Metabolic Regulation of the Arginyl and Valyl Transfer Ribonucleic Acid Synthetases in Bacteria*

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SUMMARY

The arginyl and valyl transfer ribonucleic acid synthetases (EC 6.1.13 and EC 6.1.1.9, respectively) in various strains of Escherichia coli and Salmonella typhimurium were measured over a wide range of steady state growth rates. Both enzymes were found to increase approximately 2.5-fold in specific activity over a 7-fold increase in growth rate. Appropriate tests indicated that this change was not the result of stimulation or inhibition of enzyme activity. Use of antibodies prepared against purified valyl-tRNA synthetase revealed differences in the amount of this enzyme in cell-free extracts of cultures grown at different growth rates. By use of a sequential labeling technique it was possible to demonstrate that the low activity of these two enzymes in extracts from slow growing cultures was not caused by specific degradation.

During transitions in growth rates brought about by shifts in media, the activity of each of these enzymes changed similarly to the accumulation of RNA, and not in a manner one would predict for enzymes regulated by amino acid supply. By sequential labeling it was shown that the increased differential rate of activity accumulation seen for these enzymes after a shift up was the result of an increased differential rate of synthesis.

The pattern of regulation of the arginyl- and valyl-tRNA synthetases seen in steady state cultures and during growth rate transitions has been termed metabolic regulation. The regulation of these two enzymes is similar under these conditions to that of other members of the bacterial cell's protein synthesizing machinery, and is difficult to reconcile with an amino acid mediated repression of these enzymes.

Most of the studies on the regulation of aminoacyl-tRNA synthetases in bacteria have explored the consequences of restricting the supply of an amino acid in growing cells (3-12). From such studies it has been learned that the formation of many of these enzymes is subject to an amino acid-specific control that resembles, in some respects, the repression of amino acid biosynthetic pathways. Growth during an amino acid restriction, for example, leads to elevated levels of both the cognate aminoacyl-tRNA synthetase and the ensemble of biosynthetic enzymes.

More recently, mutants have become available that appear to have abnormal control of individual synthetases (10, 12-15). These strains will undoubtedly be valuable in establishing the genetic and biochemical basis of the amino acid-mediated control of these enzymes.

For the most part, both of these approaches are directed at the same facet of synthetase regulation, and both grow out of the same reasonable assumption that amino acids or their tRNA derivatives ought to play some role in controlling the formation of these enzymes. It is also reasonable, however, to guess that these indispensable enzymes are subject to a control system at least as complex as that for catabolic and biosynthetic enzymes, and to search for other approaches to the problem. Struck by the control which enteric bacteria have evolved for other parts of their translation apparatus, such as ribosomal proteins (16) and RNA (17), tRNA (18), and elongation factors G and T (19), we have asked whether the level of some or all synthetases might be adjusted to match the demand for protein synthesis in cells growing at different rates.

In this paper we describe how the specific activities of two aminoacyl-tRNA synthetases vary with growth rate in various strains of Escherichia coli and Salmonella typhimurium; we present evidence that this variation is not trivial; and we show that it reflects a growth-linked control on the synthesis of these two enzymes.

MATERIALS AND METHODS

Organisms—E. coli NC1 and NC2 are prototrophic strains of E. coli B/r. Each was derived by simple, single colony isolation from a strain of B/r received from S. Cooper of The University of Michigan. Their only known phenotypic difference is that strain NC1 gives rise to large and small colonies on acetate as a sole carbon source, while NC2 gives rise to only one size colony. E. coli NP4 is the prototrophic strain of E. coli B kept in this laboratory. E. coli strain HP1802 is an arginine auxotroph.
previously described by Williams and Neidhardt (4). *S. typhimurium* NTI is a prototrophic strain derived from strain LT2 by selection for smooth growth during nitrogen limitation. It was derived from a strain of LT2 received from H. E. Umbarger (Purdue University).

**Media and Methods of Cultivation—**Growth studies were carried out in media based on a solution (designated MOPS) consisting of 40 mm potassium morpholinopropanesulfonate, pH 7.5, 4 mm Tricine (N-tris(hydroxymethyl)-methyl glycine), pH 7.5, 5.6 mm NaCl, 0.276 mm K$_2$SO$_4$, 5 x 10$^{-4}$ mm CaCl$_2$, 0.176 mm MgCl$_2$, 0.01 mm FeSO$_4$, 50 mm NaCl, 1.32 mm K$_2$HPO$_4$, 3 x 10$^{-4}$ mm (NH$_4$)$_2$Mo$_7$O$_{24}$, 4 x 10$^{-5}$ mm H$_2$BO$_3$, 3 x 10$^{-5}$ mm CoCl$_2$, 1 x 10$^{-5}$ mm CuSO$_4$, 8 x 10$^{-5}$ mm MnCl$_2$, 1 x 10$^{-5}$ mm ZnSO$_4$. The final pH of this solution was 7.3. This solution (with the exception of the K$_2$HPO$_4$) was prepared at 10 times concentration and then sterilized by filtration through a 0.2 μm membrane filter (Nalgene Filter Unit, Nalgene Sybron Corp., Rochester, New York). For use, it was diluted with sterile (autoclaved) distilled H$_2$O after which filter-sterilized K$_2$HPO$_4$ (0.132 mM) was added to bring the solution to 1.32 mm K$_2$HPO$_4$.

Four minimal media were prepared by adding to MOPS-G either 0.4% (w/v) potassium acetate, 0.4% (w/v) sucinic acid, disodium salt, 0.4% (v/v) glycerol, or 0.4% (w/v) β-galactose. These media are abbreviated as MOPS-A, MOPS-S, MOPS-glycerol, and MOPS-G, respectively.

For richer media the basic minimal medium containing glucose, MOPS-G, was commonly supplemented with either 1.5% Cae amino acids (MOPS-G-CA) or 1.0% bacteriotryptone with 0.5% yeast extract (MOPS-G-TRYE). In some experiments a mixture of 20 l-amino acids was added to the MOPS-G medium. This medium (MOPS-G-20aa) contained 0.1 mm cysteine and tryptophan; 0.2% histidine, methionine and tyrosine; 0.4 mm arginine, asparagine, aspartate, isoleucine, leucine, lysine, phenylalanine, proline, serine, and threonine; 0.6 mm glutamine, glutamate, and valine; 0.8 mm alanine and cysteine. Cysteine was always made up fresh and added just before use.

Other amino acid supplements, as described in the text, were made using the L isomer. Amino acids containing radioactive isotopes were also used only as the L isomer.

All solutions of supplementary nutrients were sterilized by filtration as described above.

Carrier cells were grown in a phosphate-buffered glucose minimal medium containing 50 mm Na$_2$HPO$_4$, 50 mm KH$_2$PO$_4$, 0.1 mm MgSO$_4$, 0.2% (w/v) (NH$_4$)$_2$SO$_4$, and 0.4% (w/v) β-galactose.

Bacterial cultures used for measuring enzyme activities were grown aerobically on a rotary action shaker (Gyrotary Water Bath Shaker, model G76, New Brunswick Co., New Brunswick, New Jersey) at 37°C. All cultures were grown in Erlenmeyer flasks with a minimum 1:5 ratio of culture volume to flask volume at a shaker speed of six. Bacterial mass was measured by optical density at 420 nm in a Zeiss PMQ2 Spectrophotometer using a 1-cm light path. Samples of growing cultures were removed by means of a sterile pipette and diluted with appropriate buffer to obtain readings in the range 0.05 to 0.5 absorbance unit. Growth rates are expressed as $k$, the first order rate constant, in units of hour$^{-1}$, as calculated from the expression

$$k = \frac{\ln 2}{\text{mass doubling time in hours}}.$$  

To shift cells from medium of one composition to another, either the culture was rapidly chilled, centrifuged, and the cells resuspended in prewarmed medium of the desired composition, or the culture (not more than 100 ml at an $A_{600}$ of 1.0) was filtered through a 142-mm membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Massachussetts), the cells were rinsed with an equal volume of prewarmed MOPS medium minus carbon source and supplements, and resuspended in prewarmed medium of the desired composition.

**Determination of Total Protein and RNA Content of Cultures—**The methods described by Neidhardt and Magasanik (20) were followed to prepare samples for assay of protein by the method of Lowry et al. (21) and RNA by the orcinol method of Schneider (22).

**Preparation of Cell-free Extracts—**Crude extracts for the measurement of enzyme activity were prepared as previously described (1) except that when glucose 6-phosphate dehydrogenase was to be assayed the extraction buffer was 0.01 M Tris-HCl, pH 7.6, with 0.01 mM MgCl$_2$, and the dialysis step was omitted. Protein content of the extracts was determined by the method of Lowry et al. (21), using bovine serum albumin as a standard.

**Assay of Aminoacyl-tRNA Synthetases—**These enzymes were assayed by the attachment of L-14C-labeled amino acid to tRNA in reaction mixtures identical to those previously described (23). Mixtures were incubated at 37°C and the reactions terminated between 3 and 10 min. The precipitates were collected on glass fiber filters (Grade 934A, Chevi Angel, Chifton, New Jersey) and washed with two 5-ml portions of cold 5% (w/v) trichloroacetic acid and two 5-ml portions of cold 67% (v/v) ethanol. Samples were dried and counted in a Beckman LS-230 scintillation counter, using a scintillation cocktail containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per liter of toluene.

Duplicate assays at a single protein concentration gave constant values for the arginine and the valine enzymes within ±2% variation. Zero time points (or other suitable background measurements) were run for each extract in all experiments. In all experiments except those dealing with small quantities (5 to 20 μl) of purified enzyme, two protein concentrations were assayed. This technique gave specific activities within approximately ±5% for each extract.

Specific activity is expressed in units per mg of protein, 1 unit being the amount of enzyme calculated as attaching 1 μmole of amino acid to tRNA per hour under the conditions employed. Relative specific activity is normalized to that of extracts from cells grown in MOPS-G.

**Assay of Ornithine Transcarbamylase—**This enzyme activity was assayed by the method of Jones et al. (24) using cell-free extracts. Citrulline was determined by the method of Archibald (25). Specific activity is reported as micromoles of citrulline formed per hour per mg of protein under the reaction conditions employed.

**Assay of Glucose 6-Phosphate Dehydrogenase—**The rate of appearance of NADPH in the assay system described by Lessie and Neidhardt (26) was used to measure this enzyme. Specific activity is reported as nanomoles of NADPH formed per min per mg of protein under the reaction conditions employed.

**Purification of Labeled Enzymes—**Samples (usually 50 ml) from radioactive cultures were rapidly chilled and the cells were collected by centrifugation at 12,000 x g for 10 min at 3°C. Each pellet was mixed with 5 to 6 g of carrier cells and resuspended in 200 ml of extraction buffer (6 mm potassium phosphate, pH 7.3, containing 6 mm 2-mercaptoethanol). This mixture was then centrifuged as above. Each pellet was resuspended in 24 ml of

$$k = \frac{\ln 2}{\text{mass doubling time in hours}}.$$
extraction buffer and treated with a Branson Sonifier for three 1-min pulses at a power setting of seven. Cell debris and unbroken cells were removed by two centrifugations at 17,000 \( \times g \) for 10 min. These extracts were made up to 1 mm MgCl\(_2\) and then centrifuged in a Beckman model L2-65B ultracentrifuge (Beckman Instruments Inc., Fullerton, California) at 165,000 \( \times g \) in a 50 Ti head for 4 hours at 3\(^\circ\). The supernatant extract was carefully removed for further enzyme purification. The pellet of ribosomes was processed according to the method of Shaw and Armstrong (27) to separate the 30 S and 50 S subunits and purify them on sucrose gradients.

The ribosomal-free extract was then resolved into three fractions by treatment with \((NH_4)_2SO_4\). The fraction that was precipitated between 35 to 70% saturated \((NH_4)_2SO_4\) was collected by centrifugation and resuspended in 10 mm Tris-HCl buffer, pH 7.8, containing 80 mm NaCl and 10 mm 2-mercaptoethanol. This suspension was dialyzed against two changes of buffer, pH 7.8, containing 80 mm NaCl and 10 mm 2-mercaptoethanol. This suspension was dialyzed against two changes of 400 volumes of the same buffer; it contained over 90% of each of the enzymes being studied.

The enzyme fraction from the \((NH_4)_2SO_4\) process was further purified on DEAE-Sephadex A 50 (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) run at 4\(^\circ\). The samples (150 mg of protein) were applied to a column (2.6 cm X 40 cm) of DEAE-Sephadex and washed with 340 ml of the buffer just described; the protein was then eluted with a linear gradient of NaCl and allowed to clot. The serum was separated from the clot by centrifugation and frozen.

Preparation of Antibodies—Following the removal of a sample of blood for nonimmune control serum, a white rabbit was immunized with 240 \( \mu \)g of approximately 95% pure valyl-tRNA synthetase from \( E. coli \) NP4. The enzyme sample (0.4 ml) was mixed with an equal volume of complete Freund's adjuvant prior to intramuscular injection. Six weeks later an identical injection was administered. Seven weeks after the first injection, 15 ml of blood were collected from the rabbit's ear vein and allowed to clot. The serum was separated from the clot by centrifugation and frozen.

RESULTS

REGULATION DURING STEADY STATE GROWTH

Specific Activities at Different Growth Rates— Cultures of \( S. typhimurium \) NT1 and \( E. coli \) B/r were grown at different steady states using different carbon sources and supplements to the minimal medium. The panels of Fig. 1 present a compilation of all such experiments. Cultures of either glucose (MOPS-G) or glucose plus Casamino acids (MOPS-G-CA) were grown to serve as standards in almost all experiments. The specific activity of extracts from MOPS-G cultures was defined as 100. That of MOPS-G-CA, when used as a control, was made equal to the average value of such an extract obtained in all those experiments in which both kinds of cultures were grown. A few measurements were also made in \( E. coli \) B (NP4) and \( E. coli \) HP1802. All of these measurements indicated that arginyl- and valyl-tRNA synthetases varied approximately 2.5-fold over a 7-fold range in growth rate. The implications of the phenomenon, coupled with the small magnitude of the change, made it necessary to explore several possible sources of error.

Examination of Possible Systematic Errors—to eliminate the possibility of a growth cycle effect (29, 30) cultures were maintained in logarithmic growth for a minimum of 10 generations before measurements were made. Several samples at different A values were taken from a single logarithmically growing culture. For all cultures and all media tested, no change in activity versus \( A \) was seen. Commonly, cultures were harvested at an absorbance of 1.0 (3 x 10\(^8\) cells per ml). This density pro-
Fig. 1. Level of arginyl-tRNA synthetase (ARS) and valyl-tRNA synthetase (VRS) at different growth rates in *S. typhimurium* NT1 and *Escherichia coli* B/r. A, the relative specific activity (ARS) is plotted as a function of the first order rate constant for growth \( k \) of *S. typhimurium*. Each • represents an independent measurement of growth rate and synthetase level in a different culture; ○ indicate the coincidence of two or more values. The values clustered about \( k = 0.3 \) are for cells growing in MOPS-A; \( k = 0.55 \), MOPS-S; \( k = 0.8 \), MOPS-glycerol; \( k = 1.0 \), MOPS-G; \( k = 1.25 \), MOPS-G-20 sa; \( k = 1.7 \), MOPS-G-CA; \( k = 2.1 \), MOPS-TGYE. The absolute level of synthetase in cells growing in MOPS-G was 0.20 unit per mg of protein. B, the relative specific activity (VRS) is plotted as a function of the growth rate in *S. typhimurium*. For explanation, see legend to A. The absolute level of synthetase in cells growing in MOPS-G was 0.50 unit per mg of protein. C, the specific activity of arginyl-tRNA synthetase is plotted as a function of the growth rate in *E. coli* B/r. The manner of plotting and the nature of the growth media are as described for A. These results include data from both strain NC1 and NC2. D, the relative specific activity of valyl-tRNA synthetase is plotted as a function of the growth rate in *E. coli* B/r. The manner of plotting and the nature of the growth media are as described for A. These results include data from both strain NC1 and strain NC2. The absolute level of synthetase in cells growing in MOPS-G was 0.60 unit per mg of protein.

The differences in specific activity were not caused by differential recovery or measurement of protein from the cultures at different growth rates. The recovery of protein in cell-free extracts showed no dramatic relationship to growth rate. To rule out a systematic error in measuring protein by the method of Lowry et al. (21), some measurements were made in which protein was assayed by means of [3H]leucine incorporated during growth of the cells. The results resembled those obtained by the Lowry method. Further, glucose 6-phosphate dehydrogenase, an enzyme reported to be regulated in a constitutive manner (31–33) was also measured over a wide range of growth rates. Its specific activity changed very little, indicating that the change in synthetase activity was unlikely to be the result of a differential recovery of soluble proteins.

The extraction process itself was not responsible for the observed differences. No systematic errors were uncovered by studies of the several processes employed in making extracts. It was noted that cells from slow growing cultures were more difficult to disrupt than cells from faster growing cultures, but no changes in specific activity were found to be related to this phenomenon (results not shown).

**Tests for Presence of Inhibitors or Stimulators**—Graf (34) has reported that partially purified aminoacyl-tRNA synthetases can be stimulated 1.5- to 2.0-fold by the addition of ribosomes. Since fast growing cells contain more ribosomes than slow growing cells, we had to examine whether or not the increase in activity observed was caused by such a stimulation. Removing the ribosomes from crude extracts did not result in a loss of activity. Instead, the specific activity of the enzymes increased to the extent expected if one had merely removed protein having no enzymatic activity.

It was possible that the extracts contained an inhibitor or stimulator of aminoacyl- and valyl-tRNA synthetases, which varied with growth rate like ribosomes but which could not be so easily removed. To test for such a factor, crude extracts from acetate and glucose-Casamino acids grown cells were mixed in several proportions. The activity from the two extracts was strictly additive (results not shown).

**Test for in Vivo Substrate Stabilization**—Williams and Neidhardt (4) have reported that the aminoacyl-tRNA synthetases may become specifically inactivated by limitation of the appropriate amino acid. We found that there was no difference in the specific activity of either the arginyl- or valyl-tRNA synthetase when cells were grown in a particular medium with or without arginine or the branched chain amino acids.

**Independent Measure of Enzyme Protein**—Antibodies were prepared against purified valyl-tRNA synthetase. Because the pure enzyme had been prepared from *E. coli* NP4, the antibody was first checked against crude extracts prepared from this organism. (The specific activities of arginyl- and valyl-tRNA synthetases vary with growth rate in strain NP4 in the same manner they do in *E. coli* B/r and *S. typhimurium* NT1.) Neutralization curves of different amounts of antibody against fixed amounts of the valine enzyme from crude extracts of MOPS-A and MOPS-G-CA are shown in Fig. 2. Since the neutralization curves have very similar initial slopes, the enzyme preparations must contain about the same amount of enzyme protein per unit of activity. The antibody was found to be about equally effective against the *E. coli* and the *S. typhimurium* enzyme.

![Fig. 1](http://www.jbc.org/)
then shifted to MOPS-A containing L-[3H]leucine. After one generation of growth the cells were shifted to a chase medium containing nonradioactive L-leucine (0.4 mM) and harvested after 1 hour. After extraction and preliminary purification the protein was applied to a DEAE-Sephadex column as described under "Materials and Methods." The material was eluted as described and assayed for various enzymatic activities. This graph shows that part of the elution profile containing all the protein. 0, micrograms per ml of protein which was estimated by A280/A360 readings. □, the 14C-amino acids attached to tRNA in the corresponding synthetase assays, i.e. arginyl-tRNA synthetase (ARS) peak, [14C]arginine attached; seryl-tRNA synthetase (SRS) peak, [14C]serine attached; and valyl-tRNA synthetase (VRS) peak, [14C]valine attached.

These experiments suggest that the change in valyl-tRNA synthetase activity with growth rate corresponds to a change in the amount of enzyme protein.

**Direct Measurement of Enzyme Turnover**—It was still possible that the low amounts of arginyl- and valyl-tRNA synthetase at low growth rates were the result of a specific, growth-linked in vivo destruction of these synthetases (35). Degradation could lead to the observed results if there were, for example, a continuous and monotonic variation of enzyme protection with growth rate. The antibody experiments reported above ruled out conversion of the active enzyme to an immunologically cross-reacting molecule. It was possible, however, that these enzymes might be completely degraded or in some other way rendered unreacting with antibody. If the 2.5-fold difference in specific activity between fast and slow growing cells was chiefly the result of destruction, it should be possible to grow cells in MOPS A and show that these enzymes were preferentially destroyed.

The method chosen was a modification of the one used by Arias et al. (36) to study synthesis and degradation of protein in mammalian systems. In our method, [14C]leucine was administered for a long growth period to achieve near complete labeling of the cell's proteins, and then a short period of [3H]leucine incorporation was used to label newly made proteins. The 3H:14C ratio is a measure of the rate of synthesis of any particular protein expressed relative to the steady state amount of that protein in the cell. During steady state growth, all stable proteins will have the same 3H:14C ratio at all times during the brief period of 3H incorporation; proteins that are undergoing significant rates of turnover will have higher 3H:14C ratios than stable proteins, and the higher the ratio the greater the rate of degradation.

A culture of *S. typhimurium* NT1 was grown for several generations in MOPS-A containing L-[14C]leucine. The cells were then shifted to MOPS-A containing L-[3H]leucine. After one generation of growth the cells were shifted to a chase medium containing nonradioactive L-leucine. After 1 hour of growth in this medium, the cells were harvested. No change in growth rate occurred during the course of the experiment and there were no noticeable lags in growth following medium shifts. The sample was then processed and analyzed by the methods already described.

Fig. 3 illustrates the elution pattern from the DEAE-Sephadex column, and Fig. 4 shows the analysis by electrophoresis on a Tris-glycine buffered gel of the pooled valyl-tRNA synthetase fractions from the column. A separate test (not shown) was made of the reliability of the gel elution technique by mixing uniformly 3H- and 14C-labeled extracts in a known ratio and analyzing the mixture on a similar gel. The reproducibility of the results and the near constant 3H:14C counting ratio assured us that if any protein in our samples had a ratio differing more than 15% from that of the average protein, it could be easily detected.

The fractions containing the highest valyl-tRNA synthetase activities from the Tris-glycine gels were pooled and analyzed on gels with a different buffering system to attempt to remove any comigrating contaminant. Fig. 5 depicts the elution pattern from a phosphate-buffered gel. The material from the Tris-glycine gel was fractionated, as shown, into two protein peaks with the same 3H:14C ratio, but only one of which had valyl-tRNA synthetase activity.
Stained for protein with Amido black and the other, frozen, THNA synthetase (vIzS)-containing fraction. Protein (200 µg) was run on each of two identical Tris-glycine 7.5% polyacrylamide gels as described under "Materials and Methods." One gel was stained for protein with Amido black and the other, frozen, sliced into 80 1-mm slices and assayed for valyl-tRNA synthetase and radioactivity as described under "Materials and Methods." Measurements from the two gels have been aligned in this figure by knowledge of their lengths. The solid line is an A_{260} scan of the stained gel. □, 14C counts found in the radioactive protein. △, the H:14C ratios calculated for each slice having more than 5 cpm of either. A, valyl-tRNA synthetase activity in 50 µl of the fraction as 10^{-2} × cpm [14C]valine attached in the standard valyl-tRNA synthetase assay.

![Fig. 4. Tris-glycine 7.5% polyacrylamide gel pattern of valyl-tRNA synthetase (VRS)-containing fraction. Protein (200 µg) from the pooled valyl-tRNA synthetase fraction (see Fig. 3) were run on each of two identical Tris-glycine 7.5% polyacrylamide gels as described under "Materials and Methods." One gel was stained for protein with Amido black and the other, frozen, sliced into 80 1-mm slices and assayed for valyl-tRNA synthetase and radioactivity as described under "Materials and Methods." Measurements from the two gels have been aligned in this figure by knowledge of their lengths. The solid line is an A_{260} scan of the stained gel. □, 14C counts found in the radioactive protein. △, the H:14C ratios calculated for each slice having more than 5 cpm of either. A, valyl-tRNA synthetase activity in 50 µl of the fraction as 10^{-2} × cpm [14C]valine attached in the standard valyl-tRNA synthetase assay.](image)

Fig. 5. Phosphate buffered 7.5% polyacrylamide gel electrophoresis of partially purified valyl-tRNA synthetase (VRS). Three gels of the pooled valyl-tRNA synthetase fraction (Fig. 3) each containing 200 µg, were run on the Tris-glycine 7.5% polyacrylamide gels as described under "Materials and Methods." The middle 4-cm segment was cut from each gel, sliced, eluted, and assayed for valyl-tRNA synthetase. The peak fractions were pooled, lyophilized as described under "Materials and Methods" and applied to two phosphate buffered 7.5% polyacrylamide gels. One was stained for protein and the other sliced into 40 1-mm slices and assayed for valyl-tRNA synthetase activity and radioactivity. This figure represents that portion of the gel containing all measurable protein. Open circles represent 11 counts; triangles, 14C counts; squares, H:14C ratios; solid circles, valyl-tRNA synthetase activity in 50 µl of sample as 10^{-2} × cpm [14C]valine attached in the standard valyl-tRNA synthetase assay.

It is, of course, possible that the ratios obtained for arginyl- and valyl-tRNA synthetases have been artificially lowered by their contamination with a comigrating stable protein, present in very large amounts. The purification data (Table I) and the relative constancy of the ratios throughout the experiment make this possibility very unlikely.

The H:14C ratios of the various protein fractions measured throughout the entire course of this experiment are given in Table I. The values for arginyl- and valyl-tRNA synthetases are close to the H:14C ratio of the total protein. The rate of degradation for these enzymes must then be very near that of an average protein. By use of the equations given previously (4) one can calculate from the labeling periods and growth rate of the culture that the H:14C ratio of these enzymes would have had to be approximately four times the average to account for the low enzyme activity of acetate grown cells solely by degradation.

### Table I. Summary of ratios from sequential label experiment

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Changes in Enzyme Activity following a Shift-up—Cells of strain NT1 were grown in either MOPS-A or MOPS-S and shifted to MOPS-G-CA. Valyl-tRNA synthetase activity was measured in samples removed at various times before and after the shift. These values were compared to those in control cultures grown at the steady state growth rate in preshift and post-shift media. Fig. 6 shows the differential rate of accumulation of valyl-tRNA synthetase. Similar data for arginyl-tRNA synthetase has already appeared in a preliminary publication (1). Both arginyl- and valyl-tRNA synthetase assume their new steady state differential rates of synthesis very soon after the shift. In this respect they behave similarly to stable RNA and not to bulk protein. It is also clear that the addition of large amounts of amino acids did not prevent this acceleration, even transiently.

Independent Measure of Enzyme Synthesis—A shift-up experiment was designed to provide a measurement of arginyl- and valyl-tRNA synthetase formation independent of enzyme activity measurements. A sequential double label experiment was performed, as in the study of degradation, but this time one label was administered to a culture before, and the other after a shift-up.

The experimental protocol was much like that described in the experiment exploring degradation in steady state growth, except that in this experiment the cells were resuspended in MOPS-G plus 20 amino acids including L-[14C]leucine after their growth in MOPS-A plus [14C]leucine. The experimental protocol is given in the legend to Fig. 7, which presents the growth of the orga-
Synthetase (VRS) in S. typhimurium NT1 at slow growth rate, and one was transferred at the time indicated by the arrow from patterns and amounts of protein recovery and enzyme activity, these cells were diluted 1:100 with cold carrier cells.

Methods.

Fig. 7 (right). Growth of S. typhimurium NT1 during a shift-up from MOPS-acetate (MOPS-A) to MOPS-glucose plus 20 amino acids (MOPS-G-20 aa). A culture of S. typhimurium NT1 growing in steady state in MOPS-A plus leucine (0.2 mM), isoleucine (0.2 mM), and valine (0.4 mM), was diluted into the same medium but containing [14C]leucine (0.14 mM, 15 μCi per pmole). (Growth points do not fit on the scale of the figure.) This culture was shifted by filtration to MOPS-G-20 aa containing [3H]leucine (0.2 mM, 30 μCi per pmole), at time zero. Samples were taken at the indicated times by filtration, washing, and resuspension in medium without isotope. These cultures were harvested after 1 hour and extracts made as described under "Materials and Methods."

Synthetase (VRS) in S. typhimurium NT1 at slow growth rate, and one was transferred at the time indicated by the arrow from patterns and amounts of protein recovery and enzyme activity, these cells were diluted 1:100 with cold carrier cells.

In this experiment, the ratio of 3H:14C varied considerably among individual proteins, as would be expected from our understanding of the regulation of gene expression in bacteria. This great variation introduced a new experimental difficulty. In the steady state experiment, a contaminant in the enzyme peaks would not have interfered with the interpretation of the results unless it were present in very large amounts. In this experiment, however, even small amounts of a contaminant, if it had a ratio much different than that of the protein of interest, could lead to an inaccurate estimate of the real ratios. For this reason, changes were made in the gel electrophoresis procedure to help achieve better separation. The percentage of polyacrylamide was increased (37), the gels were run longer, and the amount of protein added was halved. A large section of such a gel containing the valine enzyme activity is shown in Fig. 8.

Arginyl-tRNA synthetase and other proteins were examined in a similar fashion. Table II shows the 3H:14C ratios obtained from various enzymes and protein fractions from the three samples at various stages of purification. As can be seen, the isotopic ratio of the ribosomes increased considerably as they were fractionated into 30 S and 50 S subunits. This increase in the specific activity of the 15,000 × g centrifugation. One protein was noted which had very little total radioactivity compared to the amount of protein-staining material present; this protein was therefore present in low amounts in the preshift cells (acetate-grown) compared to the glucose-grown carrier cells. For this reason it was called "glucose" protein, and its interesting behavior in the table. Another protein had the reciprocal property; it was high in radioactivity per protein-staining material, and it was called "acetate" protein to designate its greater abundance in acetate-grown cells. Its behavior in the shift is also shown.

Fig. 9 shows a curve made by plotting part of this data versus the A of the culture just prior to the shift into chase medium. The differential rate of valyl-tRNA synthetase synthesis in-
creases immediately after the shift. (The line is drawn to the last point as it is the one in which the $^3$H counts are highest and hence most accurately determined.) One can also show that this is probably true for the arginine enzyme. The best data obtainable for these two enzymes (Point 3) gave ratios very close to the ones that would be predicted from activity measurements. Ribosomal protein behaved as expected from the preferential increase in stable RNA accumulation.

This experiment shows that the higher activity for both arginyl- and valyl-tRNA synthetases seen in fast growing cultures is probably the result of an increased differential rate of synthesis, and that this increased rate is established very soon after a shift-up.

**Changes in Enzyme Activity following A Shift Down**—Cells of strain NT1 were shifted by filtration from MOPS-TGYE to MOPS-G. The choice of this particular pair of media was made with the following reasoning. Although this shift would show smaller effects than one could see in shifting, for example, from MOPS-TGYE to MOPS-A, the extremely long lag due to adjustments to growth on acetate would be omitted. Also, in the shift actually performed, one might expect to see an effect caused by amino acid deprivation.

Samples were taken for extraction and for determination of protein and RNA. The differential rate of RNA accumulation calculated from these latter measurements is plotted in Fig. 10. In this same figure, data obtained from the control culture is shown. The differential rate of RNA accumulation ceased abruptly on the shift-down, and when it resumed it did so at a rate expected for the new medium.

The arginyl-tRNA synthetase data from this experiment is plotted in Fig. 11. This enzyme activity behaved similarly to RNA, although the resumption of increase began slightly earlier. The valine enzyme behaved identically (not shown).

Fig. 12 is a differential plot of the accumulation of an arginine biosynthetic enzyme, ornithine transcarbamylase, measured in the same experiment. The scale of this plot has been changed to emphasize the transition period of the shift. This biosynthetic enzyme was greatly derepressed very soon after the shift. One would expect such behavior because of the deprivation of arginine. The synthetase did not respond in this way. Rather, while these cells were apparently experiencing an arginine limitation, the synthesis of arginyl-tRNA synthetase was completely shut off.

This data has been replotted in Fig. 13 in relative values to

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**FIG. 9.** Change in $^3$H:$^1$C ratios with growth after a shift-up. The ratios of several protein fractions given in Table II have been plotted here as a function of the optical density of the sample at the time it was removed from the isotopic labeling conditions. $\square$, the $^3$H:$^1$C ratio found in the glucose protein; $\blacksquare$, acetate protein; $\triangle$, valyl-tRNA synthetase; $\blacktriangle$, arginyl-tRNA synthetase; $\odot$, 30 S ribosomal subunits; $\bullet$, total protein.

**FIG. 10 (left).** Differential rate of accumulation of RNA in *S. typhimurium* NT1 at fast growth rate, slow growth rate, and after a shift-down. RNA (microgram per ml) is plotted as a function of protein (microgram per ml). One culture was in steady state growth in MOPS-TGYE, $\bullet$; one culture was in steady state growth in MOPS-G, $\blacksquare$; one culture growing in MOPS-TGYE was transferred at the time indicated by the arrow to MOPS-G, $\triangle$.

**FIG. 11 (center).** Differential rate of accumulation of arginyl-tRNA synthetase (ARS) in *S. typhimurium* NT1 at fast growth rate, slow growth rate, and after a shift-down. Arginyl-tRNA synthetase (units per ml) is plotted as a function of protein (microgram per ml). For explanation, see legend to Fig. 10. The values given in Fig. 11 for arginyl-tRNA synthetase (ARS) during the shift-down have been replotted here. $\odot$. The protein scale has been changed from those of Figs. 10 and 11 to emphasize the time of the shift. All the points defining the steady state ornithine transcarbamylase differential rate do not fit on this scale. The values at fast growth rate (circles) represent a maximum.

**FIG. 12 (right).** Differential rate of accumulation of ornithine transcarbamylase (OTC) in *S. typhimurium* NT1 at fast growth rate, slow growth rate, and after a shift-down. Ornithine transcarbamylase (units per ml) is plotted as a function of protein (microgram per ml). For explanation, see legend to Fig. 10. The values given in Fig. 11 for arginyl-tRNA synthetase (ARS) during the shift-down have been replotted here. $\odot$. The protein scale has been changed from those of Figs. 10 and 11 to emphasize the time of the shift. All the points defining the steady state ornithine transcarbamylase differential rate do not fit on this scale. The values at fast growth rate (circles) represent a maximum.
show that the cells behaved as expected with regard to protein and RNA accumulation, and that arginyl- and valyl-tRNA synthetase behaved more like RNA than like bulk protein or ornithine transcarbamylase.

All experiments involving both shifts-up and shifts-down have shown that the levels of the arginyl- and valyl-tRNA synthetases parallel the changes in total RNA, and behave neither in a constitutive manner nor in a manner expected of biosynthetic enzymes.

**DISCUSSION**

There seems little possibility for our principal findings to be in error. The changes in specific activity of the arginyl- and valyl-tRNA synthetases with growth rate in E. coli and S. typhimurium have been shown not to be the result of any obvious systematic measuring error. On the basis of immunological analysis of the valine activity, the variation appears to be a reflection of the amount of enzyme protein present in the cells. Since the sequential labeling results remove the possibility of a significant growth rate-dependent degradation of these enzymes, the simplest interpretation of our data is that the differential rate of synthesis of these two activating enzymes is regulated concomitantly with growth rate. Until the biochemical basis of this phenomenon is uncovered and a more precise name can be suggested, we propose to call it metabolic regulation, in harmony with previous uses of this phase to designate growth-rate related control of macromolecular synthesis (38, 39).

A review of the literature reveals generally supporting evidence for the findings reported here, though as previously mentioned, no similar systematic study has been previously made. One report by Anderson and Neidhardt (23) contains results indicating no change in arginyl-tRNA synthetase in E. coli strain NP2 between rich broth and glucose minimal medium. Preliminary results indicate that this result may be related, in an as yet unclear manner, to the fact that strain NP2 is a pyrimidine bradytroph, a fact not known during the original work. Another report, by Cassio et al. (40), contains incidental information showing no significant difference in the level of valyl-tRNA synthetase in E. coli cells in rich broth and glucose minimal medium; we do not know the basis for this difference from our results; discounting any experimental error, their strain may be a variant lacking metabolic regulation of the valine enzyme. Data more consistent with those reported by us are to be found in several other papers (arginyl- and glutamyl-tRNA synthetases in E. coli K12 (41); valyl-tRNA synthetase in E. coli K12 (23), and the same enzyme in Streptococcus thermophilus (42)).

One possibility that deserves consideration is that instances of metabolic regulation are the result of differential gene dosage. Copies of genes located near the point at which replication of the bacterial chromosome begins are known to be increased, relative to genes located near the terminus, at increased growth rates (e.g. Ref. 43), because of the multiforked nature of the replicating chromosome. The structural gene (SAL2) for the valine enzyme is located in the vicinity of the initiation point, but the analogous gene (argS) for the arginine enzyme is located near the terminus (44), and so this explanation must be rejected as a general mechanism for both enzymes. Moreover, the behavior of neither synthetase during transitions from one growth rate to another can be explained by the gene dosage hypothesis. The nearly immediate rise in differential rate of enzyme synthesis following a shift-up and the prolonged repression following a shift-down are not predicted by the gene dosage hypothesis whatever the location of the genes of the enzymes involved.

The major implication of our findings is that they cannot be easily explained by any of the several mechanisms postulated to account for amino acid mediated repression of individual synthetases (3-12). During steady state growth, and during nutritionally induced shifts-up or down in growth rate, metabolic regulation of arginyl- and valyl-tRNA synthetases results in the opposite response expected for any process in which amino acids or their derivatives act as repressors.

Whatever the mechanism of metabolic regulation, it appears not to govern all 20 aminoacyl-tRNA synthetases similarly; preliminary results with the serine and methionine enzymes of S. typhimurium indicate a minimal correlation with growth rate. This difference is being explored, along with an attempt to learn how the two processes, "repression" and "metabolic regulation," interact to control synthetase level.

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