

# Isoleucine and Valine Metabolism of *Escherichia coli*

## XIV. Effect of Thiaisoleucine

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Thiaisoleucine (2-amino-3-methylthiobutyrate) completely inhibited the growth of strain K-12 of *Escherichia coli* at a concentration of  $5 \times 10^{-3}$  M. The inhibition was antagonized by growth-factor amounts of L-isoleucine. Thiaisoleucine inhibited the deamination of threonine and the transfer of <sup>14</sup>C-isoleucine to soluble ribonucleic acid and underwent transamination with  $\alpha$ -ketoglutarate as the amino acceptor. In each case, the analogue appeared to be less effective than isoleucine as either an inhibitor or substrate.

Analogues of the naturally occurring branched-chain amino acids have been of interest since the classic studies of Gladstone (4), who described inhibition of the growth of *Bacillus anthracis* by  $\alpha$ -aminobutyrate and its antagonism by valine. At the same time, Gladstone also drew attention to the possibility that the branched-chain amino acids could be analogues of each other and lead to what were termed amino acid imbalances.

Subsequent studies with analogues of these and other amino acids have demonstrated that they might gain their inhibitory role in one of four ways: by interfering with the transport of the natural amino acid into the cell (5); by mimicking the effect of the natural amino acid as an end-product inhibitor ("false-feedback"; 8); by preventing formation of enzymes necessary for the formation of the natural amino acid ("false-repression"; 13); or by interfering with its incorporation into protein (12). The use of amino acid analogues as agents for selecting mutants has proven useful in the past in studying the uptake, regulation, and incorporation of amino acids. For this reason, the description by McCord et al. (7) of the isoleucine analogue, 2-amino-3-methylthiobutyric acid (thiaisoleucine), was of interest, and studies of its effect on *Escherichia coli* have been undertaken. The present paper describes the effect of this compound on growth and on selected enzymatic activities in crude extracts of *E. coli* K-12.

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## MATERIALS AND METHODS

*Organisms, medium, and cultivation techniques.* The K-12 strain of *E. coli* was employed in these experiments. A mineral salt-glucose medium was employed which contained: K<sub>2</sub>HPO<sub>4</sub>, 0.7%; KH<sub>2</sub>PO<sub>4</sub>, 0.3%; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1%; MgSO<sub>4</sub>·6H<sub>2</sub>O, 0.01%; glucose, 0.5%; and other supplements as indicated below. The medium was sterilized by autoclaving. Glucose was autoclaved separately and added aseptically.

The growth experiments were performed in colorimeter tubes which were capped with Morton closures (Bellco Glass Co., Vineland, N.J.) at 37 C in a tissue culture roller drum (New Brunswick Scientific Co., New Brunswick, N.J.). Growth was determined turbidimetrically in a Klett-Summerson colorimeter with a no. 42 filter. With the instrument employed, a reading of 100 Klett units represented 204  $\mu$ g (dry weight) per ml.

*Preparation of cell extracts and enzymatic assays.* Cells used for preparing extracts were grown in 1-liter quantities of minimal medium in 2-liter Erlenmeyer flasks which were incubated at 37 C in a New Brunswick incubator-shaker. The cells were harvested in the exponential phase of growth by centrifuging for 8 min at 12,000  $\times$  g in a Sorvall RC-2 centrifuge at 0 C. The cell pellets were suspended in one-tenth their growth volume of 0.05 M potassium phosphate buffer (pH 8.0) with 10<sup>-4</sup> M L-isoleucine and were again centrifuged. After a second washing, the cells were suspended in eight volumes of the same isoleucine-phosphate buffer and disrupted at 0 C with a Branson model S-75 sonifier. The suspension was then centrifuged at 25,000  $\times$  g for 15 min at 0 C, and the clear supernatant fluid was decanted. Isoleucine was used in the buffer to stabilize threonine deaminase activity (2). The quantity of isoleucine used would have exhibited detectable inhibition in the assay of threonine deaminase activity only at low substrate concentra-

tions. At the concentration of 20 mM L-threonine used in the assay (*see* below), the isoleucine carried along with the extract is without effect upon activity.

Threonine deaminase activity of the crude extracts was determined immediately after centrifuging in a 1.0-ml reaction mixture containing (in micromoles) tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*pH* 8.0), 100; ammonium chloride, 10; pyridoxal phosphate, 0.1; L-threonine, 20 (omitted from control); crude extract protein, L-isoleucine, and thiaisleucine as indicated. After 10 min at 37 C, the reaction was stopped with 0.1 ml of 50% trichloroacetic acid. Keto acids were determined by the method of Friedemann and Haugen (3).

A 1-ml reaction mixture for the measurement of transaminase B activity contained (in micromoles): potassium phosphate buffer (*pH* 8.0), 100; pyridoxal phosphate, 0.1;  $^{14}\text{C}$ -L-isoleucine, 20 (25  $\mu\text{C}$ /mmole); sodium  $\alpha$ -ketoglutarate, 20; crude extract and thiaisleucine as indicated. After 10 min at 37 C, the reaction was stopped with 0.1 ml of 50% trichloroacetic acid. The extent of transamination was determined by an adaptation of the principle that was employed earlier for determining phosphoserine-glutamate transaminase activity (18); in this method, a cation-exchange resin was used to separate a labeled amino acid from a labeled keto acid. The entire reaction mixture was applied to a 2-ml column o' Dowex-50 ( $\text{H}^+$  form) to adsorb the unreacted  $^{14}\text{C}$ -L-isoleucine along with the other cations. The tube rinsings were also applied to the column with sufficient water to yield a total effluent volume of 5.0 ml. A 1.0-ml portion of the effluent, containing the newly formed  $^{14}\text{C}$ - $\alpha$ -keto- $\beta$ -methylvalerate and the other anions of the reaction mixture, was added to a counting vial containing 15 ml of Bray's solution. A control from which the extract was omitted served to correct for any non-amino, radioactive material in the isoleucine preparation. A control tube containing extract but lacking  $\alpha$ -ketoglutarate yielded an essentially identical blank value.

Isoleucyl soluble ribonucleic acid (sRNA) synthetase activity was determined by a modification of the methods of Holley et al. (6) and of Nishimura and Novelli (10). The 0.5-ml reaction mixture contained: Tris-chloride buffer (*pH* 8), 50  $\mu\text{moles}$ ; magnesium chloride, 30  $\mu\text{moles}$ ; adenosine triphosphate (ATP), 10  $\mu\text{moles}$ ; disodium magnesium ethylene diamine-tetraacetate, 2.5  $\mu\text{moles}$ ; potassium chloride, 2  $\mu\text{moles}$ ; glutathione, 2  $\mu\text{moles}$ ;  $^{14}\text{C}$ -L-isoleucine, 5  $\text{m}\mu\text{moles}$  (2.5  $\mu\text{C}$ ); *E. coli* sRNA, 1 mg; and bacterial extract as indicated. (Extracts employed for isoleucyl sRNA synthetase activity were prepared as those described above, except that L-isoleucine was omitted from the working buffer and from the disruption buffer.) The reaction mixture without enzyme was incubated at 37 C for 1 min before adding the extract. After 10 min, 0.2 ml of the reaction mixture was transferred to Whatman no. 3 filter paper (0.75  $\times$  0.75 inches). The paper was immersed in 10% trichloroacetic acid 30 sec later. The samples were subsequently treated as described in Schwartz Bio Research Inc. Technical Brochure 66TR1, and were counted. The assay

method gave results proportional to extract concentration in the range 0 to 10  $\mu\text{g}$  of protein per tube and proportional to time of incubation for up to 12 min at one-half and at two times the indicated levels of sRNA. The sRNA was prepared from *E. coli* by the method of Zubay (19).

Thiaisleucine hydrochloride was obtained from the Reef Laboratory (Lafayette, Ind.). This compound was synthesized by the method of McCord et al. (7); whether the procedure yields one or two racemic pairs has not been established. Amino acids and pyridoxal phosphate were obtained from Calbiochem (Los Angeles, Calif.). ATP was obtained from Sigma Chemical Co. (St. Louis, Mo.), uniformly labeled  $^{14}\text{C}$ -L-isoleucine was from Schwarz Bio Research Inc. (Orangeburg, N.Y.), and "Liquifluor" was obtained from Nuclear-Chicago Corp. (Des Plaines, Ill.). Other chemicals used were reagent grade from several sources.

Radioactivity was counted in a Nuclear-Chicago model 725 liquid scintillation spectrometer in Bray's solution (1) or in a 4.2% solution of Liquifluor in toluene as indicated.

## RESULTS

*Effect of thiaisleucine on growth of E. coli.* Preliminary experiments with seeded agar plates demonstrated that thiaisleucine was markedly inhibitory to the growth of *E. coli* K-12 and that the inhibition was readily reversed by isoleucine. To determine the level of thiaisleucine that was inhibitory, a series of 5-ml cultures in Klett tubes containing minimal medium supplemented with several different levels of thiaisleucine was incubated at 37 C in a roller drum (Fig. 1).

A concentration of thiaisleucine as low as  $10^{-4}$  M caused only a slight inhibition in the rate of growth (Fig. 1A). The inhibition was greater at higher concentrations; complete inhibition of growth was observed when the medium contained  $5 \times 10^{-3}$  M thiaisleucine. When that concentration of inhibitor was added to growing cultures (Fig. 1B), there was an immediate cessation of growth as judged from measurements of turbidity.

*Reversal of the growth inhibition by L-isoleucine.* The inhibition of growth due to thiaisleucine was reversed by the addition of L-isoleucine to the medium. The inhibitory effect of  $5 \times 10^{-3}$  M racemic thiaisleucine was prevented during the entire growth period by  $5 \times 10^{-4}$  M L-isoleucine (Fig. 2A). Even one-tenth that concentration of L-isoleucine was effective in preventing the inhibition; however, at this concentration, there was a gradual onset of inhibition owing, presumably, to the utilization of the isoleucine.

When an excess of isoleucine was added to cultures containing  $5 \times 10^{-3}$  M thiaisleucine, the onset of growth occurred only after a lag of about 1 hr (Fig. 2B). By 2 hr after the addition of L-

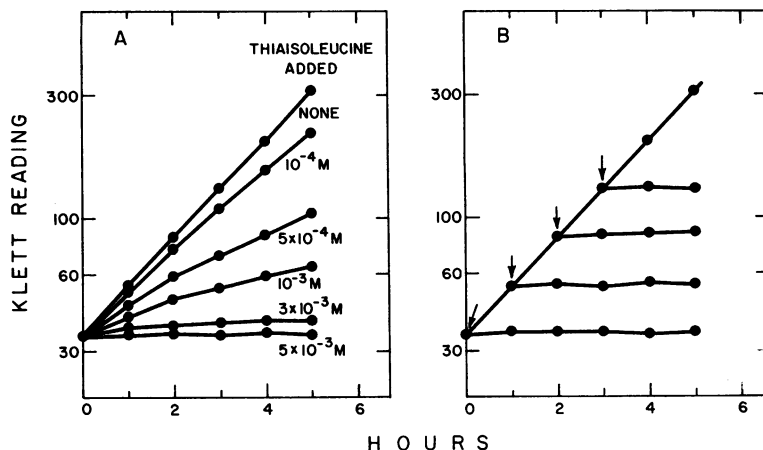


FIG. 1. Effect of thiaioleucine on the growth of *Escherichia coli*. (A) Thiaioleucine added at zero time at concentrations indicated. (B) Thiaioleucine ( $5 \times 10^{-3}$  M) added at time indicated by arrow. A reading of 100 Klett units represents 204  $\mu$ g of dry cells per ml.

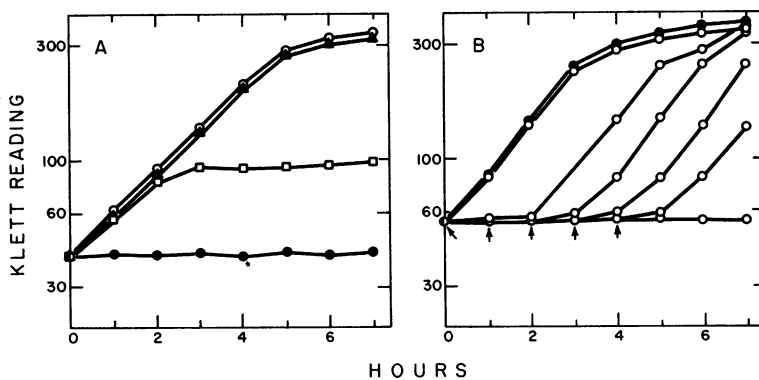


FIG. 2. Reversal by L-isoleucine of the inhibition of growth due to thiaioleucine. Symbols (A):  $\circ$ , minimal medium;  $\bullet$ , minimal medium containing  $5 \times 10^{-3}$  M thiaioleucine;  $\square$ , minimal medium containing  $5 \times 10^{-3}$  M thiaioleucine and  $5 \times 10^{-5}$  M L-isoleucine;  $\triangle$ , minimal medium containing  $5 \times 10^{-3}$  M thiaioleucine and  $5 \times 10^{-4}$  M L-isoleucine. Symbols (B):  $\bullet$ , minimal medium;  $\circ$ , minimal medium containing  $5 \times 10^{-3}$  M thiaioleucine;  $5 \times 10^{-4}$  M L-isoleucine added at time indicated by arrow. A reading of 100 Klett units represents 204  $\mu$ g of dry cells per ml.

isoleucine, the growth rate was essentially the same as that of the control culture. The length of the lag after adding L-isoleucine appeared to be the same, whether the cells had been exposed to thiaioleucine for 1 hr or for 4 hr.

**Effect of thiaioleucine on threonine deaminase.** To test whether the inhibitory effect of thiaioleucine might be due to its mimicking the effect of isoleucine as an inhibitor of threonine deaminase ("false feedback"), the sensitivity of that enzyme to thiaioleucine was examined. Thiaioleucine did, in fact, inhibit threonine deaminase activity, but only at concentrations considerably higher than those required for L-isoleucine (Table 1).

**Thiaioleucine as a substrate for transaminase B.** One other enzyme, transaminase B (14), in the pathway to isoleucine is known to have a site for isoleucine and, therefore, might be a target for inhibition by thiaioleucine. The reaction between  $^{14}\text{C}$ -L-isoleucine and  $\alpha$ -ketoglutarate, catalyzed by transaminase B, was decreased in the presence of thiaioleucine (Table 2). It was found that, to compete significantly with isoleucine, thiaioleucine had to be present in excess. (The amount of isoleucine added to the crude extract to stabilize threonine deaminase activity was such that less than 1% dilution of the labeled substrate resulted from its presence.)

That thiaioleucine was not merely competing

TABLE 1. Effect of L-isoleucine and thiaisoleucine on threonine deamination by *Escherichia coli* extracts

| Additions                   | Specific activity <sup>a</sup> |
|-----------------------------|--------------------------------|
| None.....                   | 0.066                          |
| L-Isoleucine                |                                |
| 10 <sup>-4</sup> M.....     | 0.059                          |
| 10 <sup>-3</sup> M.....     | 0.0004                         |
| Thiaisoleucine              |                                |
| 10 <sup>-2</sup> M.....     | 0.056                          |
| 2 × 10 <sup>-2</sup> M..... | 0.029                          |
| 4 × 10 <sup>-2</sup> M..... | 0.014                          |

<sup>a</sup> Expressed as micromoles per minute per milligram of protein.

TABLE 2. Effect of thiaisoleucine on transamination between <sup>14</sup>C-L-isoleucine and α-ketoglutarate

| Conditions                  | <sup>14</sup> C-L-isoleucine disappearing (μmoles) <sup>a</sup> |                   |
|-----------------------------|---|-------------------|
|                             | 20 mM L-Isoleucine  | 5 mM L-Isoleucine |
| Without thiaisoleucine..... | 83  | 56                |
| Thiaisoleucine, 100 mM..... | 26  | 8                 |
| Thiaisoleucine, 60 mM.....  | 58  | 27                |

<sup>a</sup> Reaction mixture contained 0.3 mg of protein.

with isoleucine, but could itself participate in a transamination reaction, was shown in an experiment in which the product was isolated as the 2,4-dinitrophenylhydrazone. In this experiment, a 50-ml reaction mixture containing 55 mg of crude extract protein, 10<sup>-4</sup> M pyridoxal phosphate, 10<sup>-2</sup> M α-ketoglutarate, 10<sup>-2</sup> M thiaisoleucine, and 0.1 M potassium phosphate buffer (pH 8.0) was incubated for 2 hr at 37 C. The reaction was stopped with 5 ml of 50% trichloroacetic acid and, after centrifugation, was mixed with 100 ml of 0.2% 2,4-dinitrophenylhydrazine in 1 N HCl. The mixture was extracted three times with 150 ml of toluene. The 2,4-dinitrophenylhydrazones were extracted from the toluene three times with 4 N ammonia. The ammoniacal solution was acidified with hydrochloric acid and the mixture was extracted with ether. The ethereal solution was extracted with 4 N ammonia, and the ammoniacal solution containing the acidic 2,4-dinitrophenylhydrazones was concentrated in vacuo to a small volume.

The 2,4-dinitrophenylhydrazone mixture was placed on a column (30 × 2.5 cm) of Whatman microgranular cellulose powder which had been prepared from a slurry in butyl alcohol-con-

centrated ammonia (19:2). Elution with the same solvent resulted in a separation of the 2,4-dinitrophenylhydrazone of α-keto-β-methylthiobutyric acid from that of α-ketoglutarate which remained near the top of the column. The desired product was concentrated in vacuo and recrystallized from an ethyl alcohol-water mixture. The material thus obtained was used in other experiments as a standard when the 2,4-dinitrophenylhydrazones of the keto acid analogues of thiaisoleucine and isoleucine were formed in transamination experiments and were demonstrated by paper chromatography. The *R<sub>F</sub>* of the 2,4-dinitrophenylhydrazones of the keto acids derived from thiaisoleucine and L-isoleucine were 0.76 and 0.84 in a solvent composed of *n*-butyl alcohol-concentrated aqueous ammonia (19:2).

*Thiaisoleucine as an inhibitor of the isoleucine-activating enzyme (isoleucyl sRNA synthetase)*. A third enzymatic step with which thiaisoleucine might interfere in isoleucine metabolism is one involving the incorporation of isoleucine into protein. Such an interference might occur by thiaisoleucine itself serving as a substrate for the activating enzyme and even being incorporated into protein, as has been shown for *p*-fluorophenylalanine (9), or by its acting as an inhibitor of the activating enzyme as has been shown for α-methylhistidine (15). Accordingly, the effect of thiaisoleucine as a substrate and as an inhibitor of isoleucyl sRNA synthetase was examined.

Thiaisoleucine, at a concentration 50 times that of the substrate, inhibited isoleucyl sRNA formation by 72% (Table 3). That the analogue was probably reducing the level of isoleucine incorporation by virtue of itself being a substrate is reported in the following paper (16).

## DISCUSSION

From the data presented in this paper, it is not possible to attribute the bacteriostatic effect of thiaisoleucine to any single reaction. Perhaps not surprisingly, thiaisoleucine, which differs from

TABLE 3. Effect of thiaisoleucine on the formation of isoleucyl soluble ribonucleic acid (sRNA) by an *Escherichia coli* extract

| Thiaisoleucine added     | Isoleucyl sRNA formed <sup>a</sup> (μmoles) | Percentage of inhibition |
|--------------------------|---|--------------------------|
| None                     | 0.109                                       | —                        |
| 2.5 × 10 <sup>-4</sup> M | 0.030                                       | 72                       |
| 2.5 × 10 <sup>-3</sup> M | 0.015                                       | 86                       |

<sup>a</sup> Each 0.5-ml reaction mixture contained 20 μg of bacterial protein and was incubated at 37 C. Other conditions were as indicated in Materials and Methods.

isoleucine only in having a sulfur atom instead of a  $-\text{CH}_2-$  group, appears to compete with isoleucine for binding to threonine deaminase, transaminase B, and isoleucyl sRNA synthetase. It should be emphasized that in no case did thia-isoleucine appear to be bound as effectively, or participate in the interaction as effectively, as did the natural amino acid. Thus, the strong preference of each of the enzymatic systems examined for isoleucine over thia-isoleucine was parallel to the very efficient reversal, by L-isoleucine, of the inhibitory effect of thia-isoleucine on growth.

In view of observations that the branched-chain amino acids readily interfere with the entry of each other into cells (17), and in view of the isolation of a binding protein that is presumably part of an active transport system for the branched-chain amino acids (11), it might be anticipated that thia-isoleucine and isoleucine might also compete for entry into the cell. The relatively sharp cessation of growth noted when a limiting amount ( $10^{-5}$  M) of L-isoleucine was used to antagonize thia-isoleucine (Fig. 2A) indicated that the high concentrations of analogue used probably did not interfere significantly with the entry of isoleucine itself. If there had been significant interference with entry of the last portion of isoleucine, a more gradual decrease in growth rate would have been expected. Indirect evidence that antagonism of the growth-inhibitory effect of thia-isoleucine could involve competition for entry is provided by the observation that, in minimal medium supplemented with excess L-leucine, strain K-12 is less sensitive to thia-isoleucine (S. Dwyer, *personal communication*).

Although interference with any of the susceptible reactions could lead to cessation of growth, it is not possible, from the observations made thus far, to deduce which is the actual "target." Furthermore, examination of resistant mutants may also fail to provide information on this question directly. From the very small amount of isoleucine that is necessary to achieve reversal of inhibition, any mechanism which allowed oversynthesis of isoleucine, loss of end-product inhibition, or derepression of the isoleucine- and valine-forming enzymes would be expected to result in resistance to the inhibitor. The following paper (16) describes the properties of one class of thia-isoleucine-resistant mutants.

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