Viral Modification of the Valyl Transfer Ribonucleic Acid Synthetase of Escherichia coli*

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SUMMARY

The valyl-tRNA synthetase (EC 6.1.1.9) of Escherichia coli was compared to the modified form of the enzyme which develops during infection by bacteriophage T4. The two forms were found to differ in their stability to heat, stability to urea, ability to charge yeast tRNA, electrophoretic mobility, sedimentation rate in sucrose gradients, and molecular size. Both enzyme activities are easily purified and retain their characteristic properties during and after purification. Spectral properties of the two purified enzymes are similar and are incompatible with the presence of a bound tRNA. Surprisingly, no major differences were detected between the two enzymes in the normal charging reaction, including the relative specificity for different E. coli tRNAVal species.

Modified enzyme was discovered to contain a phage-directed component that can be dissociated from the core enzyme by treatment with either urea, guanidine hydrochloride, or sodium dodecyl sulfate. This component, called $\delta$ factor, could be resolved from the core enzyme by gel electrophoresis or agarose chromatography, and is judged to be a polypeptide on the basis of its size (approximately 10,000 molecular weight), acid precipitability, absorption spectrum, and Pronase susceptibility. Discovery of this factor explains several features of synthetase modification, including its sensitivity to inhibitors of protein synthesis, and its control by a phage gene.

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There is sufficient information in the DNA of bacteriophage T4 to code for approximately 200 proteins of molecular weight 40,000 (4). When this phage infects Escherichia coli it quickly assumes control of macromolecular synthesis by actuating the information in this DNA. Approximately 70 functions have been identified by means of conditional-lethal mutants, but the bulk of the DNA performs functions that appear to be inessential for viral growth (4). Among these auxiliary functions are many that cause alterations in the translational machinery of the host. Some species of tRNA appear changed in quality or amount (cf. 5, 6), new tRNA species are made at the direction of the phage genome (cf. 7, 8), ribosomal specificity is changed (9), and at least one aminoacyl-tRNA synthetase is converted into a new molecular form during infection (1, 10-12).

A mutant strain of E. coli possessing a temperature-sensitive L-valyl-tRNA synthetase (L-valine:tRNA ligase (AMP); EC 6.1.1.9) provided the first indication that infection by T-even bacteriophages leads to the development of a new aminoacyl-tRNA synthetase activity; infection confers upon the cell the capacity to make protein at 40°C, and a relatively heat stable valyl-tRNA synthetase can be found in extracts of the cells (10). An analogous change occurs during infection of wild type cells, and in this case the phage-specific activity could be identified by various analytical procedures such as fractionation by chromatography or by sedimentation. Centrifugation through sucrose gradients revealed that the phage-induced activity sediments 0.5 times faster than the host valyl-tRNA synthetase, and that as the phage valyl-tRNA synthetase develops the host form disappears (12). Density shift experiments confirmed the suggestion that the new valyl-tRNA synthetase activity is composed chiefly of host protein made prior to the infection, and other information established that it was the host enzyme that becomes the viral enzyme. The term enzyme modification was therefore applied to this phenomenon (1, 12).

We began the present study with the belief that changes in the translational apparatus of host cells must play an interesting role in the biology of viruses and therefore warranted a full description. Our initial approach was to compare the physical and enzymic properties of the viral and the host form of valyl-tRNA synthetase. The results emphasize the reality and the magnitude of the changes in physical and other properties of the enzyme brought about by modification, but they fail to uncover a major effect on the principal reaction catalyzed by valyl-tRNA synthetase. Modification entails an apparent increase in the size of the enzyme, probably by the addition of non-nucleic acid material, and evidence has been obtained that a protein factor made during infection becomes physically attached to the host valyl-tRNA synthetase.

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MATERIALS AND METHODS

Chemicals—The common chemicals used in these experiments were reagent grade. Tris, DEAE-Sephadex A-50, and G-200-120 Sephadex were obtained from Sigma. Di-potassium ATP was obtained from Pabst Laboratories, Inc. Hydroxyapatite (Bio-Gel HTP), DEAE-cellulose (high capacity), agarose, and Pronase were obtained from Calbiochem. 14C-Amino acids, including L-[U-14C]leucine (270 mCi per mmole), were purchased from Schwarz at the highest available specific activity, and [6-14C]glucose (6000 mCi per mmole) was obtained from American-Searle. Urea ("ultrapure" grade) and guanidine hydrochloride were obtained from Mann. *E. coli* K-12 tRNA and *Saccharomyces cerevisiae* tRNA were obtained from General Biochemicals, Inc. Organic chemicals for disc electrophoresis were purchased from Eastman Chemical Co. and used without further purification. The solubilizer, NCS, was obtained from Nuclear Chicago.

Growth of Phage and Bacteria—The bacteriophages T4Bo+ and T4 r1364, a mutant with a deletion in the rII region, were obtained from W. H. McClain at Purdue University. *E. coli* strain NP2 (a K-12 strain) was maintained at 4° by occasional transfer on rich medium (Medium A) containing 10% tryptone, 5% yeast extract, and 0.2% glucose.

High-titer lysates of T4B r1364 were prepared on the permissive *E. coli* strain NP4 (a B strain). The bacteria were grown at 37°C with stirring and aeration in 1-liter lots of M9 minimal medium (13) and infected at a density of 6 × 10^10 cells per ml with a multiplicity of 1 × 10^-3. After 6 hours, CHCl₃ was added and the lysate was stored overnight at room temperature. After filtration through Celite, lysates were titrated on *E. coli*, strain NP4. Titters were usually 1 to 3 × 10^8 plaque-forming units per ml.

To prepare large quantities of phage-infected bacteria 1-liter lots of *E. coli*, strain NP2, were grown with stirring and aeration to a concentration of 6 × 10^10 cells per ml in a broth medium containing 1.3% tryptone and 0.7% NaCl. T4B r1364 was added at a multiplicity of five. After 15 min of infection, centrifugation of a typical 45-liter batch was begun in a Sharples centrifuge. It took approximately 40 min for the entire batch to flow through the centrifuge. We obtained 2 to 3 g of infected cells per liter of medium. The cell pellet was frozen and stored at -20°C.

Uninfected bacteria were obtained from surface cultures grown on medium A solidified by 2% agar, in aluminum pans covered with aluminum foil. Sterile pans containing 500 ml of medium were each inoculated with 10 ml of an overnight culture of NP2 and allowed to incubate for 48 hours at 37°C. Bacteria were scraped from the surface, frozen without prior centrifugation, and stored at -20°C. We obtained 10 to 12 g per liter of medium with this procedure.

Extract Preparation—Small quantities of infected and uninfected *E. coli* cell-free extracts were used for many types of analysis. Extracts were prepared from cells taken from cultures (50 to 100 ml) in balanced growth in either minimal or rich media. Bacteria were pelleted by centrifugation and resuspended in 2 ml of 6 mM phosphate buffer, pH 7.3, containing 10 mM 2-mercaptoethanol, and disrupted with a Branson sonifier for 2 min at setting number 4.

Large quantities of extract for enzyme purification were prepared from the frozen paste of infected or uninfected bacteria. Typically about 300 g wet weight of cells were resuspended in about 300 ml of 6 mM phosphate buffer, pH 7.3, containing 10 mM 2-mercaptoethanol and passed through a French pressure cell (Aminco).

Synthetase Assays—One unit of synthetase activity is the amount of enzyme that catalyzes the addition of amino acid to tRNA at the rate of 1 μ mole per hour under our assay conditions. Specific activity is expressed as units per mg of protein. The protein concentration of preparations was determined with Folin reagent according to the method of Lowry (14). The routine attachment assay was performed in a reaction mixture which contained, in a total volume of 0.50 ml, 0.50 mg of tRNA, 0.10 mg of bovine serum albumin, 1.0 μ mole of GHS, 1.0 μ mole of ATP, 10 μmole of [14C]-amino acid at 10 μCi per μmole, 5 μmole of KCl, 50 μmole of Tris, and 5 μmole of MgCl₂. The final pH was 7.2 to 7.3. Enzyme extract was added to this mixture at 0°C. A zero time blank was prepared at this time by adding 3 ml of 5% trichloroacetic acid to one tube. Reaction tubes were then placed in a 37°C water bath. After 10 min the tubes were removed and placed in an ice bath. After several min 3 ml of 5% trichloroacetic acid were added to each tube. After 30 min the precipitates were collected on Fiberglas filters and counted with either a Nuclear Chicago thin window gas flow counter or a Packard liquid scintillation counter.

The ability of valyl-tRNA synthetase to charge valine to yeast tRNA was measured in the same way except that tRNA from *S. cerevisiae* was substituted for *E. coli* tRNA.

To estimate the apparent values for the Kₘ of valyl-tRNA synthetase for valine, ATP, and unfractionated tRNA, one substrate was varied while the other two were held essentially constant at an initial concentration of five times their anticipated Kₘ. In all determinations of Kₘ, reactants were previously incubated at 37°C and the reaction was initiated by the addition of purified enzyme (0.04 to 0.07 μg). Four successive 100-μl samples were removed from the mixture at 1- or 2-min intervals after the addition of enzyme and pipetted into 3 ml of 5% trichloroacetic acid. Determinations of attached amino acid were performed in the usual way. Initial reaction velocity (v₀) was calculated from the slope of the product accumulation curve.

Enzyme Purification—Valyl-tRNA synthetase from *E. coli* strain NP2, and from the same strain infected with T4B r1364, was purified by a procedure similar to that of Yaniv and Gros (15).

Crude extracts (Fraction I) prepared in 6 mM phosphate buffer, pH 7.3, containing 10 mM 2-mercaptoethanol, were incubated at 37°C for 3 hours to permit autolysis of nucleic acids. The resulting autolysate (Fraction II) was then fractionated by ammonium sulfate precipitation, adsorption to calcium phosphate gel, and chromatography on DEAE-Sephadex and on hydroxyapatite by the methods already described by Yaniv and Gros (15), to yield Fractions III, IV, V, and VI, respectively. Fraction VI was applied to a column of DEAE-cellulose and eluted with a linear phosphate gradient, 0.05 to 0.40 M, pH 7.3. The fractions containing valyl-tRNA synthetase activity were pooled to form Fraction VII.

Heat Treatment of Valyl-tRNA Synthetase—A thin-walled centrifuge tube containing 0.90 ml of 6 mM potassium phosphate buffer, 10 mM 2-mercaptoethanol, and 100 μg of bovine serum albumin was placed in a 45°C water bath. After several minutes crude extract containing approximately 50 μg of protein was added and samples (100 μl) were removed at 5 min intervals and added to tubes in ice containing the assay mix. After the last sample had been withdrawn tubes were assayed in the usual fashion.

Urea Treatment of Valyl-tRNA Synthetase—A urea sensitivity
test was performed on enzyme preparations at all stages of purity. Ten times as much enzyme as needed for an attachment assay was added to a tube containing 4 mM urea, 100 μg of bovine serum albumin, and 6 mM potassium phosphate buffer, pH 7.3, containing 10 mM 2-mercaptoethanol in a final volume of 0.50 ml. After the addition of enzyme, samples (50 μl) were withdrawn at various times and pipetted directly into tubes containing assay mix at 37°C. Later it was learned that this procedure permits some renaturation of urea-inactivated enzyme, particularly in crude extracts (see "Results"), so a 5-min incubation period is recommended for the enzyme assay in this test.

Electrophoresis—Sucrose density gradient electrophoresis was conducted as described by Seifert et al. (16). A crude extract (2.0 ml) was dialyzed overnight against a buffer (Buffer A) consisting of Tris (5 mM), NH₄Cl (11 mM), magnesium acetate (5 mM), and 2-mercaptoethanol (0.5 mM) at pH 7.5. Under these conditions all of the synthetases examined behave as anions. Electrophoresis was conducted in a vertical glass tube at 4°C, pH 7.3, against a sucrose gradient (40 ml) of 40% to 5%, by applying a potential of 500 volts for 6 hours. After electrophoresis the gradient was collected by dripping fraction from the bottom (electrode end) of the tube.

Disc gel electrophoresis was conducted in 7.5% polyacrylamide with a 2.5% polyacrylamide stacking gel. Glycine was used as the counter-ion. All gels were run at room temperature. A constant current of 1.25 mA per gel was maintained until the bromphenol blue had entered the separating gel, and then increased to 2.5 mA per gel. Gels were stained with Amido blue black and destained electrophoretically in 7.5% acetic acid. The procedures used are only slightly modified from standard techniques (17, 18). Electrophoresis in the presence of sodium dodecyl sulfate was conducted according to the method of Weber and Osborn (19).

Sucrose Gradients—Sucrose density gradients were prepared with a two-chambered mixing device using 20% and 5% sucrose in 6 mM potassium phosphate buffer, pH 7.3, containing 10 mM 2-mercaptoethanol. The volume of the gradients was 4.6 ml. The sample, and enough 5% sucrose to make a volume of 0.4 ml, was layered on the gradient before centrifugation.

Sephadex G-200 Chromatography—Preparation of the gel and pouring of the column were performed essentially according to Andrews (20). For molecular weight studies the column (5 cm x 100 cm) employed was fitted with pistons at both ends and the chromatography was conducted from bottom to top. A Mariotte flask was used to maintain constant hydrostatic pressure of 10 cm. Approximately 5 ml of a crude extract containing 1 mg of protein per ml was the usual sample applied. All operations were conducted at 4°C.

-Benzoylated DEAE-cellulose Chromatography of tRNA—Preparations of tRNA labeled by in vitro charging with either ¹⁵N or ¹³C-amino acids were fractionated by chromatography on benzyolated DEAE cellulose (Schwarz/Mann). The chromatographic methods were based on those described by Gilliam et al. (21). Samples to be fractionated were suspended in 0.5 ml of a solution containing 0.1 M sodium chloride, 0.01 M sodium acetate (pH 5.75), 0.01 M magnesium chloride, and 0.01 M mercaptoethanol. They were applied to a column (1.1 x 80 cm) and washed with approximately 180 ml of the same solution. Elution was achieved with the buffer just described but containing a gradient of sodium chloride from 0.4 to 0.9 M (total volume, 1.5 liters). Fractions of 12-ml volume were collected. Yeast RNA (1 mg) was added to each fraction, and then sufficient trichloroacetic acid was added to give a concentration of 5%. The samples were filtered to catch the precipitated RNA on glass fiber filters which were then counted in a scintillation counter with a toluene-based scintillation fluid.

Preparation of Doubly Labeled Synthetases—A culture of E. coli NP4 was grown for several generations in glucose minimal medium supplemented with 10 μg of L-leucine per ml. Cells from this culture were used to inoculate 500 ml of this medium at a concentration of 2 x 10⁸ cells per ml. L-[¹⁴C]leucine, 1.0 mCi, was added to the flask, and growth was allowed to continue at 30°C with aeration. At a cell concentration of 2 x 10⁸ bacteria per ml, the culture was rapidly chilled in ice and a small sample was removed for protein determination. The culture was then centrifuged and washed twice in cold medium without glucose to remove both the [¹⁴C]leucine and the unconsumed glucose. The pellet was resuspended in a small volume of medium without glucose and added to previously warmed medium containing 10 μg of L-leucine per ml and 0.003% glucose. A sample was taken to count viable cells. Phage (T4B r1364) was added at a ratio of five per cell, and 1 min later 15 mCi of L-[¹⁴C]-leucine and 8 mCi of L-[¹⁵N]-leucine were added. Five minutes after the addition of phage a sample was taken for counting viable cells, and 20 min after infection the culture was rapidly chilled. A small sample was taken for measurement of protein. The remainder of the chilled culture was centrifuged. The cells were resuspended in 6.0 mM phosphate buffer (pH 7.3) containing 20 mM 2-mercaptoethanol and disrupted with a Bronson sonifier. Analysis of the different samples revealed that approximately 4% of the cells survived the exposure to phage, and that the total protein of the culture increased from 37.50 mg to 41.65 mg during infection. The labeled extract was added to a carrier extract made from 300 g (wet weight) of strain NP4 cells. From measurements of the amount of carbon 14 and tritium in the spent media it was found that 93% of the former and 83% of the latter had become associated with the cells. By measuring the label in the hot trichloroacetic acid residue of a small portion of the extract it could be told that of the label taken up, 63% of the carbon 14 and 17% of the tritium was recovered in protein.

The procedure of Yaniv and Gros (15), modified in the manner just described, was used to purify the host and viral forms of valyl-tRNA synthetase. In addition, isoleucyl-tRNA synthetase was purified by following the suggestion of Yaniv and Gros (15) that it elutes as a distinct peak separable from valyl-tRNA synthetase during the DEAE-Sephadex step. At this stage the fractions containing isoleucyl-tRNA synthetase activity were pooled and subjected to the subsequent purification steps separately from the pooled valyl-tRNA synthetase fractions. The ¹⁴C and ¹³C content of the various fractions was determined by means of a Packard Tri-Carb liquid scintillation counter during the course of the purification. Samples were counted within a range of 5 to 50 μg of precipitated protein. A résumé of the purification is presented in Tables VII and VIII. They present the specific enzyme activity and the ¹⁴C:¹³C ratio of samples at different stages of purification.

Chromatography on Agarose—To examine the purified enzyme for the presence of associated factors, chromatography on agarose columns in the presence of either 6 M guanidine HCl or 8 M urea was employed. The procedure was essentially that of Davison (22). Two types of column were used. For analytical purposes a glass column (0.8 x 100 cm) was calibrated by chromatographing a mixture of ¹³C-labeled trypsin, aldolase, bovine serum albumin, and ribonuclease. Blue dextran and nitrotyrosine were used to determine the void and the column volumes, respectively. Purified synthetases were then chromatographed...
If a polynucleotide were responsible for the change in valyl-tRNA synthetase during infection, it must be buried in the enzyme in such a way as to be protected from RNAse.

Exposure of crude extracts from infected and uninfected cells to high concentrations of urea revealed that the viral enzyme is extremely resistant to inactivation compared to the host form of the enzyme. The results are shown in Fig. 1. The half-life of the viral form of the enzyme is very long in 4 M urea (approximately 30 to 60 min), whereas the half-life of the cellular form is quite short (approximately 1 min) when assayed under our standard conditions, and therefore this concentration of urea can be used to perform a differential assay of the two forms of the enzyme. Mixtures of the two forms yield urea decay curves that are biphasic and easily interpretable in terms of the components. A considerable fraction (nearly half) of the urea-inactivated host synthetase can become reactivated during the actual assay, and 5-min assay times were adopted to minimize this effect.

As might be expected from the results with urea treatment, the increased stability of valyl-tRNA synthetase after viral modification could be demonstrated also by incubation of crude extracts at 45°C (results not shown). Heat stability of the modified enzyme could be inferred also from the ability of T4 phage to stabilize the valyl-tRNA synthetase activity, both in vivo and in vitro, of the temperature sensitive mutant, strain NP29 (10).

Extracts of normal and of infected E. coli cells were examined by electrophoresis through linear gradients of sucrose. By this technique Seifert et al. (16) were able to demonstrate heterogeneity of E. coli leucyl-tRNA synthetase, one portion of which migrates more rapidly than the rest as a result of having tRNA bound to it. We confirmed their result with respect to the leucine enzyme, and found that the viral modified valyl-tRNA synthetase migrated more rapidly than the normal host enzyme (results not shown).

Heterologous charging of yeast tRNA was tested in an otherwise standard assay system for amino acid charging. Extracts of uninfected cells were able to attach valine to yeast tRNA only to a limited extent (less than 5% of the rate, and less than 1% of the extent, with E. coli tRNA); extracts of cells infected either with normal phage (T4Bc+) or with an rIL mutant (T4 rIL304) were able to catalyze a much more extensive charging of yeast tRNA, albeit less than with tRNA from E. coli (Fig. 2).

Finally, molecular sieving was employed to resolve the two forms of the enzyme. Estimates of apparent molecular weight of 160,000 to 180,000 for the viral form, compared to 110,000 for the unmodified form, had been made on the basis of sedimentation through sucrose gradients (12). A sample consisting of an equal mixture of crude extracts of infected and uninfected cells was applied to a column of Sephadex G-200. Eluted fractions were assayed for valyl-, phenylalanyl-, and arginyl-tRNA synthetase activities. In addition, the fractions containing valyl-tRNA synthetase activity were tested for urea stability and for the ability to attach valine to yeast tRNA. The results were somewhat surprising. The valyl-tRNA synthetase activity migrated as a single broad peak, even though molecules of molecular weight 110,000 (valyl-tRNA synthetase) and 181,000 (phenylalanyl-tRNA synthetase) were completely resolvable. The tests for urea stability and for the ability to charge yeast tRNA revealed that the modified enzyme was present in the forward section of the valyl-tRNA synthetase peak (Fig. 3). On the basis of the known molecular weights of the synthetases measured, the modified enzyme appeared to be no more than 10% larger than the normal host enzyme.

![Fig. 1. Inactivation of host and viral valyl-tRNA synthetase activities by urea. This experiment employed fractions of a crude extract that had been fractionated by sucrose gradient electrophoresis into host enzyme (•) and viral enzyme (○). A portion (0.05 ml) of each fraction was added to a tube containing 4 M urea, 100 μg of bovine serum albumin, and 6 mM potassium phosphate buffer (pH 7.3) and 10 mM 2-mercaptoethanol in a final volume of 0.5 ml. Samples (50 μl) were removed at the times indicated and pipetted into the standard assay mix (0.5 ml). The reaction was terminated at 10 min. Crude extracts and purified enzymes gave equivalent results to those shown here.](http://www.jbc.org/)

**RESULTS**

*Studies with Crude Extracts*

Before extensive purification of the enzymes could be attempted it was important to learn whether the viral modified enzyme was likely to become demodified during purification and to learn what properties other than sedimentation rate through sucrose gradients could be used to identify the two forms of the enzyme during purification.

Crude extracts from infected cells were treated with sufficient pancreatic RNAse to hydrolyze RNA effectively. No evidence was found that the viral enzyme was altered by this treatment. If a polynucleotide were responsible for the change in valyl-tRNA synthetase during infection, it must be buried in the enzyme in such a way as to be protected from RNAse.

Exposure of crude extracts from infected and uninfected cells to high concentrations of urea revealed that the viral enzyme is extremely resistant to inactivation compared to the host form of the enzyme. The results are shown in Fig. 1. The half-life of the viral form of the enzyme is very long in 4 M urea (approximately 30 to 60 min), whereas the half-life of the cellular form is quite short (approximately 1 min) when assayed under our standard conditions, and therefore this concentration of urea can be used to perform a differential assay of the two forms of the enzyme. Mixtures of the two forms yield urea decay curves that are biphasic and easily interpretable in terms of the components. A considerable fraction (nearly half) of the urea-inactivated host synthetase can become reactivated during the actual assay, and 5-min assay times were adopted to minimize this effect.

As might be expected from the results with urea treatment, the increased stability of valyl-tRNA synthetase after viral modification could be demonstrated also by incubation of crude extracts at 45°C (results not shown). Heat stability of the modified enzyme could be inferred also from the ability of T4 phage to stabilize the valyl-tRNA synthetase activity, both in vivo and in vitro, of the temperature sensitive mutant, strain NP29 (10).

Extracts of normal and of infected E. coli cells were examined by electrophoresis through linear gradients of sucrose. By this technique Seifert et al. (16) were able to demonstrate heterogeneity of E. coli leucyl-tRNA synthetase, one portion of which migrates more rapidly than the rest as a result of having tRNA bound to it. We confirmed their result with respect to the leucine enzyme, and found that the viral modified valyl-tRNA synthetase migrated more rapidly than the normal host enzyme (results not shown).

Heterologous charging of yeast tRNA was tested in an other-
Studies with Purified Enzymes

Purification—Valyl-tRNA synthetase was purified from E. coli strain NP2, and from strain NP2 infected with phage T4 r1364. During the early stages of purification the extract from the infected culture was monitored by the several methods that distinguish between normal and modified enzyme. These tests indicated that the original extract contained approximately equal portions of the two enzyme forms. Since modified enzyme, as measured in crude extracts, has only one-third as much activity on a molar basis as unmodified enzyme (10), a mixture containing equal enzyme units of each form indicates that modification was 75% complete. The remaining unmodified enzyme probably represented uninfected cells (strain NP2 adsorbs T4 rather poorly).

A series of three column chromatography steps (DEAE-Sephadex, hydroxyapatite, and DEAE-cellulose) completed the purification. Extracts from infected and uninfected cultures behaved identically through the DEAE-Sephadex fractionation. Hydroxyapatite chromatography resolved the two valyl-tRNA synthetase activities present in the preparation from the infected culture, as expected from previous work (10). This separation was indicated by all three indicators used: urea stability, sucrose gradient sedimentation rate, and yeast tRNA charging capacity. The pooled activities from this fractionation were dialyzed separately against potassium phosphate buffer, pH 8.0, and chromatographed on DEAE-cellulose. Again their behavior was unique in the that modified enzyme flowed through the column in approximately its applied volume, while the native enzyme adhered to the column and was shifted at a higher ionic strength. In several cases a pooled lot of enzyme from hydroxyapatite contained some of the other activity due to imprecise pooling of overlapping fractions, and in these cases the contaminating activity was resolved by the DEAE-cellulose step. The purity of the preparations is shown in Tables I and II.

Analysis by polyacrylamide disc gel electrophoresis revealed that unmodified enzyme from infected cultures had several minor contaminants not present in enzyme purified from uninfected cells, and therefore these preparations were further purified by Sephadex G-200 chromatography. Following this step, the preparations each consisted of a single protein band (revealed by Amido blue-black staining) upon gel electrophoresis. Cn-stained gels run in parallel were sliced into 1-mm pieces, eluted accurately against potassium phosphate buffer, pE1 8.0, and chromatographed on DEAE-cellulose. The molecular weight of valyl-tRNA synthetase as determined by gel filtration on Sephadex G-200. A series of experiments were performed following treatment with 4 M urea (VRS-modified), infected and uninfected cells, added so as to contain equal levels of protein were added per tube. The time course of [Wlvalyl-tRNA synthetase (VRS), valyl-tRNA synthetase following treatment with 4 M urea (VRS-modified), infected and uninfected cells, added so as to contain equal levels of protein were added per tube. The time course of [Wlvalyl-tRNA synthetase, 74,000 (23); host valyl-tRNA synthetase, 110,000 (15); and phenylalanyl-tRNA synthetase, 181,000 (24).

### Figure 2

Charging of L-valine to yeast tRNA by extracts of infected or uninfected E. coli NP2. A sample (0.0 ml; 1.0 mg of protein per ml) consisting of a mixture of extracts from infected and uninfected cells, added so as to contain equal levels of host and viral forms of valyl-tRNA synthetase, was applied to a column (5 X 100 cm) of Sephadex G200. Chromatography was conducted from bottom to top at 4° and a hydrostatic pressure of 4 cm. Fractions (0.0 ml) were collected and assayed for phenylalanyl-tRNA synthetase (PHS), arginyl-tRNA synthetase (ARS), valyl-tRNA synthetase (VRS), valyl-tRNA synthetase activity was resolved by the DEXE-cellulose step. The course of overlapping fractions, and in these cases the contaminating activity was resolved by the DEAE-cellulose step. The purity of the preparations is shown in Tables I and II.

### Figure 3

Molecular weight of valyl-tRNA synthetase as determined by gel filtration on Sephadex G-200. A sample (0.0 ml; 1.0 mg of protein per ml) consisting of a mixture of extracts from infected and uninfected cells, added so as to contain equal levels of host and viral forms of valyl-tRNA synthetase, was applied to a column (5 X 100 cm) of Sephadex G200. Chromatography was conducted from bottom to top at 4° and a hydrostatic pressure of 4 cm. Fractions (0.0 ml) were collected and assayed for phenylalanyl-tRNA synthetase (PHS), arginyl-tRNA synthetase (ARS), valyl-tRNA synthetase (VRS), valyl-tRNA synthetase activity was resolved by the DEXE-cellulose step. The course of overlapping fractions, and in these cases the contaminating activity was resolved by the DEAE-cellulose step. The purity of the preparations is shown in Tables I and II.

### Table 1

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</table>

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enzymes at 5° for periods as long as 1 year did not bring about either phage or host origin, to the host enzyme. Storage of the tRNA's are not the consequence of a tight binding of tRNA, of translation through sucrose gradients, and the ability to charge yeast tRNA. The high rate of sedimentation of similar size. Therefore, the ability of the modified enzyme contained a bound tRNA or other polynucleotide and of the modified valyl-tRNA synthetase was accomplished without the loss of features that distinguish the enzymes in crude extracts (Table III). The pure enzymes had an absorbance ratio (280:260 nm) of 1.7 to 1.9, indicating that neither form of the enzyme contained a bound tRNA or other polynucleotide. The pHe optima were indistinguishable for the two forms of the enzyme (not shown). The pH optima were indistinguishable for the two forms of the enzyme (not shown). The pH optima were indistinguishable for the two forms of the enzyme (not shown).

\[ \text{Retention of Enzyme Properties} \]

The enzyme was made on the basis of tests for urea stability, sedimentation rate in sucrose gradients, and rate of charging yeast tRNA. The RF values of stained bands and activity peaks were indistinguishable. The second value in each column is for the viral modified activity.

**Table II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Yield</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract...</td>
<td>18,000</td>
<td>2,930</td>
<td>100</td>
<td>0.162</td>
</tr>
<tr>
<td>II. Autolysate.....</td>
<td>18,000</td>
<td>2,820</td>
<td>96</td>
<td>0.157</td>
</tr>
<tr>
<td>III. Ammonium sulfate precipitation...</td>
<td>6,600</td>
<td>1,770</td>
<td>60</td>
<td>0.265</td>
</tr>
<tr>
<td>IV. Calcium phosphate adsorption...</td>
<td>2,840</td>
<td>1,380</td>
<td>47</td>
<td>0.480</td>
</tr>
<tr>
<td>V. DEAE-Sephadex chromatography...</td>
<td>194</td>
<td>350</td>
<td>12</td>
<td>1.810</td>
</tr>
<tr>
<td>VI. Hydroxyapatite chromatography...</td>
<td>37.6* / 13.0*</td>
<td>191* / 91*</td>
<td>9.6</td>
<td>5.07* / 17.00*</td>
</tr>
<tr>
<td>VII. DEAE-cellulose chromatography...</td>
<td>10.9* / 3.4*</td>
<td>150* / 68*</td>
<td>7.7</td>
<td>14.4* / 21.2*</td>
</tr>
</tbody>
</table>

* The viral and host enzymes are resolved by the hydroxyapatite chromatography; the first value in each column is for the host enzyme activity. Identification of the two enzyme forms was made on the basis of tests for urea stability, sedimentation rate in sucrose gradients, and rate of charging yeast tRNA.

**Table III**

<table>
<thead>
<tr>
<th>Form of enzyme</th>
<th>Stability in pM area*</th>
<th>Sedimentation in sucrose*</th>
<th>Yeast tRNA charging†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>%</td>
<td>0.52</td>
<td>0.12</td>
</tr>
<tr>
<td>Viral-modified</td>
<td>71</td>
<td>0.68</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* The value is the percentage of original activity remaining after 10 min incubation at 37° in 4 nM area.
† The value is the distance each form sedimented relative to the total length of the gradient.
‡ The value is the initial velocity of charging L-valine to yeast tRNA.

10 mM 2-mercaptoethanol, and the eluates were assayed for attachment activity. The RF values of stained bands and activity peaks were indistinguishable.

**Retention of Enzyme Properties**—Purification of the normal and of the modified valyl-tRNA synthetase was accomplished without the loss of features that distinguish the enzymes in crude extracts (Table I). The pure enzymes had an absorbance ratio (280:260 nm) of 1.7 to 1.9, indicating that neither form of the enzyme contained a bound tRNA or other polynucleotide of similar size. Therefore the stability of the modified enzyme to denaturation by urea or by heat, the high rate of sedimentation through sucrose gradients, and the ability to charge yeast tRNA are not the consequence of a tight binding of tRNA, of either phage or host origin, to the host enzyme. Storage of the enzymes at 5° for periods as long as 1 year did not bring about any change in the properties of the two forms of the enzyme.

**Table IV**

<table>
<thead>
<tr>
<th>Form of enzyme</th>
<th>L-valine</th>
<th>ATP</th>
<th>tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.6 × 10^-5</td>
<td>7.0 × 10^-4</td>
<td>2.8 × 10^-6</td>
</tr>
<tr>
<td>Viral-modified</td>
<td>3.3 × 10^-5</td>
<td>6.5 × 10^-4</td>
<td>2.8 × 10^-4</td>
</tr>
</tbody>
</table>

**Enzymological Properties**—The purified preparations were examined by the charging reaction for pH dependence, for capacity to charge E. coli tRNA to completion, and for the $K_m$ values for valine, ATP, and tRNA. The pH optima were indistinguishable for the two forms of the enzyme (not shown). The $K_m$ was found to be 2 to 3 × 10^-4 M for L-valine, 0 to 7 × 10^-4 M for ATP, and 2 to 3 × 10^-4 M for total tRNA; there were no significant differences in these properties between the two forms (Table IV). Both enzymes exhibited some substrate inhibition by ATP and by tRNA at concentrations above the respective $K_m$ values. There was no apparent difference in the maximum amount of valine that could be charged to E. coli tRNA by the two enzymes.

To learn whether the specificity of the modified enzyme for L-valine was as selective as that of the host enzyme, two different tests for specificity were performed. In the first the effect of adding a mixture of 19 amino acids to a reaction mixture containing L-[3H]Valine, yeast tRNA, and the purified viral enzyme was measured. No lowering of the rate of valine attachment to tRNA by the other amino acids was observed (Table V). In the second experiment, a group of amino acids selected on the basis of structural or metabolic similarity to L-valine was tested directly as possible substrates in the charging reaction. As the results shown in Table VI indicate, no charging of these amino acids by the modified enzyme could be detected.

The absence of a marked difference in apparent $K_m$ value for
Saturating amounts of purified viral-modified valyl-tRNA synthetase (25 to 27 μg per 0.5 ml) were incubated at 37°C in a reaction mixture containing normal concentrations of buffer, ATP, GSH, and tRNA. Where added, other components were at the following concentrations: L-[14C]isoleucine, leucine, or threonine, 10 μCi per μmole, 20 μm; nonradioactive L-isoleucine, 0.2 mM; crude extract, 20 μg of protein per ml.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[14C]-amino acid</th>
<th>Excess isoleucine</th>
<th>Time of incubation</th>
<th>Radioactivity</th>
<th>Amino acid attached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified viral</td>
<td>Valine</td>
<td>+</td>
<td>10 min</td>
<td>436</td>
<td>4084</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>-</td>
<td>0 min</td>
<td>14</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>+</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>-</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>+</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>+</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>-</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
<td>+</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
<td>-</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td>Crude extract</td>
<td>Isoleucine</td>
<td>+</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>+</td>
<td>10 min</td>
<td>37</td>
<td>302</td>
</tr>
</tbody>
</table>

* The addition of excess nonradioactive isoleucine was designed to test whether the apparent slight activation of isoleucine was real. Since there was no reduction in activity, we assume that these few counts are the result of contaminating [14C]valine.

* This column is the radioactivity minus the radioactivity at a zero time blank.

To learn whether the rate of charging of the two peaks occurs similarly by both enzymes, the charging reactions in vitro with the purified preparations were repeated, but the reactions were terminated at 0 min under the charging conditions of Fig. 6. Again, as shown in Fig. 7, no difference in the pattern of charging by the two enzymes was observable.

In another experiment the tRNA used was isolated from T4-infected cells because of the possibility that phage infection alters the tRNAVal species, even though tests for the de novo synthesis of T4-coded tRNAVal (7, 8) or of T4-modification of tRNAVal (3) have been negative. No difference in the pattern of charging was observable (results not shown).

**Discovery of a Modifying Viral Factor**

In an earlier study, density labeling of proteins made after phage infection had established that the phage enzyme was in fact a modified form of previously existing host enzyme (12). To learn whether any phage product becomes associated with the valyl-tRNA synthetase in the course of modification, we...
OV I I I I I I
0 5 10 15 20 25
TIME (min)

FIG. 6. Time course of tRNA charging with subsaturating amounts of enzyme. Reaction mixtures (1.0 ml) contained 100 μmoles of Tris (pH 7.3), 10 μmoles of MgCl₂, 10 μmoles of KCl, 2 μmoles of glutathione, 2 μmoles of dipotassium ATP, 1.2 mg of stripped tRNA from Escherichia coli strain K-12 (General Biochemicals), 0.2 pmole of L-[14C]valine (5 μCi per pmole), and either 0.12 μg of purified valyl-tRNA synthetase from uninfected E. coli strain NP 2 (○), or 0.8 μg of purified valyl-tRNA synthetase from T4-infected strain NP2 (△). The incubation and processing of samples was done as described in the legend to Fig. 4.

FIG. 7. Benzoylated DEAE-cellulose column chromatography of partially charged tRNA. The tRNA was charged in reaction mixtures identical with those described in Fig. 6. The mixture with normal enzyme contained 0.2 μmole of L-[14C]valine (80 μCi per μmole); the mixture with viral-modified enzyme contained 0.2 μmole of L-[3H]valine (400 μCi per μmole), and either 0.12 μg of purified valyl-tRNA synthetase from uninfected E. coli strain NP 2 (○), or 0.8 μg of purified valyl-tRNA synthetase from T4-infected strain NP2 (△). The incubation and processing of samples was done as described in the legend to Fig. 4.

Table VII

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Isoleucyl-tRNA synthetase</th>
<th>Valyl-tRNA synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>0.05</td>
<td>0.11</td>
</tr>
<tr>
<td>II. Autolysate</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>III. Ammonium sulfate precipitation</td>
<td>1.12</td>
<td>1.12</td>
</tr>
<tr>
<td>IV. Calcium phosphate adsorption</td>
<td>1.62</td>
<td>1.62</td>
</tr>
<tr>
<td>V. DEAE-Sephadex chromatography</td>
<td>5.00</td>
<td>3.26</td>
</tr>
<tr>
<td>VI. Hydroxylapatite chromatography</td>
<td>15.6</td>
<td>11.1/12.6</td>
</tr>
<tr>
<td>VII. DEAE-cellulose chromatography</td>
<td>20.0/17.6</td>
<td>12.0/17.6</td>
</tr>
<tr>
<td>VIII. Sephadex G-200 chromatography</td>
<td>17.5</td>
<td>22.0/19.3</td>
</tr>
</tbody>
</table>

* This value was not determined on this extract, but represents the average value found in other similar extracts.

† The viral and host valyl-tRNA synthetase activities are resolved by the hydroxylapatite chromatography; the first value in this column is for the host enzyme.

‡ The second value in this column is for the viral-modified enzyme.

Labeling Infected Cells—The plan chosen was to previously label the host valyl-tRNA synthetase with L-[14C]leucine and then to infect the cells in the presence of [3H]glucose. The choice of the latter label was made in order to achieve the incorporation of a large amount of radioactivity without making major assumptions about the nature of the phage-related component of the synthetase. The labeling protocol described under “Materials and Methods” resulted in a crude extract that contained, following the addition of carrier extract, approximately 33 g of total protein, with a radioactivity of 40 dpm (carbon 14) and 140 dpm (tritium) per pg of total protein. Protein made before infection contained carbon 14 label only, whereas substances made after the addition of phage to the culture were labeled with tritium. A delay in infection of the cells in the minimal medium employed resulted in some incorporation of tritium into typically host proteins.

Purification of Doubly Labeled Synthetases—Table VII shows the specific enzyme activity at each of the major steps in the purification (see Reference 6), and indicates a minimum purification of 200-fold for the valine enzymes and 350-fold for the isoleucine enzyme. Table VIII indicates the [3H]:[14C] ratios of these preparations at the same steps of purification. The points to be noted are (a) that the purification of the two forms of the valyl-tRNA synthetase proceeded in the usual manner, resulting in their complete resolution from each other on the hydroxylapatite columns, and (b) that during purification the [3H]:[14C] ratio of the two host synthetases dropped from that characteristic of total protein in the crude extract, while the [3H]:[14C] ratio in the viral modified synthetase rose. The values of 1.86 and 1.89, respectively, for the [3H]:[14C] ratios of the host isoleucyl-tRNA synthetase and valyl-tRNA synthetase indicate the degree of tritium labeling to be expected in host proteins; in contrast, the viral form of the synthetase has a ratio of 4.05, indicating the presence of considerably more material made after addition of virus to the culture.
Radioisotope activities during purification from E. coli NP4 infected with bacteriophage T4B r1304.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>( {\text{H}}: {\text{C}} ) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>3.49^a 3.49^a</td>
</tr>
<tr>
<td>II. Autolysate</td>
<td>3.69^a 3.69^a</td>
</tr>
<tr>
<td>III. Ammonium sulfate precipitation</td>
<td>3.39^a 3.39^a</td>
</tr>
<tr>
<td>IV. Calcium phosphate adsorption</td>
<td>3.07^b 3.07^b</td>
</tr>
<tr>
<td>V. DEAE-Sephadex chromatography</td>
<td>2.90 2.81</td>
</tr>
<tr>
<td>VI. Hydroxylapatite chromatography</td>
<td>2.70 2.70/2.78^c</td>
</tr>
<tr>
<td>VII. DEAE-cellulose chromatography</td>
<td>2.70/3.43^c</td>
</tr>
<tr>
<td>VIII. Sephadex G-200 chromatography</td>
<td>1.86 1.89/4.05^c</td>
</tr>
</tbody>
</table>

^a These values were obtained on fractions before the separation of isoleucyl- from valyl-tRNA synthetase.

^b The viral and host valyl-tRNA synthetase activities are resolved by the hydroxylapatite chromatography; the first value in this column is for the host enzyme.

^c The second value in this column is for the viral-modified enzyme.

![Fig. 8](image)

**Fig. 8.** Polyacrylamide disc gel electrophoresis of purified, labeled aminoacyl-tRNA synthetases. Samples of the final preparations (Table II) of the host isoleucyl- and valyl-tRNA synthetase and the viral valyl-tRNA synthetase were each subjected to disc electrophoresis in 7.5% polyacrylamide (with a 2.5% stacking gel) at pH 9.3 in glass tubes (7.5 x 0.5 cm inner diameter) at a constant current of 2.5 mA per gel for approximately 1 hour with bromophenol blue as a tracking dye. Approximately 14 μg of each of the host enzymes, and 4 μg of the viral enzyme were applied. Following electrophoresis 0.75-mm slices were made, solubilized with Nuclear-Chicago Solubilizer, and assayed for \(^3\)H (●) and \(^14\)C (▲).

![Fig. 9](image)

**Fig. 9.** Chromatography of purified, labeled aminoacyl-tRNA synthetases on agarose columns in the presence of guanidine hydrochloride. Approximately 200 μg of each of the purified synthetases described in Table II were denatured in 6 M guanidine HCl and chromatographed by the procedure described under "Materials and Methods" using the 0.8 x 100 cm column. Fractions of approximately 0.2 ml volume (10 drops) were collected, and portions (50 μl) were precipitated with 5% trichloroacetic acid and assayed for \(^3\)H and \(^14\)C. The upper left panel depicts the chromatographic behavior of three \(^14\)C-labeled standard proteins, blue dextran (O) and nitrotyrosine (▲). The remaining three panels depict the behavior of the host isoleucyl-tRNA synthetase (IRS), host valyl-tRNA synthetase (VRS), and viral valyl-tRNA synthetase (VRS^*); the dextran and nitrotyrosine peaks in these panels are indicated by arrows labeled D and N, respectively. O, \(^14\)C; ●, \(^3\)H.

The preparations of the three synthetases were examined by electrophoresis in polyacrylamide gels. The results are shown in Fig. 8. Each preparation contained a single major protein band containing \(^3\)H and \(^14\)C radioactivity in the expected ratios.

**Chromatography of Denatured Enzyme on Agarose**—To examine the nature of the excess tritium in the modified enzyme, enzyme preparations were denatured with guanidine hydrochloride and chromatographed separately on an agarose column equilibrated with the denaturing agent. The column was calibrated by chromatographing \(^14\)C-labeled standard proteins of known molecular weight, together with dextran and nitrotyrosine. The upper left panel of Fig. 9 shows the behavior of the standard compounds. The host valyl-tRNA synthetase and isoleucyl-tRNA synthetase (Fig. 9, lower panels) chromatographed as expected for molecules of their size. The preparation of viral valyl-tRNA synthetase (Fig. 9, upper right panel) contained a doubly labeled component similar in chromatographic behavior to the host synthetase, and a tritium-labeled component eluting as a relatively low molecular weight (approximately 8,000 to 10,000) substance. This result was confirmed by electrophoretic analysis of the enzyme preparations in the presence of 0.1% sodium dodecyl sulfate (results not shown).

The association of phage-specific material with the modified valyl-tRNA synthetase therefore appeared fairly certain.

**Preparative Chromatography of \(\tau\)-Factor**—The next experiments were undertaken to prepare sufficient amounts of \(\tau\)-factor for preliminary analysis, and to measure carefully the amount of this factor that was associated with the modified enzyme.

An attempt to scale up the guanidine-agarose procedures failed because of the formation of precipitate. Larger portions...
FIG. 10. Chromatography of purified, labeled aminoacyl-tRNA synthetases on Sephadex G-200 with sodium dodecyl sulfate. Approximately 1.0 mg of the purified host valyl-tRNA synthetase (VRS) and of the viral enzyme (VRS*) were incubated for 1 hour at 37° in 6 mM phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate. These samples were then applied to a Sephadex G200 column (5 X 70 cm) equilibrated with the same buffer. Fractions (5.0 ml) were collected and portions (0.5 ml) of these were precipitated with 5% trichloroacetic acid, filtered, and assayed for 3H (●) and 14C (○).

Fig. 11. Chromatography of purified, labeled aminoacyl-tRNA synthetases on agarose with urea. Approximately 1.0 mg of the purified host valyl-tRNA synthetase (VRS) and of the viral enzyme (VRA*) were incubated in 8 M urea for 1 hour at 37°. The denatured samples were then applied in a volume of 1 ml to an agarose (1.5 M) column (2 X 40 cm). The column was developed from bottom to top, and 1-ml fractions were collected. Portions (0.1 ml) were precipitated with 50% trichloroacetic acid, collected on filters, and assayed for 3H (●) and 14C (○).

TABLE IX
Radioisotope activities of purified enzymes following various analytical procedures

<table>
<thead>
<tr>
<th>Analytical procedure</th>
<th>Viral valyl-tRNA synthetase</th>
<th>Host valyl-tRNA synthetase</th>
<th>Host isoleucyl-tRNA synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>Denatured†</td>
<td>Native</td>
<td>Denatured†</td>
</tr>
<tr>
<td>None</td>
<td>4.05</td>
<td>1.80</td>
<td>1.86</td>
</tr>
<tr>
<td>Disc gel electrophoresis</td>
<td>3.50</td>
<td>1.50</td>
<td>1.70</td>
</tr>
<tr>
<td>Disc gel electrophoresis</td>
<td>3.80</td>
<td>1.67</td>
<td>1.40</td>
</tr>
<tr>
<td>Disc gel electrophoresis</td>
<td>4.00</td>
<td>1.67</td>
<td>1.70</td>
</tr>
<tr>
<td>Disc gel electrophoresis</td>
<td>1.90</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate gel electrophoresis</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate gel electrophoresis</td>
<td>1.25</td>
<td>1.12</td>
<td>1.85</td>
</tr>
<tr>
<td>Sephadex G-200 Guanidine-HCl agarose</td>
<td>1.20</td>
<td>2.29</td>
<td>1.85</td>
</tr>
<tr>
<td>Urea agarose</td>
<td>1.35</td>
<td>1.75</td>
<td>1.85</td>
</tr>
<tr>
<td>Average†</td>
<td>4.05</td>
<td>1.22</td>
<td>1.65</td>
</tr>
</tbody>
</table>

† All values are presented as 3H:14C ratios.
‡ These values are taken from the final line of Table VIII.
§ Only the peak containing the "core" enzyme, not the cr factor, is given in this column.
‖ These values are not as reliable as the others. Low counts, together with difficulty in eluting the samples, led to slightly atypical (low 3H) results. These figures have been included in the averages, but omitting them does not substantially change the general results.
¶ These values do not include the values in line 1.

Unfortunately, the removal of sodium dodecyl sulfate from cr factor prepared in this manner proved difficult. Several additional trials led to a procedure that proved more suitable for separating cr factor from core enzyme. The results shown in Fig. 11 were obtained by denaturing enzyme preparations with 8 M urea and chromatographing them on agarose. Not only was the resolution of cr factor effective, but also the urea could easily be removed by dialysis.

Table IX presents a compilation of the 3H:14C ratio of many native and dissociated preparations of the three purified enzymes. As can be seen from the average values, the modified enzyme contained 23 times as much tritium as the two host enzymes (4.05:1.60); 70% of the tritium could be easily dissociated from the modified enzyme, but none of the tritium was removable from the host enzyme.

It should be recalled that during the tritium labeling of the phage-infected culture there was an over-all increase of 117% in the total protein of the culture. Since this increase resulted in a 3H:14C ratio of 3.48 (Table VIII), the protein made de novo in the presence of 3H glucose must have had a tritium content (dpm per µg of protein) approximately 31.5 times the carbon 14 content (dpm per µg of protein) of the previously existing protein. Therefore the tritium present in the host synthetases (3H:14C ratio of 1.6 to 2.0) represents a de novo synthesis of 5.1 to 6.4% of these enzymes during the labeling period. Approximately 4% of the cells remained uninfected in this experiment, so one can ascribe a small part of the tritium content of the host enzymes to continued synthesis by uninfected cells; in 20 min at 30° this number of cells would account for an increase of 1% of the same doubly labeled preparations were therefore chromatographed on Sephadex G-200 equilibrated with phosphate-buffered 0.1% sodium dodecyl sulfate. The results shown in Fig. 10 indicate an excellent separation of the tritiated material (cr factor) from the core of the modified enzyme. The fractions containing the core enzyme (carbon 14 labeled) were pooled and rechromatographed (not shown). No additional removal of tritiated material occurred.
in total enzyme. The remaining 4 to 5% increase must have occurred in the 96% of the cells that were phage-infected, and must therefore represent the completion of nascent chains plus the expression of any host mRNA made as a result of delayed infection or delayed shut-off of host function. The dissociated core of the modified valyl-tRNA synthetase had a $^{3}$H:$^{14}$C ratio of approximately 1.22 (Table IX); this ratio reflects a de novo increase of 3.9%. Since modified enzyme could come presumably only from an infected cell, this increase agrees reasonably well with the 4 to 5% increase estimated for the isoleucyl-tRNA synthetase and unmodified valyl-tRNA synthetase in the infected cells.

Of greatest importance, however, was the tritium-labeled material that could be dissociated from the modified enzyme. Removal of this material lowered the $^{3}$H:$^{14}$C ratio of the core enzyme from 4.05 to 1.22 (Table IX). If the tritium-labeled material is polypeptide in nature, then this much reduction corresponds to the removal of a mass of polypeptide corresponding to 9.0% of the enzyme (2.83:31.5 x 100). This finding is consistent with the amount of newly synthesized material detected by the density labeling technique. Furthermore, if one accepts the approximation of 10,000 as the molecular weight of $\tau$ factor judged by its behavior on Sephadex G-200, agarose, and polyacrylamide gels, then there would seem to be 1 molecule of $\tau$ factor per molecule of modified enzyme.

Preliminary Analysis of $\tau$ Factor—Chromatography on agarose in the presence of 4 mM urea (Fig. 11) was used to prepare $\tau$ factor free of the core of modified valyl-tRNA synthetase. Fractions containing the tritiated $\tau$ factor were dialyzed against 6 mM phosphate buffer to remove urea and were then analyzed to establish the general chemical nature of this material.

The first observation was that the tritium-labeled material was nondialyzable, in accord with its estimated molecular size from chromatography. Second, treatment with Pronase rendered 60% of the labeled $\tau$ factor, as well as a bovine serum albumin control, dialyzable; yeast tRNA was unaffected by the Pronase treatment (Table X).

Third, the ultraviolet absorption spectrum of a dialyzed sample of $\tau$ factor was determined. The spectrum (not shown) was characteristic of protein rather than of nucleic acid.

### Table X

<table>
<thead>
<tr>
<th>Sample –––––––––––</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>355 µg/ml</td>
<td>355 µg/ml</td>
<td>0.0</td>
</tr>
<tr>
<td>Bovine serum albumin plus Pronase</td>
<td>335 µg/ml</td>
<td>119 µg/ml</td>
<td>66.5</td>
</tr>
<tr>
<td>tRNA plus Pronase</td>
<td>30 µg/ml</td>
<td>40 µg/ml</td>
<td>0.0</td>
</tr>
<tr>
<td>$\tau$ factor plus Pronase</td>
<td>6300 dpm/ml</td>
<td>5660 dpm/ml</td>
<td>0.0</td>
</tr>
<tr>
<td>$\tau$ factor plus Pronase</td>
<td>6300 dpm/ml</td>
<td>2540 dpm/ml</td>
<td>60.0</td>
</tr>
</tbody>
</table>

$^a$ Samples (3 ml) in 6 mM phosphate buffer (pH 7.2) containing the substances indicated were incubated at room temperature in dialysis bags. Where indicated Pronase was added at 100 µg per ml.

$^b$ After 24 hours, tRNA and bovine serum albumin were assayed by absorbance at 260 and 270 nm. $\tau$ factor was assayed by precipitation with trichloroacetic acid (5%), collection on glass fiber filters, and counting.

From this information it appears that $\tau$ factor is largely polypeptide in nature.

Activity of $\tau$ Factor—To test the effect of $\tau$ factor on normal host valyl-tRNA synthetase, a sample of $\tau$ factor was prepared by treatment of modified enzyme with 4 mM urea and chromatography on agarose. The sample was dialyzed to remove urea and then added to a crude extract made from uninfected *E. coli* NP4 cells. After 1 hour at 5°C the valyl-tRNA synthetase activity was assayed and then tested for urea stability. The addition of $\tau$ factor reduced the valyl-tRNA synthetase activity by 80% and, as shown in Fig. 12, converted it into a urea-stable form.

A separate portion of the $\tau$-treated extract was analyzed by centrifugation through a sucrose gradient. The results shown in the upper panel of Fig. 13 were obtained with the untreated extract and are typical of the behavior of host valyl-tRNA synthetase. The enzyme activity sedimented as expected for a substance of 105,000 molecular weight, was sensitive to urea, and was recovered in approximately 40% yield from the gradient. In the lower panel of Fig. 13 are shown the results with the $\tau$-treated extract. A major portion, possibly 50%, of the activity sedimented at a rate characteristic of modified enzyme, while the rest formed a broad band sedimenting between that...
Yaniv and Gros (15), in their extensive study of the valyl-tRNA synthetase of *E. coli*, described the tendency of this enzyme to aggregate under certain ionic conditions. Self-aggregation could easily account for some of the characteristics of the viral modified enzyme, including its increased rate of sedimentation through sucrose gradients and its increased stability to thermal and urea-induced inactivation. Therefore an important conclusion to emerge from our studies on the purified host and viral forms of the synthetase is that modification is not simply the self-aggregation of the host valyl-tRNA synthetase into dimers or other polymeric forms as a result of some change in the internal environment of the cell brought about by viral infection. The ease with which the two forms of the enzyme are totally resolved by purification, the retention of their respective properties during and after purification, and the similarity in size of the two molecular forms as judged by sieving argue strongly for a specific modification of the structure of the enzyme whether or not aggregation occurs under certain conditions as a secondary consequence of the viral-induced event.

Two observations require further comment. The first is the difference in apparent molecular weight indicated by the sedimentation properties of the modified enzyme and its behavior during molecular sieving. The simplified method of Martin and Ames (20) to relate relative sedimentation distance to molecular weight yields a value of 170,000 to 190,000 for the viral form of the enzyme (12).

The behavior on Sephadex G-200, however, indicates that the viral form of the enzyme can be no more than approximately 10% larger than the host form. Of the several conceivable explanations for this observation, the simplest would seem to be that modification adds mass to the enzyme and also changes its conformation to a more compact and hence more rapidly sedimenting form.

The second issue is the apparent absence of marked changes in the enzymological properties of the modified enzyme despite the great change in its several physical properties. Perhaps it is to be expected that no large change occurs in the value of the $K_m$ for valine or ATP since the enzyme obviously must continue to function in the phage-infected cell. On the other hand a qualitative change in the specificity of the enzyme for either its amino acid substrate or for its several tRNA$^{\text{Val}}$ substrates might be expected if modification of the synthetase affects the normal process of translation in the cell. Our evidence with both crude extracts and with purified enzymes indicates, however, that the viral form of the enzyme continues to select L-valine from among related amino acids with the same fidelity as the host enzyme, and that the several tRNA$^{\text{Val}}$ species are all charged by both forms of the enzyme. Only through the use of yeast tRNA has it been possible to discover an alteration in substrate specificity as a result of viral modification. The increased ability of the viral form to charge yeast tRNA can be accepted as an indication of a structural change in the synthetase, but it does not help us to understand the biological significance of modification.

The discovery of $\tau$ can be combined with the other information about modification to construct a fairly precise model of the process by which $T$-even bacteriophages modify the valyl-tRNA synthetase of *E. coli*.

Two or three minutes after infection the $\psi$ gene of the infecting T4 DNA is expressed. Its product, $\tau$ factor, is a polypeptide with a molecular weight of roughly 10,000. Very shortly after
its synthesis, each molecule of \( \tau \) combines with a molecule of host valyl-tRNA synthetase. The association is highly specific and is largely if not exclusively by noncovalent bonds. The \( \tau \)-enzyme complex is enzymatically active, but has greatly altered physical properties. By the end of the early period of infection (approximately 15 min) sufficient \( \tau \) has been made to convert the cell's entire complement of valyl-tRNA synthetase into the viral form.

This model is consistent with all of the facts that are known about T4 modification of the valine enzyme. The host enzyme forms the bulk of the viral enzyme as judged both by density labeling (12) and by the fact that the viral enzyme reflects mutant characteristics when developed in a host with a mutant synthetase (1). Modification occurs throughout the early period of infection and requires continued protein synthesis (12). Phage mutants exist that modify poorly and others that fail completely to modify (1); the latter are suppressible by amber suppressors. The modified enzyme contains material made after infection as judged by density labeling and radioactive labeling (Table IX), and this material amounts to approximately 10\% of the mass of the host enzyme. Material made after infection can be dissociated by urea or guanidine from the modified enzyme (e.g. Fig. 10). This material has a molecular weight in the 10,000 range, and is chiefly polypeptide (Table X).

Several questions suggested by this model have not yet been answered. First, the complete chemical nature of \( \tau \) is not known, and its molecular weight is known only roughly. Larger quantities of \( \tau \) must be prepared to permit biochemical and physical characterization. Only then will it be possible to speak with certainty about the stoichiometry of the \( \tau \)-enzyme interaction.

Second, the experiments performed with \( \tau \) in vitro (Figs. 12 and 13) do not unambiguously establish that normal modification of host valyl-tRNA synthetase consists simply of the addition of \( \tau \). It is possible that the partially stable product formed in vivo would have been normal if the preparation of \( \tau \) had not involved an extensive denaturation step. An alternative that cannot be ruled out, however, is that modification as it normally occurs in vivo involves some processing of the host enzyme in addition to the formation of a complex with \( \tau \). It is necessary now to examine the product of the in vivo addition of \( \tau \) to synthetase under a variety of conditions to learn whether a normally modified product can be formed; also, the core obtained by the dissociation of \( \tau \) from modified enzyme can be examined to learn whether it is essentially normal host synthetase.

In addition to these questions about the nature of the modification process, there remain several areas that deserve future attention. These issues include (a) the consequences of valyl-tRNA synthetase modification, (b) the possibility of other sites for \( \tau \) factor binding, and (c) the nature of the control of the phage gene governing the production of \( \tau \).

It has already been shown that the most striking changes brought about by viral modification are in the physical properties of the enzyme; enzymatically, only the increased capacity to charge yeast tRNA distinguishes the viral from the host form of the synthetase. No changes are detectable in the in vivo charging pattern of valine tRNA species as a consequence of enzyme modification.1 Furthermore, amber-suppressible mutants of T4 that appear devoid of modifying capability exhibit no reduction in their ability to grow (1, 3). Discovering the biological significance of valyl-tRNA synthetase modification has therefore become a major challenge. Interestingly, T4 phage are now known to bring about many changes in the translation machinery of E. coli. Some host tRNA species are modified by infection (e.g. 27–29), some enzyme activities that modify tRNA nucleotides are changed (30, 31), several viral tRNA species are synthesized in the infected cell (7, 8, 32–34), and one or more factors alter the ability of ribosomes to translate various mRNA species in vitro (35–38). Some of these changes (e.g. the formation of suppressor tRNA (34) and the modification of the valyl-tRNA synthetase) can be shown to affect phage protein synthesis under highly selected circumstances, but for none of them has a biological role been established.

\( \tau \) factor has been discovered through its association with host valyl-tRNA synthetase. It is not necessary that this be the only, or indeed the chief, binding site for this factor; there is a possibility that an important function of \( \tau \) factor involves its operation on some component of the cell other than valyl-tRNA synthetase. This possibility can be explored by developing an assay for \( \tau \) to test for its presence in cell extracts.

Finally, we know about the synthesis of \( \tau \) only through its modification of the valine activating enzyme. From the fact that the addition of chloramphenicol stops modification quite promptly, one can deduce that there is not a large pool of completed but uncomplexed \( \tau \) molecules present during early times in infection. Once the cell's complement of enzyme has been modified, however, there is no indicator for \( \tau \) synthesis. Learning what happens to the synthesis of \( \tau \) after this time will depend on the development of an in vitro assay for \( \tau \).

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Viral Modification of the Valyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*

George L. Marchin, M. Margaret Comer and Frederick C. Neidhardt


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