

Leucyl-Transfer Ribonucleic Acid Synthetase from a Wild-Type and Temperature-Sensitive Mutant of *Salmonella typhimurium*¹

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Leucyl-transfer ribonucleic acid (tRNA) synthetase was purified 100-fold from extracts of *Salmonella typhimurium*. The partially purified enzyme had the following K_m values: leucine, 1.1×10^{-5} M; adenosine triphosphate, 6.5×10^{-4} M; tRNA^{Leu}_I, 4.1×10^{-8} M; tRNA^{Leu}_{II}, 4.3×10^{-8} M; tRNA^{Leu}_{III}, 5.3×10^{-8} M; and tRNA^{Leu}_{IV}, 2.9×10^{-8} M. The tRNA^{Leu} fractions were isolated from *Salmonella* bulk tRNA by chromatography on reversed-phase columns and benzoylated diethylaminoethyl cellulose. The enzyme had a pH optimum of 8.5 and an activation energy of 10,400 cal per mole, and was inactivated exponentially at 49.5 C with a first-order rate constant of 0.064 min^{-1} . Strain CV356 (*leuS3 leuABCD702 ara-9 gal-205*) was isolated as a mutant resistant to DL-4-azaleucine and able to grow at 27 C but not at 37 C. Extracts of strain CV356 had no leucyl-tRNA synthetase activity (charging assay) when assayed at 27 or 37 C. Temperature sensitivity and enzyme deficiency were caused by mutation in the structural gene locus specifying leucyl-tRNA synthetase. A prototrophic derivative of strain CV356 (CV357) excreted branched-chain amino acids and had high pathway-specific enzyme levels when grown at temperatures where its doubling time was near normal. At growth-restricting temperatures, both amino acid excretion and enzyme levels were further elevated. The properties of strain CV357 indicate that there is only a single leucyl-tRNA synthetase in *S. typhimurium*.

Branched-chain amino acid biosynthesis in *Salmonella typhimurium* is controlled in part by end product repression (5). The fact that mutations affecting leucyl-transfer ribonucleic acid (tRNA) synthetase sometimes lead to altered regulatory patterns (1) suggests that the synthetase may be one of the elements involved in regulation. As part of a continuing investigation into the mechanism of repression, we have purified leucyl-tRNA synthetase and studied some of its properties. In addition, we isolated a mutant strain having a temperature-sensitive leucyl-tRNA synthetase. The latter strain will be valuable in the further genetic characterization of regulatory mutants.

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MATERIALS AND METHODS

Materials. The following materials were purchased from commercial sources: L-[¹⁴C] leucine, uniformly labeled, and dipotassium adenosine triphosphate (ATP; Schwartz BioResearch, Orangeburg, N.Y.); *Escherichia coli* bulk tRNA, strain B, lot no. 652972 (General Biochemicals Corp., Chagrin Falls, Ohio); Sephadex G-150 and G-25 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.); diethylaminoethyl (DEAE) cellulose, 0.84 meq/g (Schleicher & Schuell Co., Keene, N.H.); hydroxyapatite HTP (Bio-Rad Laboratories, Richmond, Calif.); glass fiber filters, type E (Gelman Instrument Co., Ann Arbor, Mich.); membrane filters, 25-mm diameter, 0.45- μ m pore size (Matheson Higgins Co. Inc., Cambridge, Mass.); serum albumin, 10 mg of protein N/ml (Armour Pharmaceutical Co., Chicago, Ill.); streptomycin sulfate and ammonium sulfate, enzyme grade (Mann Research Lab., New York, N.Y.); Chromosorb W, DMCS-treated, mesh size 100/120 (Applied Science Labs, State College, Pa); 1,1,1,3-tetrachlorotetrafluoropropane (Peninsular Chemresearch, Gainesville, Fla.); tricaprylmethylammonium chloride (General Mills Inc., Chemical Div., Kankakee,

III.); benzoylated DEAE cellulose (Serva, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.). All other chemicals were of reagent grade quality. ICR-191 was a gift from H. J. Creech.

Strains, media, and culture techniques. All *Salmonella* strains used in this study were derivatives of *S. typhimurium* LT2 (LT2 and *ara-9 gal-205* are referred to as the wild type). A minimal salts solution (SSA) contained per liter of distilled water: K_2HPO_4 , 10.5 g; KH_2PO_4 , 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate dihydrate, 0.47 g; and $MgSO_4$, 0.05 g. SSA supplemented with 0.2% glucose and 1.5% agar served as liquid and solid medium, respectively. Nutrient broth and nutrient agar were used as complete media. Plates for the assay of leucine excretion were prepared as described previously (5). Cells were grown aerobically, usually at 37 C. Aeration was accomplished by bubbling air through the medium or by shaking on a rotary shaker.

Genetic methods. Transductions were mediated by P22 phage or the L4 variant of P22 (20); techniques described by Margolin (12) were used. For transductions involving the temperature-sensitive strain CV357 as recipient, the transduction mixture was incubated for 15 min at 30 C instead of 7 min at 37 C.

Isolation of strain CV356. One milliliter of an exponentially growing culture of *S. typhimurium ara-9 gal-205 leuABCD702* was inoculated into 20 ml of minimal medium containing 8 μ g of L-leucine per ml. One hour after growth ceased (Klett ca. 100), 10^{-2} M azaleucine was added, and the cells were incubated at 27 C for 10 hr. The cells were centrifuged, washed once with SSA, and added to 20 ml of minimal medium containing 12 μ g of L-leucine per ml. The cycle of growth on leucine, starvation, incubation with 10^{-2} M azaleucine at 27 C, harvesting, and washing was repeated two more times. After the third cycle, the remaining viable cells were grown for 14 generations at 27 C in minimal medium containing 800 μ g of L-leucine per ml. Approximately 10^7 cells was added to 20 ml of nutrient broth, and the culture was incubated at 37 C. Penicillin (2,000 units per ml) was added 60 min later, and the incubation was continued at 37 C for 90 min. Samples (0.1 ml) were plated onto 100 nutrient agar plates and incubated at 27 C until colonies could be seen (ca. 24 hr). Individual colonies (ca. 10^4) were picked onto two nutrient agar plates and screened for temperature sensitivity by incubating one plate at 27 C and the other at 37 C. After incubation for 8 hr, any clone which grew at 27 C but not 37 C was picked for further study.

Determination of growth rates. One milliliter of a stationary-phase culture was inoculated into 20 ml of the same medium in a 300-ml side arm flask (Bellco Glass, Inc., Vineland, N.J.) and shaken for 2 hr at the appropriate temperature. A 10-ml sample of the culture was removed and replaced with 10 ml of fresh medium. Turbidity measurements were made with a Klett-Summerson colorimeter (Klett Mfg. Co. Inc., New York, N.Y.) with the appropriate filter (blue for minimal medium, red for nutrient broth). A standard curve of cell number in minimal medium versus Klett value was obtained using a

model B Coulter counter with a 30- μ m aperture (1 Klett unit equals 5×10^6 cells). A standard curve for broth (nonlinear) was determined from viable counts.

Analysis of amino acid excretion. Excretion of leucine was qualitatively determined by an auxanographic technique (5). For quantitative determination of amino acid excretion, 1 ml of a stationary-phase minimal culture was inoculated into 200 ml of minimal medium in a 500-ml Erlenmeyer flask, and the flask was shaken on a gyratory shaker. Immediately after determination of the cell number by turbidimetry, the cultures were chilled in an ice bath and centrifuged in a refrigerated centrifuge at 18,000 $\times g$ for 15 min, and the resulting supernatant fluids were passed through a sterile membrane filter under suction to remove any remaining cells. The pH of the filtrate was then adjusted to 2.2 with concentrated HCl, and samples were analyzed on a Beckman model 120C amino acid analyzer. With the high-sensitivity modification, 0.0125 μ mole of an individual amino acid was readily detected. Generally, analyses were carried out only with the long column.

Enzyme assays. Cells were grown to late-log phase (ca. 7×10^8 cells/ml, inoculation adjusted to allow at least four doublings) in 500 ml of minimal or supplemented minimal medium in a 2-liter Erlenmeyer flask. They were harvested by centrifuging for 10 min at 18,000 $\times g$ in a refrigerated centrifuge and stored at -20 C. Frozen cells (0.5 to 1.0 g) were suspended in 8 to 10 ml of buffer and sonically treated for a total of 1.5 min at tap 3 using a Branson sonifier, model S110 (Heat Systems Co., Melville, N.Y.). For the assay of β -isopropylmalate (IPM) dehydrogenase and α -acetoxy acid synthetase, cells were suspended in 0.05 M phosphate, pH 8.0; for the leucyl-tRNA synthetase assay, the buffer was 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.0; 0.01 M $MgCl_2$, 0.0001 M dithiothreitol. The temperature, controlled with an ice bath, was never allowed to exceed 8 C. Cellular debris was removed by centrifuging at 18,000 $\times g$ for 10 min, and the extract was assayed immediately.

β -IPM dehydrogenase activity was measured by the procedure of Burns et al. (4). α -Acetoxy acid synthetase activity was measured by the procedure of Størmer et al. (22), except that arginine was substituted for creatine in making up the colorimetric reagents. Leucyl-tRNA synthetase [L-leucine: tRNA ligase (adenosine monophosphate), EC 6.1.1.4] was assayed after Sephadex G-25 treatment by the tRNA-charging reaction as described previously (1) and by the pyrophosphate exchange assay described by Rouget and Chapeville (16). Except where noted, all synthetase assays employed the tRNA-charging reaction. Standard assays for the latter contained (in a total volume of 1 ml): *E. coli* B bulk tRNA, 0.4 mg (A_{260} equal to 4); 50 mM Tris-hydrochloride, pH 7; 5 mM $MgCl_2$; 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7; 2.5 mM K_2H_2ATP , pH 7; 0.1 mM dithiothreitol; 5 mM NH_4Cl 0.02 mM ^{14}C -leucine, 10 mCi/mole. With the exception of tRNA, all reactants were at concentrations at least several times their K_m value. Protein

was usually determined by the method of Lowry et al. (11) with serum albumin as standard. Elution patterns from chromatographic columns were monitored by determining absorbance at 280 nm. Specific activity equals micromoles of product per minute per milligram protein.

Purification of leucyl-tRNA synthetase. All operations were carried out at 4 C, and all buffers contained 10^{-4} M dithiothreitol unless otherwise stated. Cells of strain CV123 were grown to early-log phase in minimal medium at 37 C in a 40-liter fermentor, harvested by centrifugation (yield ca. 1.5 g wet weight per liter), and stored at -20 C. Forty-five grams of frozen cells was suspended in 140 ml of 0.02 M potassium-phosphate buffer, pH 6.8, and sonically treated for a total of 4 min at tap 8 using a Branson sonifier. The temperature was controlled with an ice bath and was never allowed to exceed 8 C. Cell debris was removed by centrifuging at $15,000 \times g$ for 20 min. Solid streptomycin sulfate (2.64 g) was added to the resulting supernatant fluid (134 ml) with constant stirring over a period of 10 min. The solution was stirred for 30 min and then centrifuged at $15,000 \times g$ for 20 min. Solid ammonium sulfate (27.3 g) was added to the supernatant fluid (120 ml) over a period of 15 min to bring the concentration to 40% saturation (6). The solution was stirred for 30 min and then centrifuged at $15,000 \times g$ for 20 min (pellet discarded). The supernatant fluid was brought to 55% saturation by the slow addition of 16.5 g of solid ammonium sulfate, stirred for 30 min, and centrifuged. The pellet was suspended in 396 ml of 0.02 M phosphate buffer, pH 7.3, and the resulting solution was reduced to a volume of 130 ml in an Amicon model 400 ultrafiltration cell (Amicon Corp., Lexington, Mass.) fitted with an XM-50 membrane and run at an N_2 pressure of 30 psi. It was then diluted to a volume of 200 ml with the same buffer to bring the conductivity of the solution to 3.5 millimhos (4 C). The diluted solution was applied to a DEAE cellulose column (1.8 by 40 cm) equilibrated with 0.02 M phosphate, pH 7.3, at a flow rate of ca. 200 ml/hr. After washing with an additional 100 ml of buffer, a linear gradient consisting of 600 ml each of 0.02 M phosphate, pH 7.3, and 0.3 M phosphate, pH 6.8, was employed. The flow rate was maintained at 60 ml/hr by using a peristaltic pump, and 15-ml fractions were collected. The enzyme eluted at a conductivity of ca. 7 millimhos. Fractions containing synthetase activity were pooled, and the enzyme was precipitated by the addition of solid ammonium sulfate to 65% saturation. After centrifugation, the pellet was dissolved in 0.8 ml of 0.02 M phosphate buffer, pH 6.8, and applied to a Sephadex G-150 (1.8 by 90 cm) column equilibrated with 0.02 M phosphate, pH 6.8, followed by 2 ml of 40% sucrose in the same buffer. The column was eluted by ascending chromatography at a flow rate of 19 ml/hr, and 2.6-ml fractions were collected. The enzyme eluted shortly after the void volume. Fractions having synthetase activity were pooled and applied at gravity to a hydroxyapatite column (2 by 11 cm) equilibrated with 0.02 M phosphate, pH 6.8. A linear gradient consisting of 250 ml each of 0.02 M phosphate, pH 6.8, and 0.2 M phosphate, pH 6.8, was employed. The column was run at a flow rate of 11 ml/hr, and

2.6-ml fractions were collected. The enzyme eluted at a conductivity of ca. 4.5 millimhos.

Preparation and chromatography of tRNA^{Leu}. Cells were grown in minimal medium at 37 C to stationary phase in a 40-liter fermentor and harvested by centrifugation with a yield of ca. 2.5 g wet weight per liter. Bulk tRNA (150 mg per 100 g wet weight of cells) was isolated by the procedure of Silbert et al. (19). Bulk tRNA was chromatographed by the procedure of Weiss and Kelmers (24). The support material and gradient buffers were thoroughly deaerated before use since failure to do so resulted in irreproducible chromatographic patterns. The concentration of tRNA^{Leu} in effluent fractions was determined by completely esterifying a limiting amount of tRNA with ¹⁴C-leucine during 10 min of incubation under the assay conditions previously described. An excess of synthetase was supplied either as a crude extract or 60-fold purified preparation of enzyme. Up to 0.4 ml of a fraction could be assayed without interference from the salt present in the fraction. To insure complete precipitation of the tRNA, 0.2 mg of carrier RNA was added after trichloroacetic acid addition.

The pooled fractions from each peak of tRNA^{Leu} (150 to 300 ml) were applied to a DEAE cellulose column (0.8 by 15 cm) equilibrated with 0.01 M Tris-hydrochloride, pH 7.4, at a flow rate of ca. 200 ml/hr. The tRNA was eluted with 16 ml of 1 M NaCl in the same buffer, precipitated with three volumes of cold 95% ethanol, and allowed to stand overnight at -20 C. After centrifuging, the tRNA was washed once with 95% ethanol, recentrifuged, dried under vacuum, and stored at -4 C. The DEAE cellulose step allowed concentration of tRNA to the point where it could be precipitated efficiently by ethanol.

The individual tRNA^{Leu} species isolated from reversed-phase chromatography were chromatographed on benzoylated DEAE cellulose by the procedure of Gillam et al. (7). Benzoylated DEAE cellulose was washed before use with 1 M sodium acetate, pH 4.6, containing 2 M NaCl and 10% ethanol to remove A_{260} -absorbing impurities. The column (0.8 by 84 cm) was equilibrated with acetate-EDTA buffer (0.01 M sodium acetate, pH 4.6; 0.001 M EDTA) containing 0.04 M NaCl. Samples of the individual species combined from three reversed-phase columns were dissolved in 6 ml of 0.4 M NaCl in acetate-EDTA buffer and applied to the column by gravity. Elution was carried out at room temperature by using a linear gradient from 0.4 to 1.2 M NaCl in acetate-EDTA buffer, in a total volume of 500 ml. The column was run at a flow rate of 24 ml/hr, and 2.2-ml fractions were collected. Fractions containing tRNA^{Leu} were pooled, precipitated with three volumes of cold 95% ethanol, allowed to stand overnight at -20 C, and centrifuged. After washing once with 95% ethanol, the samples were centrifuged, dried under vacuum, and stored at -20 C.

RESULTS

Partial purification of leucyl-tRNA synthetase. Leucyl-tRNA synthetase was partially purified from *S. typhimurium* by using

standard procedures. The purification scheme summarized in Table 1 is for enzyme prepared from strain CV123 (*ara-9 gal-205 flr-123*); however, essentially the same results were obtained for preparations from the parent *ara-9 gal-205*, and the latter enzyme was used in the studies described below. The procedure resulted in an 8% yield of 100-fold purified material. The partially purified enzyme could be stored under argon at 4 C for up to 2 weeks with no loss in activity.

K_m determinations. The average K_m values for leucine (four determinations) and ATP (two determinations) were 1.1×10^{-5} M and 6.5×10^{-4} M, respectively. Representative Lineweaver-Burk plots from which K_m values were calculated are shown in Fig. 1 and 2. Except where noted, all leucyl-tRNA synthetase assays were carried out by the tRNA-charging assay.

K_m values for tRNA were determined by using bulk tRNA from *E. coli* and four chromatographically distinct species of tRNA^{Leu} from *S. typhimurium*. The latter were isolated by reversed-phase chromatography at pH 7.0 (Fig. 3) by the procedure of Weiss and Kelmers (24). The four peaks of tRNA^{Leu} are referred to as tRNA_I^{Leu}, tRNA_{II}^{Leu}, etc. By employing a shallower gradient, it was possible to resolve the second peak in Fig. 3 into two peaks, but this procedure was not used since it resulted in severe broadening of the last two peaks. From studies in which five peaks of tRNA^{Leu} were resolved, it is estimated that ca. 25% of tRNA_I^{Leu} is the minor species that normally chromatographs between the first and third peaks in a five-peak pattern. The material from each peak in Fig. 3 was further purified by chromatography on benzoylated DEAE cellulose. Assuming a specific activity of 1.6 nmoles of amino acid esterified per A_{260} unit of pure RNA (17), the purity of the recovered species was: tRNA_I^{Leu}, 17% tRNA_{II}^{Leu},

10%; tRNA_{III}^{Leu}, 10%; and tRNA_{IV}^{Leu}, 13%. Recoveries from reversed-phase chromatography and benzoylated DEAE cellulose chromatography were 50 to 70% and 80%, respectively.

Lineweaver-Burk plots of data obtained by using the tRNA^{Leu} preparations are shown in Fig. 4; the results of several experiments are given in Table 2, along with results obtained with *E. coli* tRNA. Leucyl-tRNA synthetase from *S. typhimurium* functioned somewhat more efficiently with homologous tRNA than with bulk tRNA from *E. coli*. Furthermore, it is interesting to note that the K_m for each of the four *Salmonella* preparations is essentially the same. As we previously mentioned, tRNA_I^{Leu} probably contains two species of tRNA^{Leu}, and, therefore, the observed K_m may reflect only one of the two species.

Effect of pH and temperature on leucyl-tRNA synthetase. The pH optimum of the enzyme in cacodylate and Tris buffers was about 8.5 (Fig. 5). The activity of the enzyme was at least twofold higher in Tris than in cac-

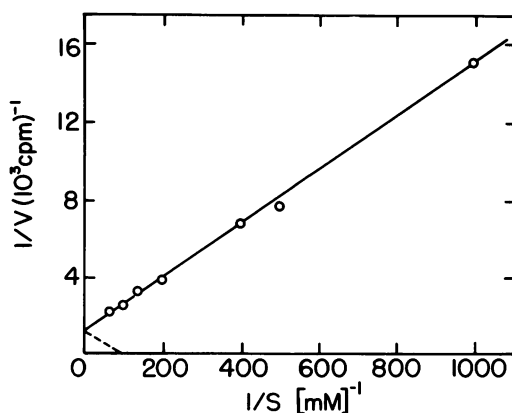


FIG. 1. Velocity (counts per min per 3 min) of leucyl-tRNA synthetase as a function of leucine concentration (Lineweaver-Burk plot). Protein, 0.27 μ g.

TABLE 1. Partial purification of leucyl-tRNA synthetase from *Salmonella typhimurium*

Fraction	Volume (ml)	Total protein (mg)	Total units ($\times 10^{-3}$)	Specific activity (units/mg)	Recovery (%)
I. Crude extract ^a	134	5,200	16	3.2	100
II. Streptomycin sulfate	120	4,100	17	4.1	106
III. Ammonium sulfate	200	1,180	9.8	8.3	61
IV. Diethylaminoethyl cellulose	117	157	3.8	24	24
V. G-150 Sephadex	23	31	2.4	76	15
VII. Hydroxyapatite	23	3.9	1.3	330	8

^a To eliminate possible interference by leucine, streptomycin sulfate, or ammonium sulfate, the supernatant fluids from this and the two subsequent fractions were passed through a Sephadex G-25 column before assay. One milliliter of supernatant fluid was applied to a column (0.8 by 15 cm) equilibrated with 0.01 M Tris-hydrochloride, pH 7.0, and eluted with the same buffer; the first milliliter past the void volume was recovered and assayed.

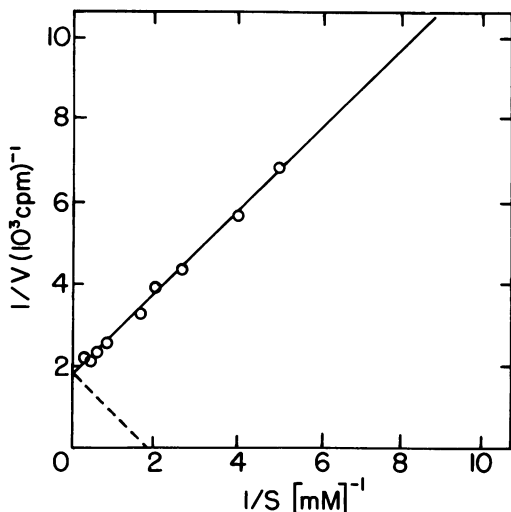


FIG. 2. Velocity (counts per min per 3 min) of leucyl-tRNA synthetase as a function of ATP concentration (Lineweaver-Burk plot). Protein, 0.27 μ g.

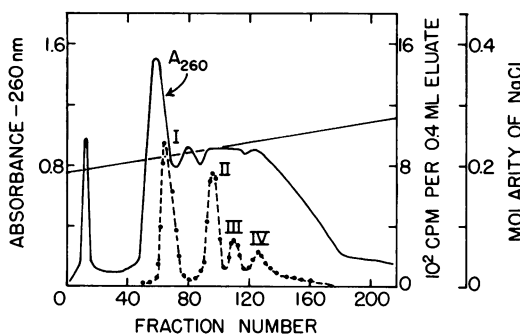


FIG. 3. Reversed-phase 2 chromatography of 100 mg of bulk tRNA from *S. typhimurium* ara-9 gal-205. The tRNA was dissolved in 10 ml of 0.01 M Tris-hydrochloride, pH 7.0, 0.19 M NaCl, 0.01 M $MgCl_2$ and applied to a jacketed column (1 by 240 cm) equilibrated with the same buffer. Elution was carried out at 37 C with a 3,800-ml linear gradient (0.19 to 0.35 M NaCl). The flow rate was 0.5 ml/min, and 10-ml fractions were collected.

odylate buffer. This is apparent in Fig. 5, from a comparison of the activities at pH 7.0, where the amount of enzyme used in cacodylate buffer was approximately twice that used in Tris buffer. In borate and carbonate buffers at pH 9, the activity of the enzyme was about one-tenth that found in Tris buffer at that same pH. The curve for activity in phosphate buffer was similar to the curve for cacodylate up to a pH of ca. 7.2, where a dramatic downward plunge of the curve occurred.

An Arrhenius plot of the increase in activity of the synthetase as a function of temperature

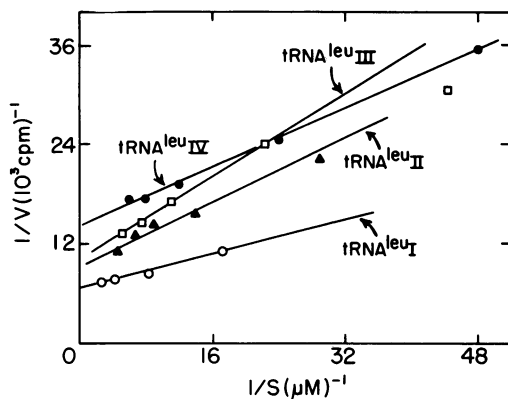


FIG. 4. Velocity (counts per min per min) of leucyl-tRNA synthetase as a function of concentration of *S. typhimurium* tRNA^{Leu}. tRNA^{Leu}, 0.18 μ g of enzyme; tRNA^{II}, 0.22 μ g of enzyme; tRNA^{III}, 0.14 μ g of enzyme, tRNA^{IV}, 0.14 μ g of enzyme.

TABLE 2. K_m values for tRNA^{Leu}

tRNA preparation ^a	K_m (moles/liter)
<i>Escherichia coli</i> tRNA ^{Leu} (bulk)	1.1×10^{-7} (2) ^b
<i>Salmonella</i> tRNA ^{Leu I}	4.1×10^{-8} (2)
<i>Salmonella</i> tRNA ^{Leu II}	4.3×10^{-8} (2)
<i>Salmonella</i> tRNA ^{Leu III}	5.3×10^{-8} (2)
<i>Salmonella</i> tRNA ^{Leu IV}	2.9×10^{-8} (1)

^a Roman numeral subscripts refer to fractions isolated from a reversed-phase column (Fig. 3).

^b Number of determinations in parentheses.

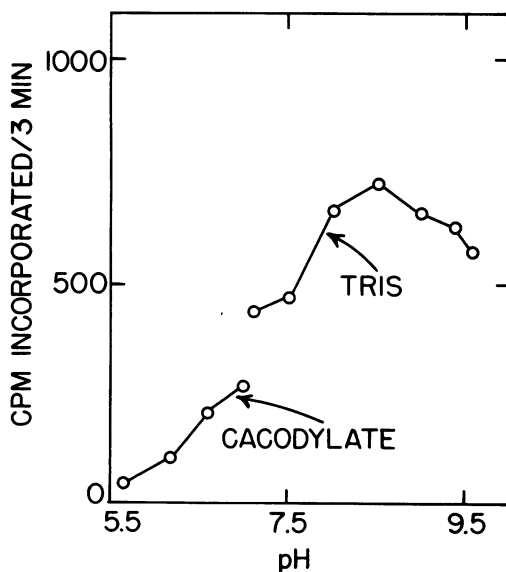


FIG. 5. Effect of pH on the activity of leucyl-tRNA synthetase. Tris buffer, 0.1 M; 0.045 μ g of protein. Cacodylate buffer, 0.1 M; 0.99 μ g of protein.

was linear to about 38 C (Fig. 6). The energy of activation calculated from this plot was 10,400 cal per mole.

The stability of the synthetase was determined by incubating the enzyme at an elevated temperature for various periods of time and then assaying at 37 C. The enzyme was completely stable after incubation for 1 hr at 42.5 C (Fig. 7). From Fig. 6, it is seen that the activity of the enzyme was reduced by about 37% when assayed at 42.5 C (in comparison with the value calculated by extrapolating the right-hand linear portion of the curve in Fig. 6 to 42.5 C). These data suggest that the reduction in activity at 42.5 C is reversible. When the temperature was raised to 49.5 C, enzyme activity decayed irreversibly by a first-order process with a rate constant of 0.064 min^{-1} . Incubation at 55 C for 5 min resulted in a loss of ca. 90% of the original activity (data not shown).

Isolation of a temperature-sensitive strain having an altered leucyl-tRNA synthetase. The incubation of $5 \times 10^{-3} \text{ M}$ DL-4-azaleucine with a culture of a leucine auxotroph starved for leucine results in death of ca. 98% of the cells (21). Death is probably caused by the incorporation of the analogue into protein. Mutants resistant to azaleucine were isolated by three cycles of growth in the presence of the analogue. Survivors of the first treatment (1.2%) were removed from azaleucine, grown in the presence of a limiting amount of leucine, starved for leucine, and then retreated with analogue. Survival was about 3% for both the second and third treatments. All operations were carried out at 27 C. Viable cells from the third cycle were subjected to penicillin treatment at 37 C (4% survival). Single-colony isolates of cells that survived penicillin were screened for those which grew at 27 C but not at 37 C on nutrient agar plates. One colony (CV356, *leuABCD702 ara-9 gal-205 leuS3*) out of 10,000 tested was temperature-sensitive. Extracts prepared from strain CV356 usually had less than 0.01% of the leucyl-tRNA synthetase activity found in comparable extracts of the parent (charging assay). Such was the case for assays carried out at 2, 27, or 37 C. In some instances, a small amount of activity (less than 10% of parent activity) was observed when the Sephadex step was omitted and the extracts were assayed immediately after preparation. The synthetase activity in wild-type extracts was not diminished upon addition of extract from strain CV356. Furthermore, the specific activities of valyl-, isoleucyl-, and phenylalanyl-tRNA synthetases were normal

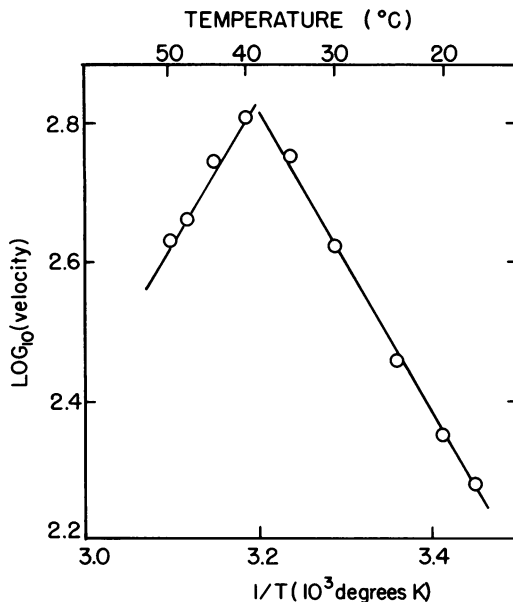


FIG. 6. Velocity of leucyl-tRNA synthetase as a function of temperature (Arrhenius plot). Phosphate buffer, (50 mM, pH 7.1) was used in the assay mixture instead of Tris buffer to avoid fluctuations in pH due to changes in temperature. Velocity equals the counts per min incorporated into trichloroacetic acid-precipitable material in 3 min. Protein, 0.99 μg .

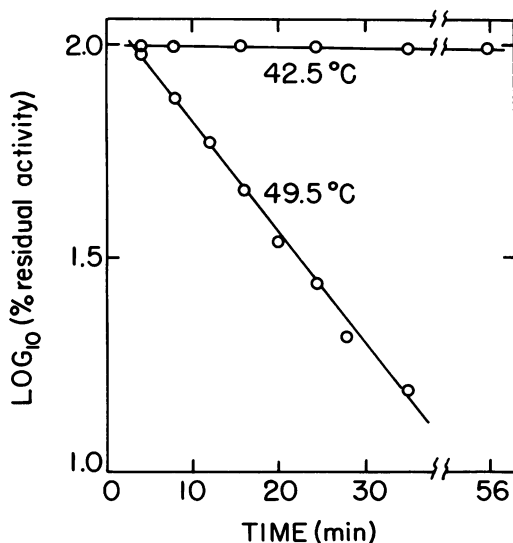


FIG. 7. Effect of temperature on the stability of leucyl-tRNA synthetase. The enzyme was incubated in the absence of substrate in 0.02 M phosphate buffer, pH 7.2, containing 0.02 M MgCl_2 and 0.0001 M dithiothreitol, at 42.5 and 49.5 C. At various time intervals, 0.1-ml samples were withdrawn and assayed at 37 C for 3 min. Protein, 1.23 μg .

in crude extracts of strain CV357. Thus, the absence of leucyl-tRNA synthetase activity is not attributable to a soluble inhibitor or to some general defect in amino acid activation. Crude extracts of strain CV357 which had been passed through Sephadex were assayed by pyrophosphate exchange. Such extracts had less than 5% of the synthetase activity observed in comparable extracts of the parent. The absence of activity probably is not attributable solely to a high K_m for leucine, because the leucine concentration in the pyrophosphate exchange assay was 2×10^{-3} M.

Is the lack of synthetase activity and temperature sensitivity caused by two mutations in widely separated genes? Strain CV356 was mixed with phage grown on the wild type, and recombinants capable of growth at 40 C on nutrient agar plates were selected. Control plates containing phage or bacteria alone had no colonies. The specific activity of leucyl-tRNA synthetase in extracts prepared from five recombinants was the same as the wild-type level, suggesting that both phenotypes are the result of either a single mutation or two closely linked mutations. Strain CV357 (*leuS3 ara-9 gal-205*) was isolated as a prototrophic recombinant from a cross between strain CV356 (*leuABCD702 leuS3 ara-9 gal-205*) and phage grown on strain *ara-9 gal-205*. When plated at 40 C on minimal medium, strain CV357 reverted, giving ca. 20 colonies per 10^9 bacteria. Nitrosoguanidine and diethyl sulfate, but not ICR-191 (acridine half mustard), increased the rate of reversion. Nine out of ten revertants excreted leucine, indicating that most of the revertants did not contain true back-mutations to the wild type.

Does the mutation in strain CV356 (*leuS3*) map in the locus coding for leucyl-tRNA synthetase? Strain CV117 (*leuS2 ara-9 gal-205*) is known to produce a leucyl-tRNA synthetase altered in its K_m for leucine and in its stability at elevated temperatures (1). This strain overproduces leucine, a phenotype which can be scored by an auxanographic test (5). Phage grown on strain CV117 were mixed with strain CV357 (*leuS3 ara-9 gal-205*), and samples were plated on minimal agar plates. Recombinants which could grow at 40 C were selected. About 98% of these recombinants had the donor phenotype (leucine excretion) indicating that *leuS2* and *leuS3* are alleles (Table 3). No excreters were observed in control experiments in which strain CV357 was transduced with wild-type phage or phage grown on strain *leuO2001* containing an operator-constitutive mutation in the leucine operon.

Properties of strain CV357. The growth rates of strains CV357 and LT2 (wild type) in minimal medium were determined as a function of temperature (Fig. 8). The parent grew faster than the mutant at all temperatures tested, the difference between them being minimal at 20 C. The growth rate of both strains increased linearly with temperature up to ca. 30 C, at which point the growth rate of strain CV357 decreased with increasing temperature until a complete cessation of growth occurred at ca. 38 C (not shown). Addition of leucine to the medium resulted in a wild-type growth rate at lower temperatures and shifted the region of severe growth restriction to higher temperature (Fig. 8).

The levels of β -IPM dehydrogenase and α -acetohydroxy acid synthetase were determined in strain CV357 grown at different temperatures. Since the leucine-forming enzymes are coordinately expressed (4), the level of β -IPM dehydrogenase serves as an indicator of the level of expression of the leucine operon. α -Acetohydroxy acid synthetase from *E. coli* is specified by one of three operons in the *ilv* cluster of genes (14). The levels of dehydrogenase and synthetase from strain CV357 grown in minimal medium were significantly derepressed at each of the growth temperatures (Fig. 9), whereas the levels of these enzymes from the wild type were low and were not affected by changes in growth temperature. At growth temperatures from 20 to 26 C, the levels of dehydrogenase and synthetase in strain CV357 were 10- to 12-fold higher than for the wild type. Growth at 28 C and above resulted in a sharp increase in the levels of dehydrogenase and synthetase to maximum values at 33 C which were ca. 40- and 100-fold higher, respectively, than those for the wild type. When strain CV357 was grown in minimal medium supplemented with leucine (100 μ g/ml), the levels of β -IPM dehydrogenase and α -acetohydroxy acid synthetase were considerably lower. At growth temperatures from 24 to 26 C, the levels of both enzymes were reduced ca. fivefold. Growth at 30 C and above increased the levels of both enzymes, but the highest levels at 34 C were only one-quarter of those found for growth at 32 C in medium lacking leucine.

The levels of leucine, valine, and isoleucine excreted by strain CV357 were significantly higher than those for the wild type (Table 4). Excretion of these amino acids by strain CV357 increased with increasing temperature, whereas the levels of excretion for the wild type remained low and relatively constant. In

TABLE 3. Cotransduction of *leuS2* (CV117) and *leuS3* (CV357)

Donor phage grown on strain	Recipient ^a strain	No. of transductants analyzed	No. of transductants excreting leucine
CV117 (<i>leuS2</i>)	CV357 (<i>leuS3</i>)	863	847 (98%)
CV6 (<i>leuO2001</i>) ^b	CV357 (<i>leuS3</i>)	50	0
LT2	CV357 (<i>leuS3</i>)	200	0

^a In each cross, selection was made for the ability to grow at 40°C on minimal agar plates. Transductants were scored for leucine excretion by an auxanographic test (5).

^b A mutation in the operator region of the leucine operon resulting in overproduction and excretion of leucine.

addition, alanine, glutamate, aspartate, glycine, and serine were excreted in small amounts by strain CV357 (not shown), but the levels were similar to those detected for the wild type at all temperatures.

DISCUSSION

Leucyl-tRNA synthetase has been purified from *Bacillus stearothermophilus* (23) and *E. coli* (2, 3, 8, 16, 18). Comparing the enzymes from *S. typhimurium* and *E. coli*, the K_m values for ATP are similar, whereas the K_m values for leucine are different (10^{-5} M for *S. typhimurium* versus ca. 10^{-4} M for *E. coli*; reference 16).

The K_m values of the *Salmonella* enzyme for four species of *Salmonella* tRNA^{Leu} were very similar, suggesting that the latter share some common structural feature. This common feature is unlikely to be the anticodon, because isoaccepting tRNA^{Leu} species from *E. coli* have different coding properties (9). Similar conclusions were reached by Roy and Söll (17), who found that seryl-tRNA synthetase from *E. coli* had nearly identical K_m values for five isoaccepting species of tRNA^{Ser}.

Genetic and biochemical data indicate that strain CV356 (and CV357) has a mutation in the structural gene coding for leucyl-tRNA synthetase. The rationale underlying the isolation of strain CV356 was as follows. Addition of azaleucine to a leucine auxotroph starved for leucine leads to death of ca. 98% of the cells in the culture (21). Because such death is probably caused by the incorporation of analogue into protein, mutants resistant to azaleucine should be either impermeable to the analogue or unable to incorporate it into protein. Mutations leading to an elevation in the rate of leucine biosynthesis are excluded because

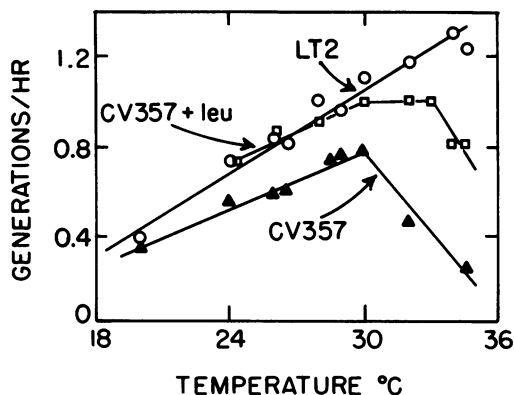


FIG. 8. Growth rates of strains CV357 and LT2 as a function of temperature. The growth rates in minimal medium represented the average of two determinations, and the growth rates of strain CV357 in minimal medium supplemented with leucine (100 µg/ml) represented the results of single determinations.

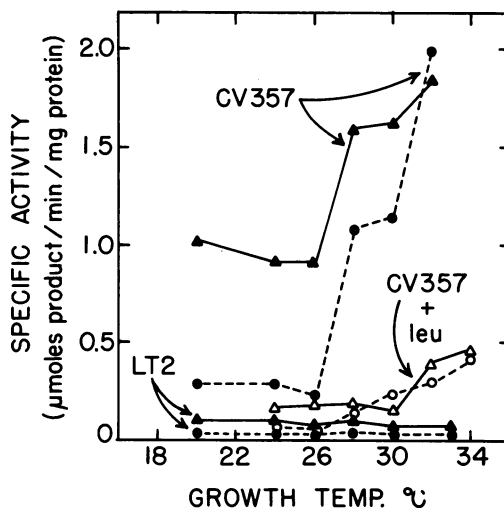


FIG. 9. Specific activity of β -IPM dehydrogenase (Δ — Δ) and α -acetoxy acid synthetase (\circ — \circ) from strains CV357 and LT2 as a function of growth temperature. Enzyme levels for strain CV357 grown in minimal medium and minimal medium plus leucine (50 µg/ml) were determined on stationary and log-phase cultures of cells, respectively. A repetition of this experiment using log-phase cells throughout gave essentially the same results.

the parent is a leucine auxotroph. The survivors after three cycles of azaleucine treatment, enriched for mutants having an altered leucyl-tRNA synthetase, were then screened for temperature-sensitive clones after penicillin selection at a high temperature.

The large amounts of branched-chain amino

TABLE 4. Amino acid excretion by strains CV357 and LT2 as a function of growth temperature

Temp (C)	Strain	Cells/ml	Amino acid excreted per cell ($\mu\text{moles} \times 10^{-12}$)		
			Valine	Leucine	Isoleucine
20	LT2	2.8×10^9	10	<1	<1
24	LT2	9.8×10^8	15	<1	<1
26	LT2	9.8×10^8	31	<1	<1
28	LT2	5.4×10^8	13	<1	<1
30	LT2	6.0×10^8	1.5	<1	<1
33	LT2	8.5×10^8	2.9	<1	<1
20	CV357	1.8×10^9	510	15	5.5
24	CV357	1.9×10^9	570	23	6.7
26	CV357	2.3×10^9	1,060	33	9.4
28	CV357	2.3×10^9	2,260	39	14
30	CV357	2.3×10^9	1,840	42	17
33	CV357	2.0×10^9	1,400	122	38

acids excreted by strain CV357 are probably caused by the elevated levels of pathway-related enzymes. The fact that at least two of these enzymes were derepressed in cells producing an excess of branched-chain amino acids indicates that high concentrations of the latter are not sufficient to cause repression. Rather, repression requires the interaction of leucine with leucyl-tRNA synthetase, a conclusion reached from the study of other activating enzyme mutants (1). Slow growth of strain CV357 is probably caused by reduced levels of charged tRNA^{Leu} owing to a damaged activating enzyme. Whether elevated levels of pathway-specific enzymes are also caused by a reduced amount of charged tRNA^{Leu} or by some property of the altered synthetase is not clear.

Extracts of strain CV357 had very low or zero leucyl-tRNA synthetase activity, even at low temperatures, when assayed by the tRNA-charging assay. Neidhardt (13) described similar findings for mutants of *E. coli* with a temperature-sensitive valyl- or phenylalanyl-tRNA synthetase. However, extracts of Neidhardt's mutants showed considerable pyrophosphate exchange activity, whereas extracts of strain CV357 had little of such activity. Presumably, sufficient activity is present in vivo to allow growth at nonrestrictive temperatures.

The possible existence of multiple leucyl-tRNA synthetases in enteric bacteria was raised by the reports of Yu (25) and Yu and Rappaport (26). Subsequent studies have indicated that the charging of tRNA^{Leu} from *E. coli* can be accomplished by a single enzyme (2, 8, 10, 15). The fact that crude extracts of

strain CV357 have no leucyl-tRNA synthetase activity suggests strongly that a single genetic locus in *S. typhimurium* specifies all the leucyl-tRNA synthetase activity observed in extracts of the wild type.

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LITERATURE CITED

- Alexander, R. R., J. M. Calvo, and M. Freundlich. 1971. Mutants of *Salmonella typhimurium* with an altered leucyl-transfer ribonucleic acid synthetase. *J. Bacteriol.* **106**:213-220.
- Bennett, T. P. 1969. Evidence for one leucyl transfer ribonucleic acid synthetase with specificity for leucine transfer ribonucleic acids with different coding characteristics. *J. Biol. Chem.* **244**:3182-3187.
- Bergmann, F. H., P. Berg, and M. Dieckmann. 1961. The enzymic synthesis of amino acyl derivatives of ribonucleic acid. II. The preparation of leucyl, valyl, isoleucyl, and methionyl ribonucleic acid synthetases from *Escherichia coli*. *J. Biol. Chem.* **236**:1735-1740.
- Burns, R. O., J. Calvo, P. Margolin, and H. E. Umbarger. 1966. Expression of the leucine operon. *J. Bacteriol.* **91**:1570-1576.
- Calvo, J. M., M. Freundlich, and H. E. Umbarger. 1969. Regulation of branched-chain amino acid biosynthesis in *Salmonella typhimurium*: isolation of regulatory mutants. *J. Bacteriol.* **97**:1272-1282.
- Dixon, M. 1953. A nomogram for ammonium sulfate solutions. *Biochem. J.* **54**:457-458.
- Gillam, I., S. Millward, D. Bleur, M. VonTigerstrom, E. Wimmer, and G. M. Tener. 1967. The separation of soluble ribonucleic acids on benzoylated diethylaminoethyl cellulose. *Biochemistry* **6**:3043-3056.
- Hayashi, H., J. R. Knowles, J. R. Katze, J. Lapointe, and D. Söll. 1970. Purification of leucyl transfer ribonucleic acid synthetase from *Escherichia coli*. *J. Biol. Chem.* **245**:1401-1406.
- Kan, J., M. W. Nirenberg, and N. Sueoka. 1970. Coding specificity of *Escherichia coli* leucine transfer ribonucleic acids and effect of bacteriophage T2 infection. *J. Mol. Biol.* **52**:179-193.
- Kan, J., and N. Sueoka. 1971. Further evidence for a single leucyl transfer ribonucleic acid synthetase capable of charging five leucine transfer ribonucleic acids in *Escherichia coli*. *J. Biol. Chem.* **246**:2207-2210.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Margolin, P. 1963. Genetic fine structure of the leucine operon in *Salmonella*. *Genetics* **48**:441-457.
- Neidhardt, F. C. 1966. Roles of amino acid activating enzymes in cellular physiology. *Bacteriol. Rev.* **30**:701-719.
- Ramakrishnan, T., and E. A. Adelberg. 1965. Regulatory mechanisms in the biosynthesis of isoleucine and valine. II. Identification of two operator genes. *J. Bacteriol.* **89**:654-660.
- Ray, D. K., and H. P. Rappaport. 1970. Further investigations of the multiple fractions of leucyl transfer ribonucleic acid synthetase activity from *Escherichia*

- coli*. Biochim. Biophys. Acta **199**:79-85.
16. Rouget, P., and F. Chapeville. 1970. Leucyl-tRNA synthetase. Purification of two interconvertible forms and evidence for an interconversion factor. Eur. J. Biochem. **14**:498-508.
 17. Roy, K. L., and D. Söll. 1970. Purification of five serine transfer ribonucleic acid species from *Escherichia coli* and their acylation by homologous seryl transfer ribonucleic acid synthetases. J. Biol. Chem. **245**:1394-1400.
 18. Seifert, W., G. Nass, and W. Zillig. 1968. Electrophoretic separation of tRNA-bound leucyl-tRNA synthetase from *Escherichia coli* extracts. J. Mol. Biol. **33**:507-511.
 19. Silbert, D. F., G. R. Fink, and B. N. Ames. 1966. Histidine regulatory mutants in *Salmonella typhimurium*. III. A class of regulatory mutants deficient in tRNA for histidine. J. Mol. Biol. **22**:335-347.
 20. Smith, H. O., and M. Levine. 1967. A phage P22 gene controlling integration of prophage. Virology **31**:207-216.
 21. Stieglitz, B. I., and J. M. Calvo. 1971. Effect of 4-azaleucine upon leucine metabolism in *Salmonella typhimurium*. J. Bacteriol. **108**:95-104.
 22. Størmer, F. C., and H. E. Umbarger. 1964. The requirement for flavine adenine dinucleotide in the formation of acetolactate by *Salmonella typhimurium* extracts. Biochem. Biophys. Res. Commun. **17**:587-592.
 23. Vanhumbecq, J., and P. Lurquin. 1969. Purification and some properties of leucyl-tRNA synthetase from *Bacillus stearothermophilus*. Eur. J. Biochem. **10**:213-218.
 24. Weiss, J. F., and A. D. Kelmers. 1967. A new chromatographic system for increased resolution of transfer ribonucleic acids. Biochemistry **6**:2507-2513.
 25. Yu, C.-T. 1966. Multiple forms of leucyl sRNA synthetase of *E. coli*. Cold Spring Harbor Symp. Quant. Biol. **31**:565-570.
 26. Yu, C.-T., and H. P. Rappaport. 1966. Multiple fractions of leucyl-transfer ribonucleic acid synthetase activity from *Escherichia coli*. Biochim. Biophys. Acta **123**:134-141.