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Inhibition of *Escherichia coli* Isoleucine Biosynthesis by Isoleucine Tetrazole

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Growth of a derivative of *Escherichia coli* K-10 was strongly inhibited by 2×10^{-4} M L-5(1-amino-2-methylbutyl)-tetrazole (isoleucine tetrazole). Growth inhibition was reversed by isoleucine, threonine, glycyl-L-isoleucine, or glycyl-L-threonine, and, in a valine-resistant mutant, by L-valine. Partial reversal of growth inhibition was effected by L-leucine, L-methionine, or L-homoserine. The tetrazole inhibited the activity of the biosynthetic threonine deaminase (EC 4.2.1.16 L-threonine hydrolyase [deaminating]), the inhibition being relieved by L-valine. The tetrazole also inhibited isoleucyl-transfer ribonucleic acid (tRNA) synthetase (EC 6.1.1.5 L-isoleucine: tRNA ligase [adenosine monophosphate]), but was without effect on the activities of α-isopropylmalate synthetase or acetohydroxy acid synthetase. One class of isoleucine tetrazole-resistant mutants produced biosynthetic threonine deaminases which were no longer subject to feedback inhibition by either isoleucine or the tetrazole.

In *Escherichia coli* the activities of the biosynthetic pathways leading to the formation of valine, leucine, and isoleucine are controlled in a complex way by both repression and feedback inhibition. The work of Umbarger and his colleagues, and also of other groups, has added greatly to our understanding of the relevant physiological controls and has been reviewed (31, 32). Analogues of the branched-chain amino acids have proved useful tools in these studies since they are frequently growth inhibitory and their toxicity can often be ascribed to their ability to falsely operate control mechanisms (32, 33).

A number of analogues in which the carboxyl group of a protein amino acid was replaced by a tetrazol-5-yl group have been synthesized and some of the chemical and biological properties of these 5-aminoalkyltetrazoles have been investigated (13, 21-23). We report here the inhibition of growth of *E. coli* by isoleucine tetrazole and its possible mode of action.

MATERIALS AND METHODS

Chemicals. Amino acids, dipeptides, α-keto acids, and alumina C_y were obtained from Sigma Chemical Co. Disodium adenosine 5'-triphosphate and glutathione (reduced form) were obtained from the Boehringer Corp. Sephadex G-25 was provided by Pharmacia Fine Chemicals. Diethylaminoethyl-cellulose and remaining unlabeled compounds (Analar grade where available) were obtained from British Drug Houses. Radioactive pyrophosphate, $[^{32}P]PP_i$, was produced by pyrolysis of labeled orthophosphate, $[^{32}P]P_i$, provided by the Radiochemical Centre, Amersham, England, which also supplied radioactively labeled amino acids. Preliminary experiments utilized DL-5(1-amino-2-methylbutyl)-tetrazole (isoleucine tetrazole) donated by Z. Grzonka (via L. Fowden), who also provided leucine tetrazole and phenylalanine tetrazole. All other experiments were conducted with L-isoleucine tetrazole kindly supplied by J. Morley of the Pharmaceutical Division, Imperial Chemical Industries, Macclesfield, who also provided the proline analogue L-5(2-pyrrolyl)-tetrazole (proline tetrazole).

Organisms, media, and growth conditions. *E. coli* C4, a derivative of strain K-10 (10), was used in most of the experiments described. Growth of this strain was sensitive to valine. In addition, strain DC1057, which produces high levels of the leucine biosynthetic enzymes, was used for the preparation of extracts for assay of α-isopropylmalate synthetase. This strain was kindly provided by H. E. Umbarger. *E. coli* strains AB3253 *thi⁻ arg⁻ pro⁻ his⁻ ilv⁻* and X697 *thi⁻ pur⁻ leu⁻ try⁻ his⁻ arg⁻ met⁻ ilv⁻* (only relevant markers shown) were used, as described, for detection of excretion of valine and isoleucine, respectively. These strains were provided by J. Pittard and J. Scaife, respectively. Strains MI237 *brnQ2 brnR3*, MI238 *brnQ4^{am}*, MI247 *brnR6^{am}*, and MI248 *brnS7^{am}* were kindly supplied by J. Guardiola (see reference 14 for further details). Strain LV25 was derived from strain C4 by selection for resistance to 2×10^{-4} M L-valine. This mutant was also resistant to glycyl-L-valine.

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The glucose-minerals medium (designated 232G) already described (29) was used unless otherwise stated. The glucose content was reduced to 0.5% (wt/vol) and the medium was supplemented with L-amino acids as indicated and, where necessary, with thiamine (5 μg/ml). Organisms for the preparation of aminoacyl-transfer ribonucleic acid (tRNA) synthetases were grown in 232G medium containing 0.5% (wt/vol) tryptone (Difco).

Liquid cultures were grown in Erlenmeyer flasks shaken on a reciprocating shaker at 37 C. Large quantities of organisms were produced in 1-liter cultures grown in 5- or 6-liter flasks. Solid media were produced by addition of 2% (wt/vol) agar (Difco). Analogues were added to cultures growing exponentially in 232G medium. Growth was determined by optical density measurements.

Selection of resistant mutants and excretion of amino acids by mutants. Tetrazole-resistant strains, designated ITR strains (for isoleucine tetrazole resistant), were isolated by plating between 10^8 and 10^9 organisms of strain C4 (without exposure to mutagens) on 232G agar containing 2×10^{-4} M L-isoleucine tetrazole. Resistant colonies were picked from such plates and streaked on fresh medium, and single colonies were subcultured on to 232G agar stabs.

Strains deficient in one or more of the branched-chain amino acid permeases were selected essentially by methods already described (14, 15). Organisms of strain C4 were plated on 232G agar containing valine plus methionine and leucine (VML), valine plus methionine and threonine (VMT), or valine plus methionine (VM) added at concentrations described (15). Resistant colonies were tested for ability to grow on VML, VMT, VM, and 232G agar supplemented with valine only. In those *E. coli* strains normally sensitive to valine (*val*^{s}), mutants which are resistant (*val*^{r}) due to alterations in valine transport retain their sensitivity to glycyl-valine. Sensitivity or resistance to the dipeptide was detected by inoculating parallel streaks on 232G agar. A strip of sterile Whatman 3MM paper (80 by 6 mm), moistened with filter-sterilized glycyl-L-valine solution (1 mg/ml), was laid transversely across the streaks and the plates were incubated overnight at 37 C. Sensitivity or resistance of the classes of *val*^{r} mutants to isoleucine tetrazole was detected by adding a small inoculum of an overnight culture grown in 232G medium to tubes containing 1 ml of 232G medium supplemented with 2×10^{-4} M filtered-sterilized L-isoleucine tetrazole, followed by overnight incubation at 37 C. Tetrazole-resistant (ITR) strains were also tested for their response to glycyl-valine and ability to grow in VML, VMT, VM, and valine.

Excretion of valine or isoleucine was detected by the double agar layer method (30) using, for detection of valine excretion, strain AB3253 and, for detection of isoleucine excretion, strain X697. The test organisms were suspended in 232G agar containing thiamine (5 μg/ml) and all the required amino acids (50 μg/ml) except valine (for AB3253) or isoleucine (for X697). Excretion of valine or isoleucine was detected by examining the plates for syntrophic growth of the amino acid-dependent strains.

Accumulation of amino acids. The rate of uptake of amino acids into the free amino acid pool was measured by a previously published method (30), except that the tubes contained 300 μg (dry weight) of organisms/ml.

Preparation of cellular extracts and enzyme assays. Unless otherwise stated, amino acid biosynthetic enzymes were assayed in crude extracts prepared from organisms grown to late exponential phase in 232G medium. Cultures were cooled to 0 C and harvested by centrifugation for 15 min at $15{,}000 \times g$. All further steps were conducted at 0 to 5 C. Organisms were washed and resuspended in the buffers indicated below and disrupted by treatment with an ultrasonic probe (MSE) for a total of 2 min in bursts of 30-s duration. Extracts were clarified by centrifugation for 20 min at $40{,}000 \times g$ and used immediately.

α-Isopropylmalate synthetase was assayed in *E. coli* DC1057. The cells were washed and resuspended in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.5, and extracts were prepared as described above. Before assaying for α-isopropylmalate synthetase activity, extracts were eluted with 0.05 M tris-(hydroxymethyl)aminomethane, pH 7.5, from a column (1 by 6 cm) of Sephadex G-25 previously equilibrated with the same buffer to remove phosphate ion from the extract since it has been reported that phosphotransacetylase activity results in high blank values during assay of α-isopropylmalate synthetase (7). The enzyme was assayed by determination of free —SH groups liberated from acetyl coenzyme A in the presence of α-ketoisovalerate, using the 5,5'-dithio-(bis)-2-nitrobenzoate method (18). A molar extinction coefficient of 13,600 was used for calculation of coenzyme A released (19).

Acetohydroxy acid synthetase was assayed in extracts of strain C4 after washing and resuspension of the organisms in 0.05 M potassium phosphate buffer, pH 8, containing 20 μg of thiamine pyrophosphate per ml (11). The enzyme was assayed by a published method (26) except that the concentration of flavin adenine dinucleotide was increased to 20 μg/ml (5).

Threonine deaminase was assayed by a previously published method (27) in extracts prepared from late-exponential cells of *E. coli* C4. Prior to disruption the organisms were suspended in 0.05 M potassium phosphate buffer, pH 7.5, containing 5×10^{-4} M disodium ethylenediaminetetraacetic acid and 5×10^{-4} M dithiothreitol, but lacking isoleucine (6).

Isoleucyl-tRNA synthetase was assayed in partially purified preparations obtained from exponential-phase *E. coli* C4 cells by one of two methods. The organisms were disrupted by passage through a French pressure cell (Aminco). Earlier experiments were conducted with enzyme prepared by the method of Bergmann et al. (4), except that 10% (vol/vol) glycerol was added to all solutions. Later experiments were carried out with a preparation obtained by the method of Baldwin and Berg (2). The latter procedure was followed only as far as elution from alumina C_γ, that fraction which eluted with 0.5 M phosphate buffer (pH 7.5) being used as a source of enzyme. When freshly prepared this fraction contained leucyl- and valyl-tRNA synthetase activities in addition to

isoleucyl-tRNA synthetase activity, but after storage at −20 C for more than 24 h the activities towards leucine and valine were greatly reduced. Enzyme activity was assayed by the adenosine 5'-triphosphate-[^{32}P]PP_i exchange reaction by a published method (24). Results are expressed as percentage of exchange.

RESULTS

Effect of isoleucine tetrazole on growth. Addition of 5×10^{-4} or 2×10^{-3} M L-isoleucine tetrazole to cultures of *E. coli* C4 growing exponentially in 232G medium resulted in an immediate cessation of growth. Growth inhibition by 2×10^{-4} M analogue was somewhat less pronounced, but still severe (Fig. 1). By contrast, growth was not significantly inhibited by 2×10^{-3} M DL-phenylalanine tetrazole, DL-leucine tetrazole, or L-proline tetrazole.

Reversal of growth inhibition. The growth inhibitory effect of 2×10^{-4} M L-isoleucine tetrazole was completely reversed by an equimolar amount of L-isoleucine (Fig. 1). Addition of 2×10^{-5} M L-isoleucine resulted in transitory reversal of the growth inhibition due to 2×10^{-4} M analogue; however, the growth rate decreased after about 1 h and finally ceased, presumably due to utilization of the isoleucine for protein synthesis. The growth inhibitory effect of 2×10^{-4} M L-isoleucine tetrazole was also reversed by 2×10^{-3} M L-threonine (Table 1) and was partially reversed by 2×10^{-4} M L-threonine, 2×10^{-3} M L-leucine, or 2×10^{-3} M L-methionine (Fig. 1 and Table 1). A 10-fold excess of L-homoserine effected a partial reversal of growth inhibition, but L-aspartate, L-lysine, or L-alanine were completely ineffective in reversing inhibition due to 2×10^{-4} M L-isoleucine tetrazole (Table 1).

The growth inhibitory effect of 2×10^{-4} M L-isoleucine tetrazole was also reversed by 2×10^{-3} M glycyl-L-isoleucine or 2×10^{-3} M glycyl-L-threonine, but not by the same concentration of L-prolyl-L-leucine or L-leucyl-L-tyrosine or by 10^{-3} M L-methionyl-L-methionine. In the absence of analogue, the dipeptides, with the exception of L-prolyl-L-leucine which was somewhat growth inhibitory, were without significant effect on growth (Table 1).

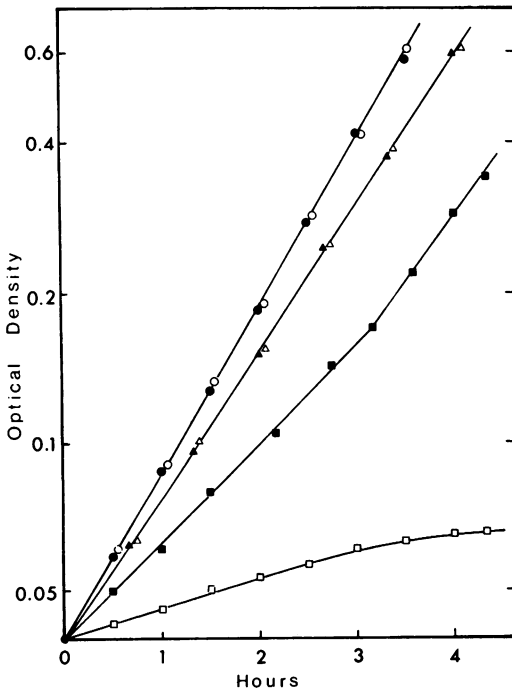

FIG. 1. *Growth of E. coli C4 in 232G medium* (●) *and in 232G containing 2×10^{-4}* M *L-isoleucine tetrazole* (□). *Growth in 232G containing 2×10^{-4} M L-isoleucine tetrazole supplemented with 2×10^{-4} M L-isoleucine* (○), *2×10^{-4} M L-threonine* (▲), *2×10^{-3} M L-leucine* (△), *or 2×10^{-3} M L-methionine* (■).

TABLE 1. *Reversal of growth inhibition due to isoleucine tetrazole by amino acids and dipeptides*

Strain	Addition (2×10^{-3} M)	Growth rate[a]: Analogue present[b]	Growth rate[a]: No analogue
C4	None	—[c]	54.4 ± 1.7[d]
	L-Ile (2×10^{-4} M)	52	
	L-Thr	56	
	L-Thr (2×10^{-4} M)	62	
	L-Leu	62	
	L-Met	88(66)[e]	
	L-Homoserine	71	
	L-Asp	—	
	L-Lys	—	
	L-Ala	—	
	Gly-L-Ile	55	55
	Gly-L-Thr	60	54
	L-Pro-L-Leu	—	72
	L-Leu-L-Tyr	—	54
	L-Met-L-Met (10^{-3} M)	—	48
LV25	None	160	48
	L-Val	57	51
	L-Val (2×10^{-4} M)	56	50
	Gly-L-Val	122	52

[a] Figures represent mean generation time in minutes.

[b] 2×10^{-4} M L-isoleucine tetrazole.

[c] —, Growth rate too low to obtain accurate estimate of mean generation time.

[d] Standard deviation of the mean of eight determinations.

[e] Figures in parentheses represent mean generation time during phase of more rapid growth (see Fig. 1).

Growth of strain LV25 was inhibited by 2×10^{-4} M isoleucine tetrazole, though the mutant was somewhat less sensitive than was the parent strain to this concentration of analogue. At 2×10^{-4} M or 2×10^{-3} M L-valine effected almost complete reversal of inhibition by 2×10^{-4} M analogue. Glycyl-L-valine (2×10^{-3} M) failed to reverse the inhibitory effect of 2×10^{-4} M analogue on growth of strain LV25 (Table 1).

Effect of isoleucine tetrazole on α-isopropylmalate synthetase activity. Activity of α-isopropylmalate synthetase in extracts of strain C4 grown in 232G was very low. Consequently the effect of the analogue on this enzyme was investigated in extracts of strain DC1057, which is derepressed for the leucine biosynthetic enzymes. Isopropylmalate synthetase was measured at pH 7.2 and 8.5 since, although the isopropylmalate synthetase of *Salmonella typhimurium* displays optimal activity at pH 8.5, maximal feedback inhibition by leucine is apparent at lower pH values (19). Although 10^{-3} M L-leucine inhibited activity of α-isopropylmalate synthetase at pH 8.5, inhibition was considerably stronger at pH 7.2. In contrast, 10^{-3} M L-isoleucine tetrazole did not inhibit the enzyme at either pH value (Table 2).

Effect on acetohydroxyacid synthetase. In common with earlier findings (34) L-valine strongly inhibited the first enzyme of valine synthesis, but at comparable concentrations isoleucine tetrazole was without effect on the activity of the enzyme (Table 2).

Effect on threonine deaminase. The reversal of growth inhibition pattern reported above suggested that isoleucine tetrazole was acting as an antagonist of isoleucine. It seemed likely that the analogue might mimic isoleucine in effecting feedback inhibition of threonine deaminase. The threonine deaminase activity of crude extracts of strain C4 was strongly inhibited by isoleucine, in agreement with observations of earlier workers (9, 12). L-Isoleucine tetrazole, when added at 5×10^{-4} M or 10^{-3} M caused, respectively, a 27 and 68% inhibition of enzyme activity, compared with about 85 and 95% inhibition brought about by isoleucine at the same concentrations (Table 3).

The inhibition of *E. coli*, *S. typhimurium*, and *Bacillus subtilis* threonine deaminases by isoleucine is antagonized by valine (9, 12, 16). Although reversal of isoleucine inhibition by valine is dependent on the relative proportions of the negative and positive effectors, the relationship is not one of simple competition. Valine also antagonized inhibition of the enzyme by isoleucine tetrazole in crude extracts of *E. coli* C4, the degree of antagonism being influenced by the relative amounts of valine and the tetrazole (Table 3).

TABLE 2. *Effect of L-isoleucine tetrazole on α-isopropylmalate (α-IPM) synthetase and acetohydroxy acid (AHA) synthetase activities in cell extracts*

Addition	Enzyme activity[a]		
	α-IPM syn[b]		AHA synthetase[c]
	pH 7.2	pH 8.5	
	0.485	0.483	10.08
10^{-4} M L-leucine	0.481	0.485	
10^{-3} M L-leucine	0	0.216	
10^{-4} M L-valine			2.90
10^{-3} M L-valine			1.81
10^{-4} M L-isoleucine tetrazole	0.477	0.516	10.09
10^{-3} M L-isoleucine tetrazole	0.444	0.477	9.74

[a] Micromoles of coenzyme A (α-IPM synthetase) or acetoin (AHA synthetase) formed per milligram of protein per hour.
[b] Strain DC1057.
[c] Strain C4.

TABLE 3. *Inhibition of threonine deaminase by isoleucine and isoleucine tetrazole*

Addition ($M \times 10^{-4}$)	Minus L-valine		Plus 10^{-2} M L-valine	
	Activity[a]	% Inhibition	Activity[a]	% Inhibition
L-Isoleucine				
	4.13	0	3.51	15
1.25	2.19	47	3.60	13
2.5	2.07	50	2.98	28
5	0.74	82	2.60	37
10	0.29	93	0.83	80
L-Isoleucine tetrazole				
5	3.02	27	4.42	(+7)
10	1.32	68	3.39	18
20	0.41	90	1.12	73

[a] Micromoles of α-ketobutyrate formed per milligram of protein per hour.

Effect on aminoacyl-tRNA synthetases. Isoleucine tetrazole strongly inhibited isoleucyl-tRNA synthetase, but the analogue did not itself promote the adenosine 5′-triphosphate-[^{32}P]PP_i exchange reaction (Table 4). At 80-fold excess of analogue over natural substrate inhibition was complete, but that the analogue was not acting as a general inhibitor of aminoacyl-tRNA synthetases was indicated by the observation that it was without significant effect on the activities of partially purified alanyl-, methionyl-, and phenylalanyl-tRNA synthetases of *E. coli* C4.

TABLE 4. *Inhibition of isoleucyl-tRNA synthetase by L-isoleucine tetrazole*

L-Isoleucine[a]	L-Isoleucine tetrazole[a]	Counts/ 100 s[b]	% Exchange
0.05		19,629	18.21
0.05	0.05	12,060	11.69
0.05	1.0	9,654	8.97
0.05	2.0	11,112	10.32
0.05	3.0	3,714	3.45
0.05	4.0	3,163	2.93
		3,149	2.92
	0.05	2,649	2.46
	2.0	2,625	2.44
	4.0	2,781	2.58

[a] Micromoles per assay tube.
[b] Average of two determinations.

Mutants resistant to isoleucine tetrazole. About 60 tetrazole-resistant (ITR) derivatives of *E. coli* C4 were selected as described. These separate isolates did not necessarily all arise as independent mutational events, but at least three classes, namely mutants possessing altered threonine deaminases and two phenotypically distinct groups of transport mutants, could be distinguished (see below). With the exception of the transport mutants, growth of all the tetrazole-resistant isolates was completely inhibited by 2×10^{-4} M L-valine. None of the resistant strains overproduced significant amounts of isoleucine or valine, as judged by their failure to cross-feed strain X697 or strain AB3253.

Some of the tetrazole-resistant mutants were chosen for further study. The growth rates of ITR 1, 5, 10, 19, 29, and 45 in 232G medium did not differ significantly from the growth rate of strain C4 in the same medium. The growth rates of strains ITR 5, ITR 10, ITR 19, and ITR 29 in 232G medium containing 2×10^{-4} M tetrazole was identical with their growth rates in 232G medium, but strains ITR 1 and ITR 45 grew somewhat more slowly in the presence of the analogue.

Threonine deaminase activity of tetrazole-resistant mutants. Preliminary screening of a group of resistant strains for sensitivity of their biosynthetic threonine deaminases to isoleucine and isoleucine tetrazole was carried out on toluene-treated organisms (Table 5). Of six strains tested, three (ITR 1, ITR 5, and ITR 19) possessed threonine deaminases which were completely resistant to inhibition by concentrations of isoleucine and isoleucine tetrazole which caused 91 and 30% inhibition, respectively, in the wild-type strain. In two mutants, ITR 10 and ITR 29, threonine deaminase retained its sensitivity to the feedback effectors and the enzyme of strain ITR 45 was still slightly inhibited by isoleucine. The observations on strains ITR 1, ITR 5, and ITR 45 were confirmed with cell-free extracts (Table 6). The specific activity of the enzyme in ITR 1 and ITR 45 was consistently lower than that observed in ITR 5 and the wild-type strain. This phenomenon has not been investigated further. Thus, one class of isoleucine tetrazole-resistant mutants possessed altered threonine deaminases which were no longer sensitive to feedback inhibition by either isoleucine or isoleucine tetrazole.

Role of branched-chain amino acid permeases. The experiments reported above showed that the growth inhibitory effect of isoleucine tetrazole was most readily reversed by isoleucine, but that a number of metabolically and structurally related amino acids could effect at least a partial reversal. Some or all of these effects could be due to competition between the analogue and the natural amino acids for entry to the organisms. This possibility was studied by investigating the ability of 5×10^{-5} M L-isoleucine tetrazole to inhibit uptake of 2 ×

TABLE 5. *Inhibition of threonine deaminase in toluene-treated tetrazole-resistant strains*

Strain	% Inhibition	
	L-Isoleucine[a]	L-Isoleucine tetrazole[a]
C4	91	30
ITR 1	0	0
ITR 5	0	0
ITR 10	88	36
ITR 19	0	4
ITR 29	87	33
ITR 45	16	4

[a] Final concentration: 5×10^{-4} M.

TABLE 6. *Threonine deaminase activity in extracts of tetrazole-resistant strains*

Addition	Sp act[a]		
	ITR 1	ITR 5	ITR 45
	2.59	4.95	2.78
5×10^{-5} M L-Ile[b]	2.59	4.57	2.64
5×10^{-4} M L-Ile	2.62	4.90	2.92
5×10^{-5} M L-IleT[c]	2.33	4.44	2.73
5×10^{-4} M L-IleT	2.39	4.65	2.92

[a] Micromoles of α-ketobutyrate formed per milligram of protein per hour.
[b] L-Isoleucine.
[c] L-Isoleucine tetrazole.

10^{-7} M L-leucine, L-isoleucine, or L-valine. In several experiments, not reported here, no significant inhibition of the initial rate of uptake of branched-chain amino acids was observed. However, since these experiments were carried out it has been reported (14, 15) that a complex of permease systems is responsible for transport of branched-chain amino acids into *E. coli* and it is evident that the substrate concentrations used in the uptake experiments were not ideal for distinguishing which, if any, of the several permeases was responsible for transport of the analogue.

Permease-deficient strains isolated and described by Guardiola and his colleagues (14, 15) were tested for their ability to grow in 232G medium containing appropriate supplements, with or without 2×10^{-4} M L-isoleucine tetrazole. Growth of strain MI248 *brnS7^am^*, which lacks the high-affinity (HA) permease inhibited by threonine (HA-2), was completely inhibited by the analogue, suggesting that this permease is not important in mediating analogue transport. Growth of strain MI238 *brnQ4^am^*, deficient in the HA permease designated HA-1 (15), was partially resistant to the tetrazole (mean generation time in absence of tetrazole, 55 min; in the presence of 2×10^{-4} M tetrazole, 164 min), indicating that HA-1 is probably partially responsible for transport of tetrazole. Strain MI237 *brnQ2 brnR3 metE* (only relevant markers shown) was completely resistant to 2×10^{-4} M isoleucine tetrazole in the presence of L-methionine (25 μg or 50 μg/ml). This strain lacks both HA-1 and HA-2 and the presence of methionine (necessary for growth) represses, and is a substrate of, the very high-affinity (VHA) permease (14). Thus, the VHA permease also appears to be involved in transport of the tetrazole.

Of 53 mutants selected for resistance to tetrazole (ITR strains), only seven, including strains ITR 10 and ITR 29 (Table 5), possessed properties consistent with their identification as transport mutants and the threonine deaminases of these strains remained sensitive to feedback control by isoleucine and isoleucine tetrazole. The seven strains were all sensitive to glycyl-valine, and from growth characteristics on media containing valine and other supplements (see Materials and Methods) and from the criteria established by Guardiola and his co-workers (14, 15), it was concluded that six of these mutants were VHA^-, HA-1^-, HA-2^+. The remaining strain displayed the phenotype VHA^+, HA-1^-, HA-2^+ and grew rather slowly in 232G medium containing 2×10^{-4} M isoleucine tetrazole.

Of 70 strains selected for valine resistance on VML, 14 were resistant to glycyl-valine and were therefore not transport mutants. Some of the VML^R, glycyl-valine-sensitive strains were plated on VM medium and 24 VM^R strains were isolated. From their phenotypic characters determined as already described, two of these secondary mutants were judged to be VHA^-, HA-1^-, HA-2^- and, as expected, were completely resistant to the tetrazole.

DISCUSSION

Due to the presence of the negatively charged tetrazol-5-yl group, the aminoakyltetrazoles can exist in zwitterionic form. The tetrazole group is a slightly weaker acid than the carboxyl group and the basicity of the amino group of aminoalkyltetrazoles is lower than that of the corresponding amino acid; nevertheless, the dissociation constants of an amino acid and its corresponding tetrazole analogue are closely comparable (21). Furthermore, molecular models indicate close spatial similarity between tetrazolyl and carboxyl groups (22).

Isoleucine tetrazole at 2×10^{-4} M severely inhibited growth of *E. coli* C4, inhibition being readily reversed by isoleucine. The transitory reversal by very low concentrations of isoleucine was ascribed to utilization of isoleucine for protein synthesis and its ultimate exhaustion from the medium. In this respect reversal of tetrazole inhibition by low levels of isoleucine exactly parallels that observed by Szentirmai and Umbarger (27) in their study of reversal of growth inhibition due to thiaisoleucine. Reversal of tetrazole inhibition by low concentrations of isoleucine suggested that the analogue was behaving as an antagonist of isoleucine. Reversal of growth inhibition by other amino acids will be discussed below.

The tetrazole was without effect on the activities of either α-isopropylmalate synthetase or acetohydroxy acid synthetase, but caused appreciable inhibition of biosynthetic threonine deaminase. Thiaisoleucine and *O*-methylthreonine also inhibit the threonine deaminase of *E. coli* (25, 27), but evidence has been presented that neither of these analogues acts by binding at the site occupied by the normal feedback inhibitor, isoleucine; hence, they cannot be acting as allosteric effectors (8, 25; J. J. Wasmuth, quoted in reference 36). The role of valine as a positive effector, antagonizing isoleucine inhibition, is well known (9, 12, 16). Valine also antagonized the inhibitory effects of isoleucine tetrazole, suggesting that the analogue was bound at the allosteric (isoleucine binding) site. On the basis of kinetic evidence other workers

have concluded that glycyl-leucine possibly binds to the isoleucine-binding site of threonine deaminase (35).

Isoleucine tetrazole also inhibited isoleucyl-tRNA synthetase. Since the analogue lacks a carboxyl group it cannot be esterified to tRNA or incorporated into proteins. Isoleucyl-tRNA synthetase of *E. coli* activates both isoleucine and valine, though the enzyme transfers only the former amino acid to tRNA (3). However, the presence of tRNA enhances the ability of the enzyme to discriminate between valine and isoleucine during formation of aminoacyl adenylate (20). It would be of interest to know whether the presence of tRNA similarly promotes a reduction in affinity of the enzyme for isoleucine tetrazole, with the possibility that, in vivo, the analogue may be a less potent inhibitor of isoleucyl-tRNA synthetase than appears from the experiment described here.

It is impossible, from the information currently available, to pinpoint precisely the primary mode of action of isoleucine tetrazole, but two lines of evidence favor the view that, in vivo, inhibition of threonine deaminase is of importance in determining the toxicity of the analogue. Antagonism of isoleucine inhibition of threonine deaminase by valine and by high concentrations of the substrate threonine and also, rather ineffectually, by methionine or homoserine has already been demonstrated (9, 12). If isoleucine tetrazole mimics isoleucine in binding at the feedback site, inhibition of threonine deaminase by the analogue may be similarly antagonized by amino acids. Such antagonism could account for the observed pattern of reversal of tetrazole-induced growth inhibition by amino acids (Fig. 1 and Table 1). The partial reversal of the growth inhibitory effect of the analogue by leucine cannot be readily explained by its effect on threonine deaminase activity. Leucine is an inhibitor of threonine deaminase, but does not antagonize isoleucine inhibition and does not display negative cooperativity with respect to substrate, as does isoleucine (35). Addition of leucine to *E. coli* K-12 growing in minimal medium results in derepression of the *ilvADE* genes, possibly due to limitation of isoleucine synthesis as a result of inhibition of threonine deaminase (35). However, derepression of threonine deaminase, the product of the *ilvA* gene, is probably not, per se, sufficient to reverse the growth inhibitory effect of isoleucine tetrazole since, although glycyl-leucine caused derepression of *ilvADE* in *E. coli*, this did not lead to resistance to the dipeptide (35).

The observation that the biosynthetic threonine deaminases of one class of tetrazole-resistant mutants were feedback resistant to both isoleucine and the tetrazole also supports the view that, in vivo, the tetrazole acts primarily by inhibition of threonine deaminase. This phenotypic property would be sufficient to imbue such strains with analogue resistance. As judged by cross-feeding experiments the resistant mutants did not overproduce appreciable amounts of isoleucine, but this does not constitute a very sensitive test for overproduction (1), and the possibility of slight overproduction with accompanying elevated intracellular levels of isoleucine cannot be discounted. If inhibition of isoleucyl-tRNA synthetase is significant in determining inhibition of growth by the analogue, even low levels of isoleucine overproduction may be sufficient to overcome inhibition of the aminoacyl-tRNA synthetase.

Available evidence suggests that the tetrazole enters *E. coli* by the VHA branched-chain amino acid permease and the HA-1 permease recently definitively described (14, 15). Identical conclusions were reached from the study of both tetrazole-resistant, transport-deficient strains isolated during the present investigation and permease-deficient strains isolated independently (14, 15) using different selection pressures. Uptake of the tetrazole thus resembles that of thiaisoleucine and *O*-methylthreonine, which are transported by the VHA permease and one, or both, of the HA permeases (14). The failure of alanine and the ability of leucine to effect only a partial reversal of growth inhibition due to the tetrazole suggests that the action of branched-chain amino acids and methionine or homoserine in reversing growth inhibition by the analogue (Table 1) is not due solely to the activities of these amino acids as substrates or effectors of repression of one or more of the branched-chain permeases (14, 15, 28). This conclusion is supported by the observation that growth inhibition by the tetrazole was reversed by glycyl-isoleucine and glycyl-threonine and since dipeptides enter *E. coli* by permease(s) distinct from the amino acid permeases, reversal by dipetides cannot be attributed to competition for entry and was presumably due to intracellular liberation of isoleucine or threonine by peptidases.

Although isoleucine tetrazole may exert toxicity by inhibition of threonine deaminase activity, other possibilities exist, such as those proposed by Wasmuth and Umbarger (36) in relation to the biological activities of thiaisoleucine and glycyl-leucine. For example, the analogue may, in conjunction with valine, retard maturation of threonine deaminase; such a retardation would result in repression of the

ilvADE genes. Another possibility is that isoleucine tetrazole could interfere with synthesis of threonine deaminase polypeptide by some form of post-transcriptional control (see reference 36).

Inhibition of *E. coli* threonine deaminase by thiaisoleucine (27) and glycyl-leucine (35) has already been mentioned. Growth and threonine deaminase activity of *Bacillus subtilis* was also inhibited by 3-cyclohexeneglycine. Growth inhibition was reversed by homoserine, threonine, or one of the branched-chain amino acids (17). Unlike isoleucine tetrazole inhibition of *E. coli* threonine deaminase, inhibition of the *B. subtilis* enzyme by cyclohexeneglycine was not relieved by valine (17). As with tetrazole-resistant *E. coli* strains, mutants resistant to thiaisoleucine and at least one mutant resistant to glycyl-leucine retained their sensitivity to valine (27, 35). Further, some glycyl-leucine-resistant strains possessed biosynthetic threonine deaminases which were no longer subject to feedback inhibition (35). Thiaisoleucine-resistant mutants of *S. typhimurium* (but not of *E. coli*) also had threonine deaminases which were less sensitive to feedback inhibition by isoleucine, compared with the wild-type enzyme (J. M. Blatt, quoted in reference 32).

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