2200 is the wild-type parent of strain AB3516. The specific activities of the wild-type enzymes have been defined as 1.0; however, AB2200 enzymes exhibit specific activities approximately 20% higher than AB1976 enzymes.

[c] The DH activity in fresh extracts is 0.0; activity slowly appears on standing at 4 C, reaching a maximum of 0.3.

Merodiploids maintained in this way remain viable for at least 1 year.

Bacteriophages. P1v is a virulent, generalized transducing phage derived from P1kc (19).

Bacterial strains. The bacterial strains employed in this investigation are listed in Table 2.

Derivation of bacterial strains. Spontaneous revertants of the *ilvD188 ochre* mutation of AB3511 were obtained by plating an overnight culture grown from a small inoculum on minimal medium lacking isoleucine and valine. Strain AB3511 contains three nonsense mutations other than the *ilvD188 ochre* mutation. Two of these mutations, *his-4* and *trp-3*, are suppressed by the same suppressors that suppress *ilvD188*, and the other nonsense mutation, *ara-9*, is suppressed by one of these suppressors (10). Strain AB3512, the chosen revertant of *ilvD188*, is prototrophic with respect to isoleucine and valine but is still auxotrophic for tryptophan and histidine and cannot utilize arabinose as a carbon source. It is, therefore, assumed to have arisen by reversion of the *ilvD188* mutation rather than by suppression.

Merodiploid strains were obtained by mating F′ males containing F-*ilv* merogenotes with the Ilv⁻ mutant strains and selecting for Ilv⁺ phenotypes. From these heterozygous diploids, homozygous diploids were obtained as spontaneous Ilv⁻ segregants at a frequency of approximately 2 to 10 per 10^8 cells. To prove that segregants were in fact homozygous diploids, and not merely Ilv⁻ strains produced by loss of the merogenote or by a second mutation, two types of tests were made: (i) crosses were made with appropriate F⁻ strains to show that they were still F′ donors, and (ii) the absence of the *ilv*⁺ allele was shown by the inability to obtain Ilv⁺ transductants of the original F⁻ recipient with transducing phage grown on the homozygous diploid.

Three homozygous diploids were obtained in this way: AB3521, homoallelic for *ilvD188*; AB3523, homoallelic for *ilvD75*; and AB3526, homoallelic for *ilvE316*.

Cross-streak test for donor ability. Recipient cultures and presumed males were centrifuged following overnight growth in supplemented minimal medium, and the pellets were resuspended in minimal buffer. Approximately 0.1 ml of the recipient cell suspension was placed in a line slightly off center and running across a plate containing a medium selective for recombinants. After this volume had dried into the agar, suspensions of the various donor cells were streaked across it with sterile toothpicks. F⁻ strains carrying the same *ilv* allele as the donor strain were also streaked across the recipient as controls. High-frequency donor (Hfr) cells gave rise to isolated colonies along the streak after 48 hr of incubation at 37 C. F′ donors, which transferred the selected marker on the episome, gave rise to solid growth along the streak after 24 hr at 37 C. Single colonies were evident at the edge of the heavy growth resulting from F′ donors. When cross-feeding occurred, growth was solid but lighter than that due to F′ marker transfer. No single colonies were evident at the edges of growth due to cross-feeding. The three types of growth were easily distinguishable.

Cross-streak test for complementation. The method was the same as that used in the test for donor ability. All males were F′ merodiploids, homozygous for a particular *ilv* allele. Complementation was evidenced by heavy growth along the streak as in the test for donor ability. When complementation was negative, heavy growth was absent at 24 and 48 hr. Occasional isolated colonies along the streak at 48 hr were the result of recombination between noncomplementing strains.

Bacterial matings. Matings were done by the method of Adelberg and Burns (1). The ratio of recipient cells to donor cells was 18:1. When the donor strain was a partial diploid, the male was grown in supplemented minimal medium, and the mating was carried out in nutrient broth. Matings were interrupted by vigorous agitation on a Vortex Genie Mixer for 30 sec. In some cases, males were lysed by treatment with T6 bacteriophage.

Genetic mapping. Genetic mapping was done by conventional three-factor crosses (17). Both conjugation and transduction were employed.

Preparation of crude extracts. Cultures in late exponential phase (5×10^8 to 8×10^8 cells/ml) were harvested from minimal medium by centrifugation, washed once, and finally suspended in 0.05 M tris-(hydroxymethyl)aminomethane (Tris) buffer to effect approximately a 100-fold concentration of the cells. Three different Tris-hydrochloride buffers were employed: 0.05 M, *p*H 8.0; 0.05 M, *p*H 7.8; and 0.05 M, *p*H 7.8, plus 5×10^{-4} M L-isoleucine and 10^{-4} M ethylenediaminetetraacetic acid (EDTA). They will be designated as "standard" Tris buffers in this paper. The choice of buffer depended upon the experiment according to the following general rationale: (i) if only TD was to be assayed, standard Tris, *p*H 8.0, was used; (ii) if TD activity was to be assayed more

TABLE 2. *Escherichia coli K-12 strains employed*[a]

Strain	*ilv*	*met*	*arg*	*thi*	*pro*	*trp*	*his*	*mal*	*xyl*	*mtl*	*ara*	*lac*	*gal*	*tsx*	*ton*	*str*	Other characters
F⁻ strains																	
AB1255	A201	1	1	1, 2	+	+	1	A1	4	+	+	+	+	5	+	8, 9, 17	
AB1514	E12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
AB1450	D16	1	1	1, 2	+	+	1	A1	4	+	+	Y1	6	7	+	8, 9, 17	
AB1976	+	E46	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	
AB2070	E12	E46	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	
AB2071	D105	E46	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	
AB2200	+	E46	+	1	+	3	4	A1	+	1	+	Z13	+	3	?	8, 9	
AB2220	D130	E46	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	λ^R
AB2221	D132	E46	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	λ^R
AB2277	D145	E46	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	λ^R
AB3501	D88	E46	H1	1, 2, 12	A2	3	4	A1?	4?	+	+	Y1, Z4	+	3	+	1	
AB3503	D138	E46	H1	1, 2, 12	A2	3	4	A1?	+	+	+	Y1, Z4	K2?	3	+	+	
AB3505	D188	E46	H1	1, 2, 12	A2	3	4	A1?	4?	1?	+	Y1, Z4	K2	3	+	+	
AB3507	D75	E46	H1	1, 2, 12	A2?	3	4	+	+	+	+	Y1, Z4	K2	3	+	+	
AB3509	D144	E46	H1	1, 2, 12	A2?	3	4	A1	+	1	+	+	K2	3	+	+	
AB3511	D188	+	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	
AB3512	+	+	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	
AB3515	E12	+	+	1	+	3	4	A1	+	1	+	Z13	+	3	1	8, 9	
AB3516	E316	+	+	1	+	3	4	A1	+	1	+	Z13	+	3	1	8, 9	
AB3517	E316	E46	H1	1?	+	3	4	?	4	?	+	+	+	3?	1?	8, 9	
AB3518	+	E46	+	1, 2, 12	A2	3	4	A1	+	1	+	Y1, Z4	K2	3	1	8, 9	*leu-23*
F′ strains																	
AB1526	+/D16	1	H1	1, 2	+	+	1	A1	4	+	+	+	6	+	+	9, 17	F-*ilvE*⁺-*ilvD*⁺-*ilvA*⁺ (F216)
AB1545	+/D16	+	+	+	+	+	1	A1?	4	+	+	Y1	6	+	+	8, 9, 17	F-*ilvE*⁺-*ilvD*⁺ (F25)
AB3519	+/D188	+	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	F-*ilvE*⁺-*ilvD*⁺ (F25)
AB3520	+/D188	+	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	F-*ilvE*⁺-*ilvD*⁺-*ilvA*⁺ (F216)
AB3521	D188/ D188	+	+	1, 2, 12	A2	3	4	A1	+	1	9	Y1, Z4	K2	3	1	8, 9	F-*ilvE*⁺-*ilvD188-ilvA*⁺ (F217)
AB3522	+/D75	+	H1	1, 2, 16	A2?	3	4	+	+	+	+	Y1, Z4	K2	3	+	?	F-*ilvE*⁺-*ilvD*⁺-*ilvA*⁺ (F216)
AB3523	D75/D75	+	H1	1, 2, 16	A2?	3	4	+	+	+	+	Y1, Z4	K2	3	+	?	F-*ilvE*⁺-*ilvD75-ilvA*⁺ (F218)
AB3524	+/E316	+	+	1	+	3	4	A1	+	1	+	Z13	+	3	1?	8, 9?	F-*ilvE*⁺-*ilvD*⁺ (F25)
AB3526	E316/E316	+	+	1	+	3	4	A1	+	1	+	Z13	+	3	1?	8, 9?	F-*ilvE316-ilvD*⁺ (F219)

Continued on p. 1183

Table 2 continued from p. 1182

Hfr strains[b]																	
AB684	D75	+															
AB1931	+	E46															
AB1953	D88	+															
AB2081	D132	+															
AB3513	D188	+															

[a] Locus symbols are those used by Taylor and Trotter (32). Allele designations are in accordance with the recommendations of Demerec et al. (8). When it is not known which of two parental alleles exist in a given strain, both allele numbers are shown (e.g., *thi-1, 2*). Both alleles are shown for loci which are heterozygous in diploid F′ strains; the F-merogenote allele is given first. Allele numbers are those assigned in this laboratory.

[b] All Hfr strains carry *argH1*, *purC1*. They inject in the order ←*str-ilv-thr-leu*.

than 3 hr after disruption of the cells, standard Tris, *p*H 7.8, plus isoleucine and EDTA, was used; and (iii) if either DH or TRB activity was to be assayed, standard Tris, *p*H 7.8, was used.

The concentrated cell suspension in standard Tris buffer was treated for 2 min in an MSE Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London, England) to disrupt the cells. Cell debris was removed by centrifugation at 12,000 × *g* for 20 min at 4 C in a refrigerated centrifuge. The supernatant fluid was designated "crude extract."

Preparation of S-100 extract. The cells were handled in the same manner as in the preparation of crude extract, through the sonic treatment step. Cells were resuspended in standard Tris, *p*H 7.8, supplemented with 5×10^{-4} M dithiothreitol and 10^{-3} M $MgCl_2$. After sonic treatment, nucleic acid was precipitated by the slow addition of streptomycin sulfate. The streptomycin-treated sonic extract was centrifuged at 25,000 × *g* for 20 min at 4 C, and the pellet was discarded. The supernatant fraction was centrifuged for 2.5 hr at an average force of 100,000 × *g* in a Beckman model L preparative ultracentrifuge to remove ribosomes and other large particulate matter from the extract. The supernatant fraction obtained was the "S-100 extract."

All TD activity was found in the S-100 extract; no activity could be detected in either the 25,000 × *g* pellet or the 100,000 × *g* pellet. The S-100 extract could be stored for 10 days at −60 C with no loss of activity.

Diffusion. The method of Fuchs and Gorin (14), utilizing a cellulose ester filter as a semipermeable membrane, was followed. A Millipore filter (Millipore Corp., Bedford, Mass.) with a pore size of 0.65 μm was used in experiments with crude extracts; for experiments with S-100 extracts, a Metricell membrane (Gelman Instrument Co., Ann Arbor, Mich.) with a pore size of 1.2 μm was used. By assaying samples from the diffusion chamber at various times, the value of $T_{0.1}$, the time at which 10% of the enzyme has crossed the membrane, can be obtained. The $T_{0.1}$ value is inversely proportional to the diffusion constant according to the equation: $T_{0.1} = 0.2/(BD)$, where D is the diffusion constant and B is a constant of the cell (14). Since the size of the diffusing molecule is inversely proportional to the diffusion constant also, $T_{0.1}$ is directly proportional to size.

Two criteria were employed to ensure against concentration-dependent breakdown: (i) the diffusion must be linear with time and the plotted line must extrapolate through the origin; and (ii) the extract must show no concentration-dependent breakdown when diluted to the various low concentrations equivalent to those expected to cross the membrane at various times, and kept at these concentrations for a time equivalent to the $T_{0.1}$ determined by diffusion. No concentration-dependent decay of activity was demonstrable by either criterion. In experiments with crude extracts, diffusion was followed until at least 10% of the enzyme had diffused across the membrane; in experiments with S-100 extracts, diffusion was followed as long as possible (until at least 30% had crossed the membrane). The diffusion data from all experiments gave linear results when either the fraction diffused or the logarithm of residual activity was plotted versus time.

Analysis of diffusion with crude extracts from strain AB3516 (E-amber). Though the altered TD in crude extracts from strain AB3516 (*E-amber*) is more stable than that of wild-type strains, and no loss of activity is evident under the conditions employed in the diffusion experiments, the measurement of the amount of enzyme diffused is complicated by a sigmoidal relationship between enzyme activity and enzyme concentration. By using a calibration curve it was possible to correct the data by converting the fraction of activity diffused to the fraction of enzyme diffused.

Gel filtration. Sephadex G-200 was swollen and packed into a Sephadex Laboratory column (2.5 cm in diameter) according to the directions of the manufacturer. The final column volume was 190 ml. The column packing was stabilized by allowing the elution buffer, standard Tris, *p*H 7.8, plus isoleucine, EDTA, and 5×10^{-4} M dithiothreitol, to percolate through for several days. Testing of the quality of the column packing and determination of the void volume were done with Blue Dextran 2000. The void volume of 64 ml varied by less than 1 ml during the course of these experiments. All column runs were carried out at 4 C. A 2-ml sample was applied to the top of the column and allowed to sink in slowly. Two milliliters of buffer was then applied to the column and, after this volume had flowed into the bed, the space above the bed was filled with buffer and the reservoir was attached to the column. The flow rate was adjusted to approximately 18 ml per hr by varying the hydrostatic pressure between 130 and 150 mm. Samples (2 ml) of effluent were collected with an automatic fraction collector. A small volume of concentrated bovine serum albumin solution (final concentration, 1.0 mg/ml) was added to the collection tubes, to aid in stabilizing the eluted enzymes.

Samples for gel chromatography were prepared by concentrating S-100 extracts approximately 1.5-fold with Aquacide 11 at 4 C. The following marker proteins were added to the concentrated S-100 extracts: cytochrome *c*, 1.0 mg/ml; yeast alcohol dehydrogenase, 0.6 mg/ml; and *E. coli* alkaline phosphatase, 0.04 mg/ml. The maximum shift in peak position for any protein in different experiments was 2 ml.

Urea treatment. Urea (10 M) in Tris, *p*H 8.0, was added to extracts in standard Tris, *p*H 7.8, to give a final urea concentration of 3.0 M. Final *p*H of the treated extract was 8.1 to 8.2. Urea treatment was carried out for 30 min at 0 C. Protein concentrations prior to the addition of urea were 12 to 13.5 mg/ml.

Derepression of enzyme synthesis. Strain AB3518, a leucine-requiring transductant of the wild-type strain, AB1976, was grown in a chemostat (27). Derepression was effected by limiting leucine such that the generation time was 2.5 hr (compared to a normal generation time in batch culture of 1 hr). Leucine deprivation causes derepression of the *ilv* operon because the operon is under the control of "multivalent repression" (11). Under these growth conditions, there was a fivefold increase in specific activity of TD, DH, and TRB.

Ammonium sulfate fractionation. Solid ammonium sulfate was added slowly with gentle stirring to sonic extracts of chemostat-grown cultures of AB3518. After equilibration for 30 min at 4 C, precipitates were collected by centrifugation at 25,000 × *g* for 15 min. The *p*H during ammonium sulfate treatment was lowered from 7.8 before treatment to 7.4 at 46% saturation.

Enzyme assays. The TD assay was a modification of that used by Ramakrishnan and Adelberg (29). The incubation mixture contained Tris-hydrochloride buffer, *p*H 8.0, 100 μmoles; pyridoxal phosphate, 50 μg; NH_4Cl, 20 μmoles; L-threonine, 80 μmoles; and 0.1 ml of crude extract in a total volume of 1 ml. L-cysteine hydrochloride (25 μmoles), adjusted to *p*H 8.0 was used when extracts of AB3516 (*E-amber*) or AB3524 (*E-amber*) were used. The substitution of NH_4Cl for cysteine was the only significant change from the method of Ramakrishnan and Adelberg (29). *E. coli* extracts contain an activity which catalyzes the breakdown of L-cysteine to ammonia, pyruvate, and hydrogen sulfide in the presence of pyridoxal phosphate (24). NH_4^+ has been found to be an activator of TD in other systems (4, 18). Keto acid production was measured by the direct method of Friedemann and Haugen (13).

DH was assayed by using α,β-dihydroxyisovaleric acid as a substrate by the method of Myers (25).

TRB was measured by a new assay employing an instantaneous extraction of the keto acid-phenylhydrazone (Duggan and Wechsler, *in preparation*).

All assays result in the production of keto acid which is linear with time for at least 1 hr when an "unrepressed" wild-type extract is used as a source of enzyme. There was no detectable disappearance of product in any of the assays during incubation times of 1 hr.

Isoleucine-valine enzyme activities are expressed as micromoles of keto acid produced per milligram of protein per hour (specific activity).

Protein concentrations in the reaction tubes for isoleucine-valine biosynthetic enzymes was 1.16 to 1.75 mg/ml. *E. coli* alkaline phosphatase was determined by measuring the absorbance (at 415 nm) due to hydrolysis of *p*-nitrophenylphosphate (15).

Yeast alcohol dehydrogenase was measured by the rate of increase in absorbance (at 340 nm) due to the reduction of nicotinamide adenine dinucleotide (33).

Cytochrome *c* was measured by absorbance at 412 nm.

Protein determinations. Protein concentrations were routinely determined by the method of Lowry et al. (23). Crystalline bovine serum albumin was used as a standard.

Reagents. α,β-Dihydroxyisovaleric acid was generously supplied by J. W. Myers, and *E. coli* alkaline phosphatase was the gift of H. P. Treffers. Ammonium sulfate (special enzyme grade) and cytochrome *c* were obtained from Mann Research Laboratories, Inc. Alcohol dehydrogenase was obtained from Worthington Biochemical Corp., Freehold, N.J. Sephadex G-200 and Blue Dextran 2000 were supplied by Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Aquacide 11 was a product of Calbiochem. All other chemicals were reagent grade.

RESULTS

Genetic complementation. As stated above, the enzymological data suggested that the *ilv-188* mutation lies in the *ilvD* gene (governing DH), and that the *ilv-316* mutation lies in the *ilvE* gene (governing TRB). These inferences were tested by using partial (F′) homozygous diploid strains as donors in cross-streak tests. The results (Table 3) support the preliminary assignments: *ilv-316* complements all *ilvD* mutant alleles, but not the one *ilvE* allele available for test; *ilv-188*, on the other hand, complements the *ilvE* allele but none of the *ilvD* alleles. The two nonsense mutations, *ilv-188* and *ilv-316*, complement each other.

As a control, a homozygous diploid, containing an *ilv* missense mutation, *ilvD75*, was also employed as a donor in cross-streak tests. This strain, which is DH-negative, complemented *ilv-316* but not *ilv-188* nor any other *ilvD* allele.

Genetic mapping. The nonsense mutations, *ilv-188* and *ilv-316*, were mapped by pairwise, three-point, reciprocal crosses. In each case, the positions of two different *ilv* mutations were determined relative to an "outside marker," *metE*. The mapping of *ilv-188* was carried out by conjugation, using an Hfr donor strain that transfers in the order *origin-ilv-metE*. The mapping of *ilv-316* was carried out by transduction, using phage P1v.

In each cross, the donor strain and recipient strain carried different *ilv* mutations; the donor was always $metE^+$, and the recipient was always

TABLE 3. *Complementation between ilv alleles*[a]

Recipient allele	Recipient enzyme deficiency	Donor strain and allele		
		AB3523 *ilv-75/75*	AB3521 *ilv-188/188*	AB3526 *ilv-316/316*
ilv-16	DH	−	−	+
ilv-75	DH		−	+
ilv-88	DH	−	−	+
ilv-105	DH	−	−	+
ilv-132	DH	−	−	+
ilv-138	DH	−	−	+
ilv-144	DH	−	−	+
ilv-145	DH	−	−	+
ilv-188	DH	−		+
ilv-12	TRB	+	+	−
ilv-316	TRB	+	+	

[a] Complementation was determined by cross-streak test. All controls were negative. Symbols: +, complementation; −, lack of complementation.

metE$^-$. For each pair of *ilv* mutations, reciprocal crosses were performed, a given *ilv* allele being in the donor in one cross and in the recipient in the other cross. In the conjugation crosses, selection was made for *Met*$^+$ recombinants, and the recombinants were picked and tested for their Ilv phenotype. In the transduction crosses, selection was made for Ilv$^+$ recombinants, and the recombinants were picked and tested for their Met phenotype.

To obtain a Met$^+$ Ilv$^+$ recombinant in either method, only two crosssovers are required when the *ilv* mutation of the recipient is closest to *metE*, but four crossovers are required when the *ilv* mutation of the donor is closest to *metE*. Thus, in each reciprocal pair of crosses the percentage of Met$^+$ Ilv$^+$ recombinants is significantly higher in one cross than in the other; the cross giving the highest percentage is that in which the recipient *ilv* allele is closest to *metE*.

The results of the mapping experiments are summarized in Table 4 and Fig. 3. The *ilv-188* (*ochre*) mutation is seen to lie between *ilvD132* and *ilvD75*. The *ilv-316* (*amber*) mutation lies to the left of the furthermost *ilvD* mutation, and to the right of *ilvE12*; it could thus be in either *ilvD* or in *ilvE*. Its assignment to *ilvE* is based on two other sets of data: the complementation data summarized in Table 3, and the enzymological data summarized in Table 1. To confirm this assignment by mapping tests, it would be necessary to find an *ilvE* mutation that maps to the right of *ilv-316*; no such mutant is presently available.

Effect of ilvE316 (amber) on DH. The nonsense mutation, *ilvE316* (*amber*), affects all three gene products of the *ilv* operon. The *ilvE* product, TRB, is completely inactive; the *ilvD* gene product, DH, and the *ilvA* gene product, TD, are affected as described below. It will be recalled that *ilvD* and *ilvA* are proximal to *ilvE*, so that the effects to be described are *antipolar*.

Early attempts to assay DH activity in extracts of AB3516 (*E-amber*) resulted in uniformly negative results. It was discovered, however, that if the extracts were allowed to remain at 0 C, DH activity appeared. The level of DH activity increased for 2 to 3 hr and then began to disappear (Table 5). Essentially all of the activity had disappeared within 8 to 9 hr after sonic treatment. The TD activity remained insensitive to feedback inhibition throughout this time period, and no TRB activity was ever observed.

Attempts to halt the appearance of DH activity with divalent cations, valine, isoleucine, or leucine, either singly or in a variety of combinations, failed, as did the addition of reducing agents. Conversely, neither dithiothreitol nor the bubbling of nitrogen through the extract prevented the subsequent disappearance of DH activity. That the observed activity was actually due to DH was attested to by the fact that the activity displayed an absolute requirement for ferrous sulfate.

TABLE 4. *Genetic mapping by reciprocal three-factor crosses*[a]

Donor allele	Recipient allele	Per cent Ilv$^+$Met$^+$	Deduced gene order
Transduction			
ilv-12	*ilv-316*	6.1	*ilv-12 — ilv-316 — metE*
ilv-316	*ilv-12*	1.1	
ilv-316	*ilv-88*	7.3	*ilv-316 — ilv-88 — metE*
ilv-88	*ilv-316*	0.4	
ilv-12	*ilv-88*	9.0	*ilv-12 — ilv-88 — metE*
ilv-88	*ilv-12*	1.8	
Conjugation			
ilv-88	*ilv-132*	1.8	*ilv-88 — ilv-132 — metE*
ilv-132	*ilv-88*	0.25	
ilv-88	*ilv-188*	3.0	*ilv-88 — ilv-188 — metE*
ilv-188	*ilv-88*	<0.5	
ilv-88	*ilv-75*	3.8	*ilv-88 — ilv-75 — metE*
ilv-75	*ilv-88*	0.7	
ilv-132	*ilv-188*	2.0	*ilv-132 — ilv-188 — metE*
ilv-188	*ilv-132*	<0.25	
ilv-132	*ilv-75*	2.5	*ilv-132 — ilv-75 — metE*
ilv-75	*ilv-132*	<0.8	
ilv-188	*ilv-75*	1.1	*ilv-188 — ilv-75 — metE*
ilv-75	*ilv-188*	0.5	

[a] In each cross, between 1,000 and 2,000 selected recombinants were tested for the unselected phenotype. The final map order is shown in Fig. 3.

TD substrate kinetics. During the course of this work, the cooperativity of the wild-type TD catalytic sites which has been defined in detail by Changeux (5, 6) was not always seen. An identical problem has been encountered with the *Salmonella typhimurium* enzyme (4) and is probably due to the carry-over of extremely low levels of valine in the extract. By acting as an activator, valine tends to mask the sigmoid shape of the substrate kinetics, and a classical, hyperbolic curve is the result. Careful washing of the cells prior to sonic disruption generally served to alleviate this problem. The fact that such difficulty is encountered, however, weakens any conclusion, based on a failure to observe cooperative interactions, that homotropic transitions do not exist.

The substrate kinetics of the TD activities from strain AB1976 (wild type) and strain AB3511 (*D-ochre*) are shown in Fig. 4. The pronounced cooperativity of the wild-type TD contrasts sharply with the lack of cooperative interactions

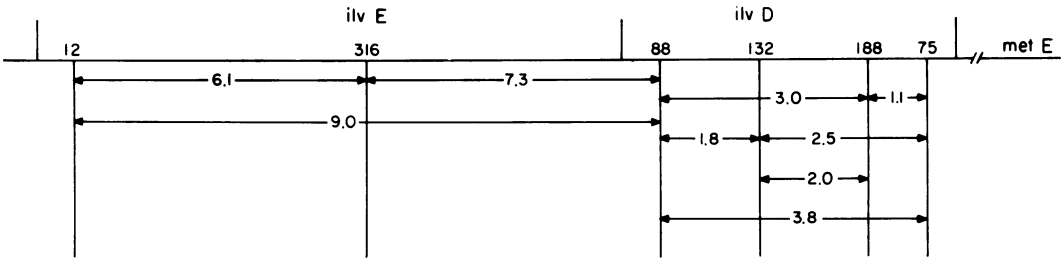

FIG. 3. *Partial map of the ilvD and ilvE genes. Recombination frequencies were determined in three-factor reciprocal crosses. Values for recombination frequencies are the per cent Ilv+ Met+ in the cross of each reciprocal pair giving the higher ratio.*

seen with the mutant enzyme. The addition of 10^{-4} M L-isoleucine, the feedback inhibitor of the enzyme, would be expected to increase cooperative interactions. Because isoleucine was found not to affect the TD of strain AB3511 (*D-ochre*), and because cooperativity in the absence of isoleucine was not always evident with the TD of wild type, it is not possible to affirm absolutely that the TD of AB3511 (*D-ochre*) cannot perform the substrate-induced homotropic transition. Cooperativity was, however, never observed with the TD of AB3511 (*D-ochre*) in spite of numerous repetitions of the substrate kinetics both with and without isoleucine.

The TD present in extracts of AB3516 (*E-amber*) exhibits kinetics very different from those of the AB3511 (*D-ochre*) enzyme (Fig. 5 and 6). The double-reciprocal plot (Fig. 6) extrapolates to at least two different values for the K_m and is strikingly similar to that reported for the *E. coli* deoxythymidine kinase at low activator concentrations (28). In the case of deoxythymidine kinase, the two different K_m values were postulated to be due to different forms of the enzyme, and evidence that this explanation is correct has recently been presented (20). As the concentration of activator was increased, the distinction between the two extrapolated K_m values for deoxythymidine kinase became unclear and the double-reciprocal plot tended to approximate classical kinetics. The addition of 2.5×10^{-3} M L-valine, an activator of wild-type TD, on the other hand, alters neither the kinetics nor the actual assay values obtained for the *E-amber* TD. Also, the addition of 10^{-4} M L-isoleucine to the reaction mixture had no effect.

TD inhibition kinetics. The response of wild-type TD to L-isoleucine was found to be similar to that reported by Changeux (5–7). At very low concentrations, isoleucine enhances TD activity; at higher concentrations, it is inhibitory. As is apparent from Fig. 7, the TD of strain AB3511 (*D-ochre*) is insensitive to isoleucine inhibition. Even when the threonine concentration is de-

TABLE 5. *Appearance of DH activity in ilvE316 extracts*[a]

Time at 4 C	Specific activity	Per cent of wild-type activity[b]
hr		
0	0.00	0.0
0.75	0.147	15.0
1.5	0.19	19.4
2.5	0.294	30.0
3.5	0.254	26.0
4.5	0.23	23.5
5.6	0.192	19.6
7.0	0.144	14.7
10.0	0.00	0.0

[a] Extract in standard Tris, *p*H 7.8.
[b] Wild-type specific activity = 0.98.

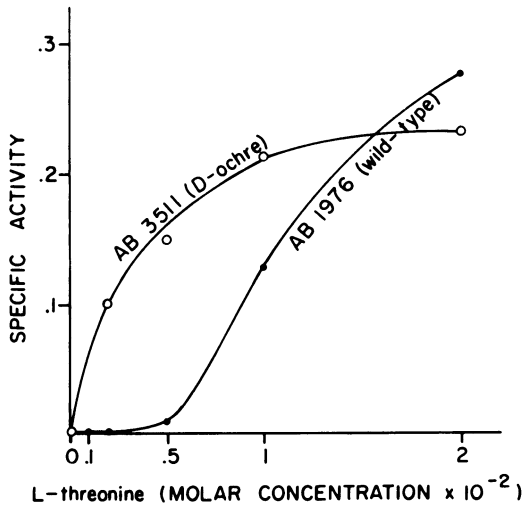

FIG. 4. *L-Threonine deaminase activity of wild type (AB1976) and of the D-ochre mutant (AB3511), as a function of substrate concentration in the presence of a constant concentration (10^{-4}M) of inhibitor. Concentration of protein in the assays: AB1976, 1.52 mg/ml; AB3511, 1.48 mg/ml. Reaction mixtures were incubated at 37 C for 30 min.*

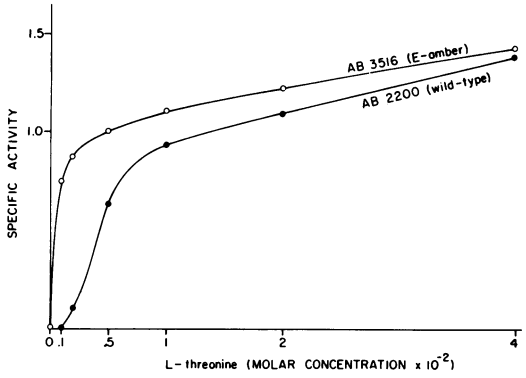

FIG. 5. *L-Threonine deaminase activity of wild type (AB2200) and of the E-amber mutant (AB3516), as a function of substrate concentration. L-Cysteine (25 μmoles) substituted for NH_4Cl in the assay of AB3516 enzyme. Concentration of protein in the assays: AB2200, 1.3 mg/ml; AB3516, 1.46 mg/ml. Reaction mixtures were incubated at 37 C for 30 min.*

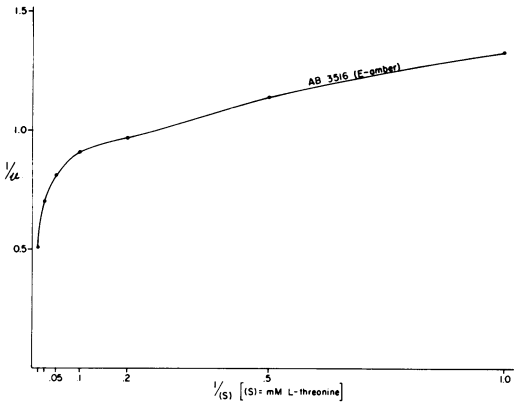

FIG. 6. *L-Threonine deaminase activity of the E-amber mutant (AB3516), as a function of substrate concentration. Data of Fig. 5 plotted by the method of Lineweaver and Burk.*

creased to very low values, the *D-ochre* TD shows no sensitivity to isoleucine.

The TD of strain AB3516 (*E-amber*) is also completely desensitized to isoleucine.

Reversal of the desensitized condition by urea. Changeux presented evidence that moderate concentrations of urea effect a polymer-to-monomer transition in the TD of wild-type *E. coli* K-12 (6). Dilution of the urea-treated enzyme into the assay mixture allowed a reaggregation to the polymerized form. If the altered characteristics of TD in the mutants were a consequence of erroneous aggregation, it might be expected that urea treatment would restore isoleucine sensitivity to the mutant enzymes.

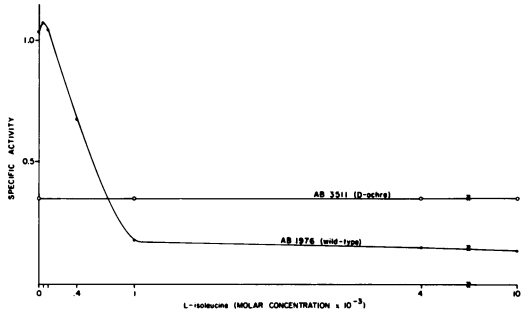

FIG. 7. *L-Threonine deaminase activity of wild type (AB1976) and of the D-ochre mutant (AB3511), as a function of inhibitor concentration. Concentration of protein in the assays: AB1976, 1.68 mg/ml; AB3511, 1.46 mg/ml. Reaction mixtures were incubated at 37 C for 15 min.*

Urea treatment was carried out as described in Materials and Methods. Following urea treatment, "reaggregation" was allowed to occur for 10 min at 37 C, after dilution of the urea-treated extract and prior to the addition of substrate to the reaction mixture. The amount of urea in the final reaction mixture was shown in control experiments to have no effect on the assay.

The TD activity of AB3516 (*E-amber*) was decreased 50% by the urea treatment, but there was a definite regain of sensitivity to isoleucine (Table 6). Under the same conditions, wild-type TD activity decreased by 90% but retained its feedback sensitivity.

To obtain positive results with the TD of AB3511 (*D-ochre*), it was necessary to subject the extract to a single freeze-thaw cycle prior to urea treatment. The decrease in activity was 75%, but the remaining activity had become sensitive to isoleucine inhibition (Table 6).

Enzymatic properties of an ilvD188 revertant. Strain AB3512 is a spontaneous Ilv^+ revertant of AB3511 (*D-ochre*). Since the *ilvD188* mutation confers only one biosynthetic block, lack of DH, a reversion to prototrophy selects only for the restoration of DH activity.

Enzyme assays of the revertant strain, AB3512, show that although DH activity is only partially restored, the level of TD activity is fully restored and the TD activity is isoleucine-sensitive (Table 7). Furthermore, the kinetics of the revertant enzyme are nearly identical to those of the wild-type enzyme with respect to substrate concentration and, more importantly, with respect to isoleucine concentration (Fig. 8).

Ammonium sulfate fractionation. As a possible explanation of the antipolarity effects of the *E-amber* and *D-ochre* mutations, it might be postulated that in wild-type *E. coli* K-12 the three

TABLE 6. *Reversal of desensitized condition by urea*

Extract	Treatment	Activity remaining after treatment	Inhibition by 4×10^{-3} M isoleucine[a]
		%	
AB1976 (wild-type)	None		87
	Urea	10	55
AB3511 (*D-ochre*)	None		0
	Urea	25	57
AB3516 (*E-amber*)	None		0
	Urea	50	44

[a] Reduction in activity (percentage).

TABLE 7. *Dehydrase and threonine deaminase levels in an ilvD188 revertant*

Strain	TD activity	DH activity	TD sensitivity to isoleucine
AB3512 (Ilv$^+$ revertant of *ilvD188*)	0.91	0.66	1.0
AB1976 (wild-type)[a]	1.0	1.0	1.0

[a] Wild-type levels defined as equal to 1.0.

products of the *ilv* operon (TD, DH, and TRB) must form an aggregate in order to function properly. Experiments were thus performed to discover whether the TD and DH activities would remain associated during partial purification by ammonium sulfate fractionation.

The results of such a fractionation are shown in Table 8. The two activities do not remain together: the bulk of the TD activity is precipitated in the 37–46% fraction, while precipitation of the majority of the DH activity is not achieved at 46% saturation with ammonium sulfate.

The salting-out profile of DH is peculiar in that most proteins begin to precipitate during salt fractionation at a very high rate and a major proportion of the protein precipitates within a fairly narrow range of salt concentration, approximately 8% (9). Other workers have had difficulty using ammonium sulfate fractionation to advantage with DH from *E. coli* (J. W. Myers, *personal communication*) as well as from green plant, *Phaseolus radiatus* (T. Satyanarayana, Thesis, Indian Institute of Science, 1964).

Diffusion experiments. The antipolar effects of the *E-amber* and *D-ochre* mutations on the enzymatic properties of TD might reflect changes in the number or arrangement of enzyme subunits. Such changes might be detected as changes in the diffusion rate of the enzyme.

The diffusion rates of the TD proteins from *E-amber* and *D-ochre* strains differed significantly from that of wild-type TD (Table 9). In contrast, the diffusion rate of TRB was not affected by the *D-ochre* mutation. As a control, extracts were made from a strain carrying the missense mutation, *ilvD132*; the TD and TRB proteins of this mutant showed normal diffusion rates.

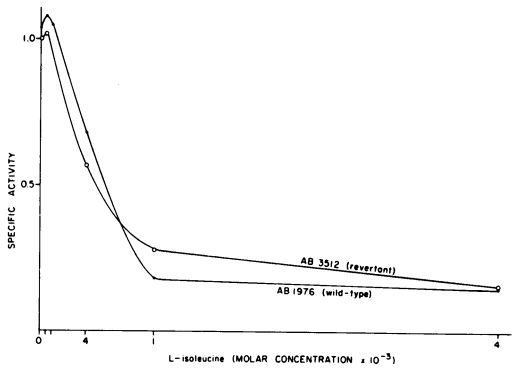

FIG. 8. *L-Threonine deaminase activity of a D-ochre reverant (AB3512) and of wild type (AB1976), as a function of inhibitor concentration. Concentration of protein in the assays: AB3512, 1.71 mg/ml; AB1976, 1.68 mg/ml. Reaction mixtures were incubated at 37 C for 15 min.*

TABLE 8. *Salting-out of TD and DH from wild-type extract*[a]

Enzyme	Enzyme activity as a percentage of total in ammonium sulfate fractions		
	Precipitate (0–37%)	Precipitate (37–46%)	Supernatant fluid (46%)
TD	7	62	31
DH	8	10	82

[a] Of the TD and DH activity present at the beginning of the experiment, 100% was recoverable after fractionation. Total error including assays and experimental errors in handling are estimated to be ±10%.

TABLE 9. *Values of $T_{0.1}$ (hr)*[a]

Enzyme	Strain and mutation			
	AB1976 (wild type)	AB3516 (*ilvE316, amber*)	AB3511 (*ilvD188, ochre*)	AB2221 (*ilvD132*, missense)
TD	22.1	29.8	15.9	22.0
DH	11.6	—[b]		
TRB	8.9		9.0	9.1

[a] $T_{0.1}$ = time in hours necessary for 10% diffusion.

[b] The unstable dehydrase of AB3516 disappeared in less than 4 hr in the diffusion chamber and could not be stabilized.

The different diffusion rates of TD, DH, and TRB of the wild-type strain confirm that these proteins do not exist in the extract as a stable aggregate. More important, however, is the finding that the *E-amber* and *D-ochre* mutations have caused large changes in the diffusion rate of TD: assuming that the $T_{0.1}$ values observed reflect differences in molecular size, the *E-amber* TD appears to be 30% larger than wild-type TD, and the *D-ochre* TD appears to be 30% smaller than wild-type TD.

The diffusion data in Table 9 were obtained with crude extracts of the wild-type and mutant strains. To verify that the results reflected true differences in the enzymes whose activities were being measured, rather than other differences in the extracts, the experiments were repeated with partially purified (S-100) extracts. The data obtained with crude extracts were shown to be comparable to those obtained with more purified material (Table 10). Furthermore, the TD enzymes in crude extracts and S-100 extracts show identical substrate and inhibitor kinetics.

Gel filtration. The different diffusion rates of the mutant TD proteins suggested that they differ in size, and might therefore move at different rates during gel filtration.

S-100 extracts of each of the two mutants and of the wild type were chromatographed on G-200 Sephadex in admixture with two or more marker proteins. Also, each mutant extract was run jointly with the wild-type enzyme and cytochrome *c*. The results of these experiments (Fig. 9) confirm that the mutant and wild-type TD proteins differ in size.

There was considerable loss of activity from AB3511 (*D-ochre*) and AB1976 (wild type) extracts during chromatography. This loss appears to be due to removal of stabilizing substances in the extract, as no AB1976 TD could be detected in the effluent in the absence of dithiothreitol and without bovine serum albumin in the collection tubes.

TABLE 10. *TD diffusion comparison of crude and S-100 extracts*[a]

Strain and Mutation	Crude extract		S-100 extract	
	$T_{0.1}$	Normalized $T_{0.1}$ ratio	$T_{0.1}$	Normalized $T_{0.1}$ ratio
AB1976 (wild type)	22.1	1	15.9	1
AB3516 (*ilvE-316, amber*)	29.8	1.35	23.4	1.47
AB3511 (*ilvD-188, ochre*)	15.9	0.72	12.0	0.75

[a] $T_{0.1}$ = time in hours necessary for 10% diffusion. The $T_{0.1}$ values of the crude extract were determined with an 0.65-μm pore filter; those of the S-100 extract were determined with a 1.2-μm pore filter.

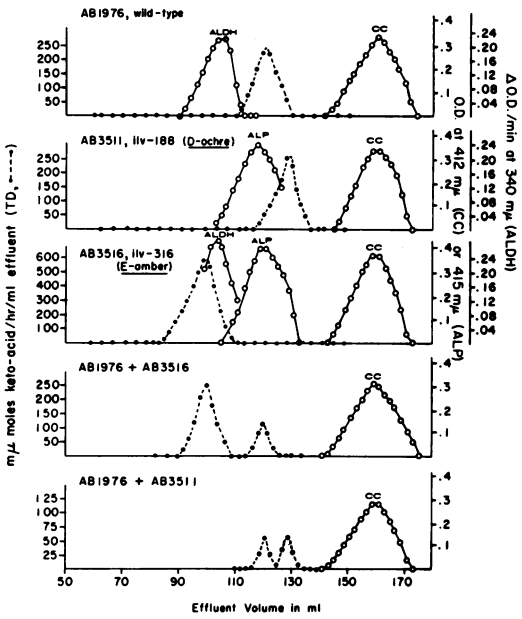

FIG. 9. *Elution diagrams for separation of proteins on Sephadex G-200 column. Each diagram represents a single column run. Threonine deaminase activity is represented by broken lines and solid circles. Abbreviations used: cc, cytochrome C; ALDH, yeast alcohol dehydrogenase; ALP, E. coli alkaline phosphatase. Wild-type and AB3511 (D-ochre) extracts were mixed at a ratio of 1:1; wild-type and AB3516 (E-amber) extracts were mixed at a ratio of 2:1.*

The various TD proteins retained their enzymological properties after emerging from the column: the wild-type TD was fully sensitive to isoleucine and activated by valine, whereas neither mutant TD responded to either isoleucine or valine. In the column runs in which each mutant TD was applied in admixture with wild-type TD, the activity corresponding to the wild-type peak was 100% sensitive to 10^{-2} M L-isoleucine and was activated by 2.5×10^{-3} M L-valine. The activities corresponding to the mutant TD peaks in these runs did not respond to either effector.

In Fig. 10, the effluent volume is plotted against the logarithm of the molecular weight according to the method of Andrews (2). The molecular weights of cytochrome *c* and *E. coli* alkaline phosphatase are 12,400 and 78,000, respectively (2). Yeast alcohol dehydrogenase has a molecular weight of 151,000 (21). Based on this analysis,

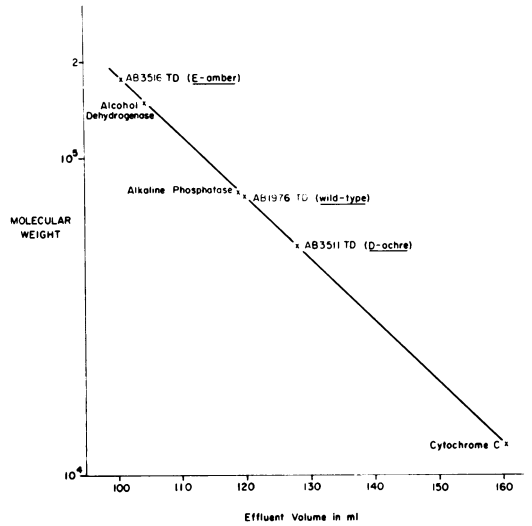

FIG. 10. *Plot, according to the method of Andrews (2), of elution volume versus log molecular weight for proteins on a Sephadex G-200 column.*

the molecular weight of the wild-type TD is approximately 76,000. The TD of AB3511 (*D-ochre*) has a molecular weight of about 53,000, and the TD of AB3516 (*E-amber*) has a molecular weight of about 176,000.

On the basis of these data, the wild-type TD from *E. coli* differs markedly from the TD of *S. typhimurium*, which has a molecular weight of 194,000 (37). The fact that the wild-type TD which emerged from the column was sensitive to isoleucine and activated by L-valine makes it unlikely that the molecular weight as observed on Sephadex G-200 is a subunit molecular weight.

Properties of enzymes from heterozygous diploid strains. Heterozygous diploids of the two nonsense mutants were derived as explained in Materials and Methods. The *ilv* regions of AB-3519, heterozygous for *ilvD188*, and of AB3524, heterozygous for *ilvE316*, are schematically diagrammed in Fig. 11. Complementation with *ilvE* and *ilvD* mutant recipients is observed in crosses with both these donors, but there is no complementation with *ilvA* mutant recipients.

Proof that AB3519 carries *ilvD188* was obtained by growing P1v on AB3518 and using the lysate to obtain *metE*$^+$ Ilv$^-$ transductants of the AB1976 wild-type strain. A lysate grown on this Met$^+$ Ilv$^-$ transductant was unable to transduce AB3511 (*ilvD188*) to Ilv$^+$. That AB3524 carries *ilvE316* was verified by obtaining the homozygous diploid, AB3526, as an Ilv$^-$ segregant of AB3524.

Cooperative interactions, characteristic of the wild-type enzyme, are apparent in the substrate kinetics of the TD from each heterozygote (Fig. 12). Furthermore, the decrease in TD activity caused by the *D-ochre* mutation in the haploid strain is abolished in the heterozygote, and the enzyme levels are at least as high as wild-type.

Strain AB3524 (*E-amber*), on the other hand, shows TD activities about 50% lower than the haploid strain carrying *ilvE316*. Furthermore, the cooperativity is accentuated in comparison to wild-type. Both the increased cooperativity and the decrease in specific activity could be explained by assuming that this TD is subject to inhibition by some component in the extract (cf. Fig. 4). It is, however, unlikely that any isoleucine was

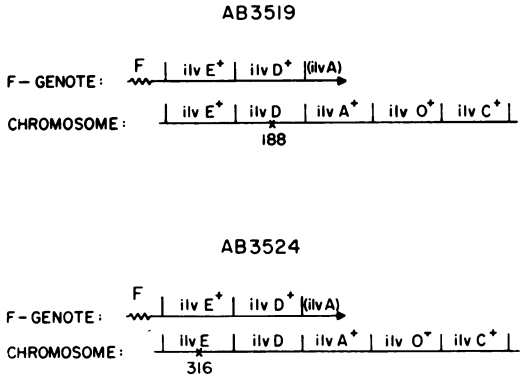

FIG. 11. *Schematic diagram of diploid condition in AB3519 and AB3524, showing the F-genote and corresponding chromosomal region.*

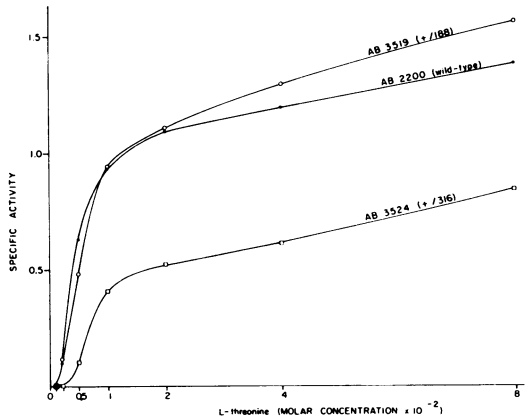

FIG. 12. *L-Threonine deaminase activity of wild-type and of the heterozygotes as a function of substrate concentration. Strains AB3519 and AB3524, heterozygous for ilvD188 and ilvE316, respectively. Strain AB2200 (wild type) is shown for comparison. Concentration of protein in the assays: AB2200, 1.52 mg/ml; AB3519, 1.48 mg/ml; AB3524, 1.56 mg/ml. Reaction mixtures were incubated at 37 C for 15 min.*

present, as the cells were grown in medium lacking isoleucine and no isoleucine was added to the cells during the preparation of the extract.

The isoleucine inhibition kinetics of the TD activities from the heterozygotes are shown in Fig. 13. AB3524 (*E-amber*) kinetics are exactly like those of wild-type; AB3519 (*D-ochre*) also exhibits inhibition kinetics quite similar to wild-type enzyme, except for the absence of activation at low isoleucine concentrations. Most importantly, both heterozygous strains produce a TD which, like the wild-type enzyme, is sensitive to isoleucine inhibition.

The TD proteins from crude extracts of the heterozygotes appear to be identical to wild-type in molecular size on the basis of diffusion studies (Table 11). All of these results indicate that TD, the product of the *ilvA* gene, is restored to a nearly normal condition in the presence of wild-type *ilvD*$^+$ and *ilvE*$^+$ alleles, even when the heterozygote still carries the *D-ochre* or *E-amber* alleles.

DISCUSSION

While this work was in progress, several reports of antipolarity appeared. In the tryptophan loci of *S. typhimurium*, a series of mutations in the phosphoribosyl transferase gene exert an apparently antipolar effect towards the anthranilate synthetase gene (3). This antipolar effect has clearly been shown to be a function of the aggregation of the protein products of these genes and is not an effect either on transcription or on translation (3).

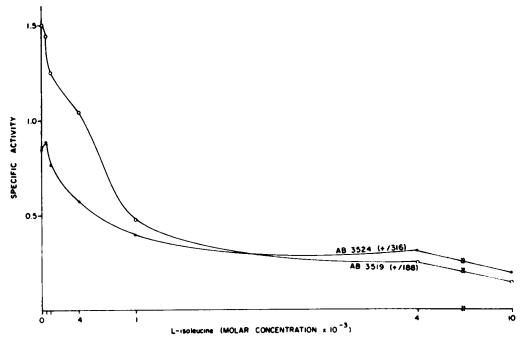

FIG. 13. *L-Threonine deaminase activity of the heterozygotes as a function of inhibitor concentration. Strains AB3519 and AB3524. Concentration of proteins in the assays: AB3519, 1.48 mg/ml; AB3524, 1.56 mg/ml. Reaction mixtures were incubated at 37 C for 15 min.*

TABLE 11. *Diffusion of threonine deaminase*[a]

Strain	*ilv*	$T_{0.1}$
		hr
AB3519	+/188	22.2
AB3524	+/316	22.2
AB1976	Wild type	22.1

[a] $T_{0.1}$ = time necessary for 10% diffusion.

In the tryptophan operon of *E. coli*, the last three genes are *C*, *B*, and *A*, in that order. Nonsense mutations in both the *A* and *B* genes show an antipolar character (36). Since it is known that the products of the *A* and *B* gene aggregate to form a functional tryptophan synthetase (35), it is logical to explain antipolarity in these two genes in terms of interference with normal aggregation. The explanation of the antipolar effect on the *C* gene product is not yet apparent.

Antipolarity has also been observed in genes coding for enzymes which form a multienzyme aggregate in the polyaromatic pathway of *Neurospora crassa* (16). The aggregation of biosynthetically related enzymes into a functional complex would seem to be an important, if not universal, factor in explaining antipolar mutations. Within the last few years, various multienzyme aggregates, aside from those mentioned above, have been reported. The *E. coli* pyruvate dehydrogenase, for example, has been shown to be a complex (22).

In view of the increasingly large number of functional multienzyme aggregates already observed, it is not unreasonable to speculate that proteins encoded by polygenic messengers from single operons may generally be aggregated. Whether the aggregation is stable or whether it is necessary for the functioning of the component parts would presumably depend on the particular aggregate. It is possible that many such multiprotein aggregates would be short-lived, even in vivo. Also, in vivo aggregates which are only moderately or weakly stable may disaggregate in cell-free extracts.

In the present work, we have characterized antipolar effects on TD of nonsense mutations in two distal genes: *ilvD*, coding for DH, and *ilvE*, coding for TRB. The mutation in *ilvE*, an *amber* mutation, causes TD to move in diffusion and gel filtration experiments as though it were much larger than the wild-type enzyme; the mutation in *ilvD*, an *ochre* mutation, causes TD to move as though it were significantly smaller than the wild-type enzyme. Both nonsense mutations alter TD so that it is completely insensitive to inhibition by L-isoleucine, the feedback inhibitor of the wild-type enzyme.

Complete lack of response to isoleucine has not been previously reported for a mutant TD of *E. coli*. Changeux (6, 7) did obtain mutants with

partially desensitized TD activities by screening for revertants of mutants lacking TD activity. Complete desensitization of *E. coli* TD has been obtained by treatment with mercurials or heat (5, 7, 12). Desensitization by either of these methods is competitively inhibited by isoleucine. Partial desensitization of TD can be obtained by growing wild-type strains in the presence of 3-fluorotyrosine (7). All the methods of desensitization alter the response of the enzyme to inhibitor more than they alter the response to substrate. Thus, feedback inhibition by isoleucine is a more labile characteristic than is the catalytic function of L-threonine deaminase.

The antipolar effects of the nonsense mutations on TD can be reversed in three ways: (i) genetic reversion of the *ilvD188* mutation restores TD completely to normal; (ii) TD regains the kinetic properties of wild type in diploid strains carrying normal $ilvE^+$ and $ilvD^+$ alleles, along with the *E-amber* or *D-ochre* mutation; and (iii) treatment of the mutant TD enzymes with 3 M urea partially restores isoleucine sensitivity.

All of these observations are compatible with the theory that the three enzymes of the *ilv* operon form a multienzyme aggregate, and that the partial peptides produced by the genes containing nonsense mutations cause abnormal aggregations in which TD is desensitized to isoleucine. The antipolar effect of the *E-amber* mutation on DH is also consistent with this theory. It is noteworthy that certain enzymes of the isoleucine-valine biosynthetic pathway appear to be aggregated in *N. crassa* (34); nevertheless, we have been unable to demonstrate such aggregation in extracts of our wild-type *E. coli* strain. The three enzymes diffuse independently of each other, and TD and DH separate readily during ammonium sulfate fractionation.

It is also possible to explain the data in terms of models which involve shared subunits of enzymes. For example, if the TD of wild-type *E. coli* contains one or more DH subunits in addition to the catalytic subunits, the effects of the *D-ochre* mutation would be explained. The effects of the *E-amber* mutation, however, would require more assumptions: either the *E* gene product is normally present as a subunit in each of the three enzymes, or else the *E-amber* mutation actually lies in a fourth gene, located between the *ilvD* and *ilvE* genes. The product of this hypothetical gene would be a subunit of both DH and TD; the inactivation of TRB by the *amber* mutation would then be a strong polar effect.

The wild-type enzyme of *S. typhimurium* contains four identical subunits (37), but neither the number of subunits nor the variation in subunit types in the *E. coli* enzyme has been determined. The data reported here, however, are consistent with the view that the TD activity of wild-type *E. coli* is associated with an aggregate of subunits, and that the presence of the partial polypeptides produced by the *D-ochre* and *E-amber* mutations causes the mutant aggregates to contain either a smaller or a larger number of subunits than is present in the wild-type aggregate.

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