# PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF MODIFICATION OF *ESCHERICHIA COLI* VALYL-tRNA SYNTHETASE BY *vs* MUTANTS OF THE BACTERIOPHAGE $T_4$.

by

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# INTRODUCTION

Bacteriophage $T_4$ is a double-stranded DNA virus with a genome size of approximately $1.66 \times 10^5$ nucleotide pairs (166 kilobases). The length of the DNA in each phage is actually closer to 170 kilobases since the linear genome is circularly permuted and terminally redundant by about 2%. The phage contains enough information to code for 160 to 170 average-sized proteins. So far approximately 140 genes have been identified, and 70 of these were discovered to be nonessential. Nonessential genes are defined by mutations that alter or prevent phage growth under some conditions, but do not prevent plaque formation on E. coli B grown in Hershey medium. (1). Nonessential, therefore, does not imply nonfunctional or unimportant.

$T_4$ infection of E. coli is initiated by a two step adsorption to a specific site on the cell wall (2). The first step involves a reversible attachment; the second an irreversible fixation (3). A complex conformational change in the sheath, tail plate, and tail fibers allows the core to penetrate the cell wall and inject its DNA (2).

Host specific DNA and RNA synthesis is stopped after infection by a complex of mechanisms. One mechanism requires protein synthesis and is independent of the multiplicity of infection (4, 5). The other can inhibit host nucleic acid synthesis even in the absence of protein synthesis, the degree of inhibition being greater at higher multiplicities (4, 5). Only after the arrest of host macromolecular synthesis is the E. coli DNA degraded (6).

Seven minutes after infection, phage specific DNA synthesis begins at a rate which is 5 to 10 times higher than the previous

rate of host DNA synthesis (7). Phage nucleic acid synthesis can be characterized by the specific incorporation of 5-hydroxymethylcytosine into the DNA and modification of the completed strands by methylation and glucosylation (2).

The phage mRNA's are composed of three classes. Immediate early mRNA is produced in the absence of protein synthesis (8). E. coli RNA polymerase without the sigma factor will indiscriminately transcribe the phage DNA, but upon the addition of the sigma factor, only the early messages are produced (9). The second class of messengers, quasi-late mRNA (2), require protein synthesis for their transcription (8) and also require phage-induced alterations of host RNA polymerase (2). Richardson discovered that E. coli termination factor rho prevents the transcription of $T_4$ quasi-late genes (10). An immediate early gene product may act as an antitermination factor that allows the switch of mRNA synthesis from the immediate early to the quasi-late class. The third class of messengers, late mRNA, requires phage DNA synthesis and protein synthesis (11).

Very early in the study of $T_4$ physiology the question was asked to what extent the virus depended upon the translational apparatus of the host cell. In 1966 Neidhardt and his colleagues developed an approach to this problem by isolating a diverse set of aminoacyl-tRNA synthetase mutants of E. coli which were incapable of protein synthesis at 42°C (12).

Among the elements of the host translational apparatus, the aminoacyl tRNA synthetases were chosen for study because of their crucial role in protein biosynthesis. In Escherichia coli these molecules select from a diverse set of 20 amino acids and approximately 60 different tRNA species to aminoacylate the appropriate tRNA.

Using ATP as their exclusive energy source, each of these syntheses catalyzes the attachment of an amino acid to its cognate tRNA (13). Aminoacylation may be described as a two step reaction (13, 14). The first step results in the activation of the amino acid by the formation of an enzyme bound aminoacyl adenylate complex from the amino acid and ATP. Pyrophosphate is released in this reaction. The second step produces a covalent linkage between the amino acid and the 2' or 3' hydroxyl group of the --C-C-A end of the tRNA molecule. AMP is then released. A second possible function for these enzymes has been demonstrated involving the deacylation of aminoacyl-tRNA (13). This reaction would permit the synthetases to correct for aminoacylation errors and, therefore, improve the accuracy of translation. This reaction also could affect the steady state level of charged versus uncharged tRNA present in the cell. Numerous additional regulatory functions for aminoacyl-tRNA synthetases have been proposed. Among these are the regulation of operons for amino acid biosynthetic enzymes, metabolic regulation of the aminoacyl-tRNA synthetases themselves (autoregulation), and control of stable RNA synthesis during amino acid restriction (12, 15). To date, however, no conclusive evidence has proven any of these putative regulatory roles for the synthetases per se.

Although the catalytic properties of the different synthetases are similar, their structural characteristics and regulatory functions in cellular metabolism may be quite varied (13). Proteolysis of various aminoacyl-tRNA synthetases has led to speculations on their tertiary structure. Results of trypsinolysis with isoleucyl- (16), leucyl- (17), and valyl-tRNA synthetases (18) suggest similar structures. The model proposed consists of two globular sections

linked covalently by a small peptide bridge with several strong non-covalent bonds forming a trough between them (16). Mild trypsin treatment appears to break the covalent bridge without a drastic effect on the overall structure. Valyl-tRNA synthetase after trypsinolysis still is able to catalyze the pyrophosphate exchange reaction and the charging of tRNA with valine, although, the Km for $tRNA^{val}$ is lower compared to the untreated form of the enzyme (18).

The selection of a temperature-sensitive valyl-tRNA synthetase mutant of Escherichia coli (19) has helped to define the role of this enzyme in cellular metabolism. The enzyme functions normally in vivo at 30°C, but a shift to 40°C results in the rapid discharging of valine tRNA coordinate with a cessation of protein synthesis (20). Neidhardt and his colleagues were thus in a position to examine whether or not bacteriophage $T_4$ utilized host valyl-tRNA synthetase for the synthesis of viral proteins. When these temperature-sensitive mutant bacteria were infected with the bacteriophage $T_4$ at 30°C for 5 minutes and then shifted to 40°C, their ability to synthesize protein was restored (21).

The previously temperature sensitive valyl-tRNA synthetase activity was discovered to be stable at nonpermissive temperatures when the E. coli cells were infected with $T_4$. This phage-induced valyl-tRNA synthetase activity thus enabled the cells to charge $tRNA^{val}$ and to synthesize protein at 40°C (21). Protein synthesis was required for the new activity to appear as an immediate-early phage function (21). To determine whether the phage-induced valyl-tRNA synthetase activity was coded for by the $T_4$ genome, density shift experiments and double labeling techniques were performed with wild-type E. coli strains. The density shift experiment showed that the new enzyme was composed

mainly of polypeptide chains made prior to phage infection (22). Double labeling techniques, on the other hand, suggested that the new activity was associated with a molecule 10% of which was synthesized *de novo* in phage-infected cells (23).

The phage-induced valyl-tRNA synthetase was investigated further to find what characteristics differentiated it from the host enzyme. Greater resistance to denaturation by heat was shown by the ability of $T_4$ to grow on *val*$^{ts}$ mutants at nonpermissive temperatures (21). The new enzyme was also more resistant to denaturation in 4 M urea. The normal host enzyme was rapidly inactivated, yet under the same conditions the phage-induced enzyme was only slowly inactivated (24). Sucrose gradients were used to demonstrate a higher rate of sedimentation for the new enzyme compared to the host enzyme (22). The molecular weights were estimated from the sucrose gradient centrifugation for both enzymes; the host enzyme had an apparent molecular weight of 105,000 while the phage-induced enzyme was estimated at 170,000 (24). The apparent net charge of the two enzymes also differed as shown by the elution of phage valyl-tRNA synthetase activity from an hydroxyapatite column at a significantly higher salt concentration than the host enzyme (24). The new enzyme had a greater ability to charge yeast tRNA (25). Both enzyme preparations, though, gave apparent Km values during aminoacylation for l-valine, ATP, and unfractionated tRNA that were similar (24).

Electrophoresis on polyacrylamide gels with SDS, chromatography on agarose columns with guanidine or urea, and chromatography on Sephadex G-200 with SDS of the phage-induced enzyme from double label experiments showed the excess $^3H$ label (added at the time of phage

infection) to dissociate as a single peak, with the approximate molecular weight of 10,000. None of the $^{3}H$ label from the host enzyme dissociated (26). This peak was further characterized as a protein because of its size, UV spectrum, susceptibility to pronase (26), and amino acid composition (27). This peptide has been named tau (26). Tau is made within the first 5 minutes after infection at 37°C along with other immediate-early enzyme functions and completed after 15 minutes (25).

The $T_4$ induced valyl-tRNA synthetase is modified by the addition of this small peptide, tau. The molecular weight of the host enzyme was determined to be approximately 105,000 and the phage-induced enzyme to be around 170,000. Since the addition of tau would only change the molecular weight by 10,000, the addition of another factor was possible (25). The sedimentation rate of purified, modified valyl-tRNA synthetase on sucrose gradients was specifically increased by the addition of purified tRNA isolated from E. coli B, as is its stability in 4 M urea (28). The modification of the enzyme by tau is thought to allow the interaction of this new enzyme with tRNA (28). The modification of valyl-tRNA synthetase is believed to be unique since $T_4$ has no effect on glycyl-, histidyl-, and phenylalanyl-tRNA synthetases (21).

To aid in the investigation of the phage-induced valyl-tRNA synthetase activity, a mutant isolation was conducted. Heavily mutagenized $T_4$ phage were screened for their ability to convert host valyl-tRNA synthetase to the urea stable form. Two mutants of $T_4$, vs1 and vs2, were analyzed at that time (26). The vs1-induced valyl-tRNA synthetase displayed an intermediate rate of denaturation in 4 M urea between the host and wild-type $T_4$ enzymes. It is thought

to possess a missense mutation (26). The vs2-induced enzyme was indistinguishable from E. coli valyl-tRNA synthetase when urea tests were performed (26). An amber mutation was believed to be the cause of the vs2-induced activity, since growth on an su$^+$ strain of E. coli resulted in the appearance of the urea-resistant $T_4$-valyl-tRNA synthetase activity (26). Both mutants were unable to repair the thermolabile enzyme from temperature-sensitive mutants; but their burst sizes were normal on the wild-type parent at nonpermissive temperatures. A revertant of vs2, vs2RF01, has been isolated recently. It grows at the nonpermissive temperatures on val S$^{ts}$ mutants of E. coli (unpublished results); however, does not regain the 4 M urea stability to the full extent that wild-type $T_4$-induced enzyme does. Genetic studies of the $T_4$vs$^-$ mutants indicate that they define a single complementation group (26) mapping between the tk and regB genes at gene position 58 kb (1). The simplest explanation for the mutants therefore, is that the vs gene codes for the tau peptide.

The function of tau and modification remains a mystery. Tau is known to be an immediate-early gene that is nonessential (1, 29). Several possible functions for the role of tau were investigated by comparing $T_4$ and $T_4$vs2 (30). The overall rate of transfer of valine into protein, for example, was reduced to a similar extent after infection. Also, modification did not alter the level of aminoacylation of the total valine tRNA pool nor of the individual species of valine tRNA. Modification did not appear to be necessary to maintain these levels after infection. No evidence was found that modification affects any valyl-tRNA synthetase reaction that yields novel valine-containing products. And, although the $T_4$-modified

enzyme was more stable *in vitro* than the *vs*2-modified enzyme, modification did not seem to facilitate phage growth at temperatures above the optimum. All of this research was based on the assumption that the *vs*2 phage forms an amber fragment of the *tau* peptide that is unable to modify the host enzyme. It seems unlikely, however, that the production of a modifying factor able to attach to the valyl-tRNA synthetase and alter some of its properties would have persisted very long in the absence of any selective pressure for its maintenance, if it had no function advantageous to the phage (30).

Additional biochemical and genetic information has accumulated since the isolation of the $T_4$*vs*$^-$ mutants. Modification is immediate early. The high molecular weight form of the virus-induced enzyme, as well as a component of urea stability, are due to its unique association with tRNA (28). The $T_4$ mutants, *vs*1, *vs*2, and *vs*2RF01, appear to produce a *tau*-like peptide (unpublished observations). The phage-induced enzyme is uniquely susceptible to trypsin inactivation (27). Finally, in the initial investigations of the *vs*2 mutant (30), the revertant, *vs*2RF01, was not available for comparative studies.

We sought initially, therefore, to compare a number of physiological parameters during infection of *E. coli* B with the available $T_4$ strains. These studies were designed to direct us toward more precise information concerning the biochemistry of modification. This investigation was extended to the growth of the $T_4$ phage in other strains of bacteria. Finally, a preliminary characterization of the partially purified valyl-tRNA synthetase from $T_4$*vs*$^+$, *vs*1, *vs*2, and *vs*2RF01, using limited proteolysis, was conducted.

## MATERIALS AND METHODS

Bacteria and bacteriophage. Escherichia coli strain NP4 (a B strain) and bacteriophage $T_{4Bc}^{+}$, vs1, vs2, and vs2RF01 were obtained from F. C. Neidhardt, University of Michigan. Shigella dysenteriae strain Sh-15 was obtained from S. E. Luria, MIT.

Chemicals. All common chemicals were reagent grade. Adenosine 5'-Triphosphate (ATP), glutathione (GSH), bovine serum albumin (BSA), potassium morpholinopropane sulfonate (MOPS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), lysozyme, trizma base (tris), DEAE cellulose, trypsin, and sodium penicillin G were obtained from Sigma Chemical Company, St. Louis, Missouri. E. coli B tRNA, $^{3}$H-valine (6 Ci/mM), $^{3}$H-uridine (8 Ci/mM), and $^{3}$H-arginine (12 Ci/mM) were purchased from Schwarz/Mann, Orangeburg, New York.

Media. Cells were grown in a variety of media specifically indicated for each experiment. M9-minimal medium containing 5.8 mg/ml $Na_2HPO_4$, 3 mg/ml $KH_2PO_4$, 0.5 mg/ml NaCl, 1 mg/ml $NH_4Cl$, $10^{-5}$ M $FeCl_3$, $10^{-3}$ M $MgSO_4$ and 0.4% glucose as carbon source was prepared according to Adams (31). Benzer broth contained 7 g NaCl and 13 g tryptone per 1000 ml $H_2O$. Benzer agar contained 5 g NaCl and 13 g tryptone per 1000 ml $H_2O$. Soft agar was Benzer agar with only half the amount of agar added. MOPS minimal medium was prepared according to Neidhardt et al. (32). Standard buffer contained 6 mM potassium phosphate and 6 mM 2-mercaptoethanol, pH 7.1.

Preparation of cell-free extracts. 50 ml cultures were grown aerobically on a Gyrotory Water Bath Shaker and their optical density measured in 5 ml cuvettes in a Klett Summerson Photoelectric Colorimeter using a red filter. At a density of $2 \times 10^8$ cells/ml the cultures were infected with bacteriophage $T_{4Bc}^+$ at a multiplicity of infection (MOI) of 5 phage per cell. After incubation at 37°C for 10 minutes, the cells were centrifuged at 10,000 rpm for 10 minutes and then resuspended in 5 ml standard buffer. Cells were sonicated for 2 minutes at 15 second intervals with a Sonifier Cell Disruptor, Model 185W, at a setting of 4. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes, and the supernatant referred to as cell-free extract. The extracts were stored at 4°C and the protein content was determined by the method of Lowry et al. (33).

Synthetase assay. Valyl-tRNA synthetase (VRS) activity was measured by attachment of $^3$H-valine to tRNA in a reaction mix containing 250 µg tRNA, 100 µg BSA, 2.0 µmoles ATP, 10 µmoles of $^3$H-valine at 15µCi/µmole, 5 µmoles of KCl, 50 µmoles of tris, and 5 µmoles of $MgCl_2$ with a final pH of 7.2. Samples containing VRS were added at 4°C to give a final volume of 0.5 ml. Blanks contained all components except enzyme. After a 5 minute incubation at 37°C the reaction was stopped by cooling quickly and adding 3 ml cold 5% trichloroacetic acid. After 30 minutes precipitates were collected on glass fiber filters and washed with 10 ml cold 5% trichloroacetic acid and 5 ml 67% ethanol. Filters were dried at 80°C for 30 minutes and counted in a Packard Tricarb Liquid

Scintillation Spectrometer with 5 ml toluene base scintillation cocktail.

Urea stability test. The resistance of enzyme to denaturation by 4 M urea was tested by incubation of valyl-tRNA synthetase activity at 37°C in a solution containing 4 M urea, 100 µg BSA, and standard buffer in a total volume of 0.5 ml. Controls were incubated without urea. After a period of 5 minutes, a 0.1 ml aliquot of the mixture was pipetted into 0.4 ml reaction mix described above, and was assayed for VRS activity. Urea stability was expressed in percentage of the control activity surviving the urea treatment.

Glycerol density gradient centrifugation. Glycerol density gradients 32% to 8% (w/v) were prepared in a Buchler two-chambered mixing device. Gradients were buffered with 6 mM potassium phosphate containing 10 mM 2-mercaptoethanol. Then, 0.2 ml of the cell-free extracts were layered on top of the 4.8 ml gradients. After centrifugation at 40,000 rpm for 16 hours, the gradients were collected from the bottom of the tube in 10 drop fractions. Every other fraction was assayed for VRS activity as described above.

Urea-treated phage. $T_4$ phage ($10^{11}$ to $1.5 \times 10^{12}$) in 0.1 ml washing fluid (0.01 M Tris, pH 7.4, 0.001 M $MgCl_2$, 0.1% NaCl, 0.01% gelatin) were added to 0.2 ml of a 9 M urea solution containing 2% BSA and 0.1 M sodium phosphate, pH 7.0. After 10 minutes at 37°C, 0.2 ml of this mixture was added to 1.8 ml of 2% BSA in 0.1 M phosphate, pH 7.0. This treatment reduced the titer on bacteria by a factor of $10^6$ or more. (34).

Spheroplasts. Spheroplasts were prepared by two separate methods. (a). One milliliter of an overnight culture of NP4 in minimal medium was subcultured in 19 ml of the same medium and aerated at 37°C (for about 4 hours) until the increase in optical density at 550 nm reached 0.6, approximately 2 x $10^8$ cells/ml. A portion (16.6 ml) of the subculture was added to 2.8 g of sterile sucrose in a 250-ml Erlenmeyer flask. Then 2 ml of V broth (3 g of meat extract, 3 g of yeast extract, 10 g of peptone, 2 g of glucose, 4 g of $MgSO_4 \cdot 7H_2O$, and 200 ml of distilled water) and 10 mg of sodium penicillin G were added. The mixture was incubated at 32°C for 2 hours. This procedure yielded about 2 x $10^8$ spheroplasts. For other Enterobacteriacae, the cells were grown in Benzer broth and when treated with the penicillin 30 mg of the drug were used.(34). (b). Cells were grown at 37°C with aeration in a defined medium, M9, until they reached a concentration of 2 x $10^8$ cells/ml. After two washings with 0.1 M tris buffer, pH 8.0, they were resuspended at 5 x $10^8$ cells/ml in 5 ml of a protoplasting medium (0.5 M sucrose in 0.3 M Tris buffer, pH 8.0). To this suspension, 50 µg of crystalline egg white lysozyme is added (0.05 ml of 0.1% lysozyme solution), and the mixture is swirled for 2 minutes at room temperature. Then 0.025 ml of a 4% EDTA solution was added with mixing. Spheroplast formation was complete in 10 minutes (35). The spheroplasts were tested for their sensitivity to osmotic shock (a method of showing the efficiency of the spheroplast preparation procedure) by diluting the cells 1/5 in 0.3 M sucrose and in distilled water, and then reading the $OD_{550}$ difference on a Beckman DU Spectrophotometer (35).

Spheroplast infection procedure. A standard infection mixture consisted of 0.5 ml of spheroplasts ($1 \times 10^8$ cells) diluted with an equal volume of 0.01 M sodium phosphate, pH 7.0, and 0.1 ml of urea-treated phage. This mixture was incubated at 37°C for set intervals of time, and titers were determined for each interval by the pour plate method described below.(34).

Pour plate technique. Benzer agar plates were overlayed with 2.5 ml soft agar containing 0.1 ml of an overnight bacterial culture (usually NP4) and 0.1 ml of the appropriately diluted phage. The phage were diluted in Benzer dilution fluid (7 g NaCl and 1 g tryptone per 1000 ml water.)

High titer lysates. One milliliter of an overnight culture of bacteria was diluted in 20 ml of minimal medium (M9 or MOPS). The cells were incubated with aeration for 1.5 hours at 37°C. The cultures were infected with the $T_4$ phage at an MOI of 0.01 phage/cell. The infection mixture was aerated at 37°C for 5 hours and then the cells were lysed with chloroform.

Mutagenesis. NP4 cells were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Tris-maleic buffer (TM buffer) consisted of M9 minimal medium in which the phosphate salts were replaced by Tris and maleic acid, each at a final concentration of M/20, pH 6. A culture of NP4 was incubated at 37°C with shaking until the cells were in logarithmic phase, $5 \times 10^8$ cells/ml. A sample of the culture was filtered rapidly on a 47 mm Millipore filter, pore size 0.45 μ. After washing on the filter with 5 to 10 ml of TM buffer, the cells

were suspended by placing the membrane in the original volume of TM buffer at pH 6.0 in a 125-ml Erlenmeyer flask, and agitating with the Vortex Mixer. The membrane was removed, and MNNG (1 mg/ml) was added directly to the suspension to give the desired concentration of 100 µg/ml. The final concentration of treated cells was 5 to 8 x $10^8$/ml. After incubation for 1 hour at 30°C a 1.0 ml sample was filtered on a Millipore filter, washed with 5 ml of cold M9 without glucose, and resuspended in 10 ml of M9 medium without glucose. The suspension was then serially diluted and plated, using the soft agar overlay method. There was no selection scheme used. (36).

Rate of protein synthesis. An overnight culture of NP4 was grown in MOPS medium, l-arginine (10 µg/ml), and l-tryptophan (50 µg/ml.) A subculture was grown in the same medium at 30°C to a density of 2 x $10^8$ cells/ml. After centrifugation for 10 minutes at 10,000 rpm, the pellet was resuspended in 5 ml of the defined medium without arginine. The $T_4$ phage at an MOI of 5 phage/cell was added to 1 ml of the resuspended culture and kept at 0°C for 5 minutes to insure good adsorption. The infected cells were then placed in a flask containing 9 ml of MOPS medium, 20 µg arginine, 25 µCi $^3$H-arginine, and 50 µg/ml tryptophan in a shaking water bath at 30°C. Each minute for 25 minutes a 0.2 ml sample was removed and put in 2.5 ml cold 5% trichloroacetic acid. These samples were filtered on glass fiber filters, dried at 80°C, and then counted in a Packard Tricarb Liquid Scintillation Spectrometer with 5 ml toluene base scintillation cocktail.

Rate of RNA synthesis. This procedure was performed as described above by replacing the arginine with uridine.

Partially purified valyl-tRNA synthetase. The tRNA was removed from cell-free extracts by column chromatography on DEAE cellulose according to Seifert et al. (37). Ten milligrams of cell-free extract were applied to a 10 cm diameter column containing 10 g of adsorbant (capacity 0.85 meq/g, medium mesh). A 200 ml gradient (0.22 M to 0.70 M) of $NH_4Cl$ separated the VRS activity from tRNA. Enzyme fractions were pooled for limited trypsinolysis (28).

Trypsin treatment. Trypsin (1 mg/ml) and the partially purified VRS pools could only be frozen once for short periods of time because of the rapid decay in enzyme activity from freezing and thawing. In a typical experiment, the appropriate amount of partially purified VRS (to give approximately 1000 cpm in the aminoacylation assay) was incubated in the presence or absence of 100 µg tRNA with 45 µg trypsin at 37°C for 1 hour. Before the addition of trypsin, 0.1 ml samples were transferred to test tubes on ice. The trypsin was added, and 0.1 ml samples were withdrawn and placed in tubes on ice. Aminoacylation activity was assayed by adding the assay mix (0.4 ml) to the chilled tubes, and by incubating at 37°C for 5 minutes. The cell-free extracts and the DEAE cellulose column were prepared in TMA/2 buffer. TMA buffer contained 10 mM Tris, 10 mM $Mg(C_2H_3O_2)_2$, and 22 mM 2-mercaptoethanol, pH 7.4.

# RESULTS

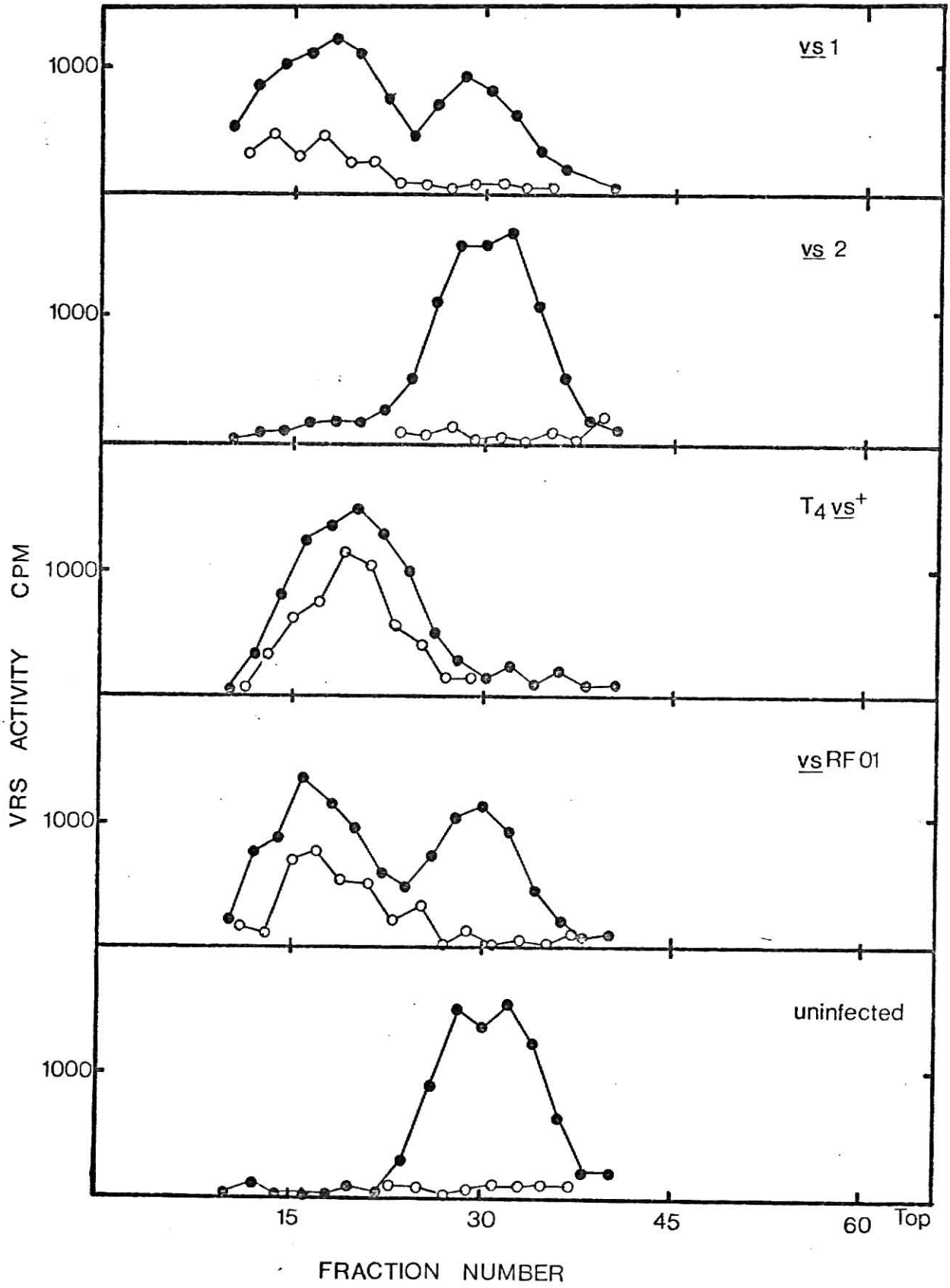
A set of $T_{4\underline{vs}}^-$ mutants isolated by W.H. McClain was utilized to investigate the physiological function of valyl-tRNA synthetase modification. An initial characterization of these mutants has been published (26). To compare the phenotypes of these mutant phage a culture of E. coli in balanced growth was split into five small cultures and infected with $T_{4Bc}^+$, vs1, vs2, and vs2RF01. One culture remained uninfected. After 3 minutes surviving bacteria in the infected cultures were less than 1%; after 10 minutes the cells were pelleted. Cell-free extracts were prepared and then were analyzed for valyl-tRNA synthetase activity after glycerol density gradient centrifugation. The results are shown in Figure 1 and are consistent with the phenotypes reported for two of these mutants; the vs2RF01 phenotype has not yet been reported.

Initially we reasoned that bacteriophage $T_4$ development in another bacterial host with a somewhat different physiology and metabolism might be altered sufficiently to demonstrate a role for modification of the synthetase. Specifically, analysis of the valyl-tRNA synthetase activity in a different host could show if the tau peptide interacted with this host's enzyme to fulfill an unknown function. Also, the behavior of the vs gene mutants, especially vs2 (which was assumed to be unable to modify the enzyme), might prove useful in determining the function of tau in $T_4$ development.

Members of the genus Enterobacteriacae such as Salmonella, Enterobacter, Proteus, Serratia, and E. coli support $T_4$ growth when spheroplasts are infected with urea-treated phage (34). The interaction between the phage and the bacteria does not require phage tail fibers

Figure 1. Glycerol density gradient sedimentation of the cell-free extracts of uninfected and $T_4$ phage-infected E. coli cells.

Glycerold density gradients, 32% to 8% w/v, were prepared and 0.2 ml of the extracts of uninfected and $T_4$-, vs1-, vs2-, and vs2RF01-infected cells were layered on top of the 4.8 ml gradients. After centrifugation at 40,000 rpm for 18 hours, the gradients were collected from the bottom in 4 drop fractions. Every other fraction was assayed for valyl-tRNA synthetase activity ( ● ). The other fractions were assayed for urea stable valyl-tRNA synthetase activity ( o ).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or the specific tail fiber receptors on the bacteria. Using the NP4 host (E. coli B) in logrithimic growth, spheroplasts were prepared by two methods: penicillin treatment, and a lysozyme-EDTA procedure. The addition of 10 mg of penicillin to the bacteria in a osmotically protecting medium and incubation for 2 hours at 32°C proved to be the most effective method of producing spheroplasts. This was shown by simultaneously diluting the spheroplast preparation in water and a 0.3 M sucrose solution to test the sensitivity of the cells to osmotic shock. The $T_4$ phage were treated with 6 M urea for 10 minutes at 37°C. The best infection of the spheroplast with the urea-treated phage resulted in a lysate with a titer of $1.5 \times 10^7$ phage/ml. Assuming a reasonable burst size of 200 phage/spheroplast (34), of the $1 \times 10^8$ spheroplasts exposed to the treated phage, only 0.1% of the spheroplasts were infected. The infection efficiency, therefore, was too low to detect modification of valyl-tRNA synthetase by urea denaturation tests.

The low infection efficiency of the spheroplast system prompted us to find a natural host for the $T_4$ bacteriophage other than Escherichia coli. A Shigella dysenteriae strain, Sh-15, that is able to support $T_4$ development under normal conditions. Shigella dysenteriae is interesting because host macromolecular synthesis continues after phage infection (4). In E. coli the complete arrest of host synthesis requires protein synthesis (4, 5), appears with other immediated-early phage gene functions (2), and may require some form of translational regulation, such as the inactivation of one or more cellular tRNA's (2).

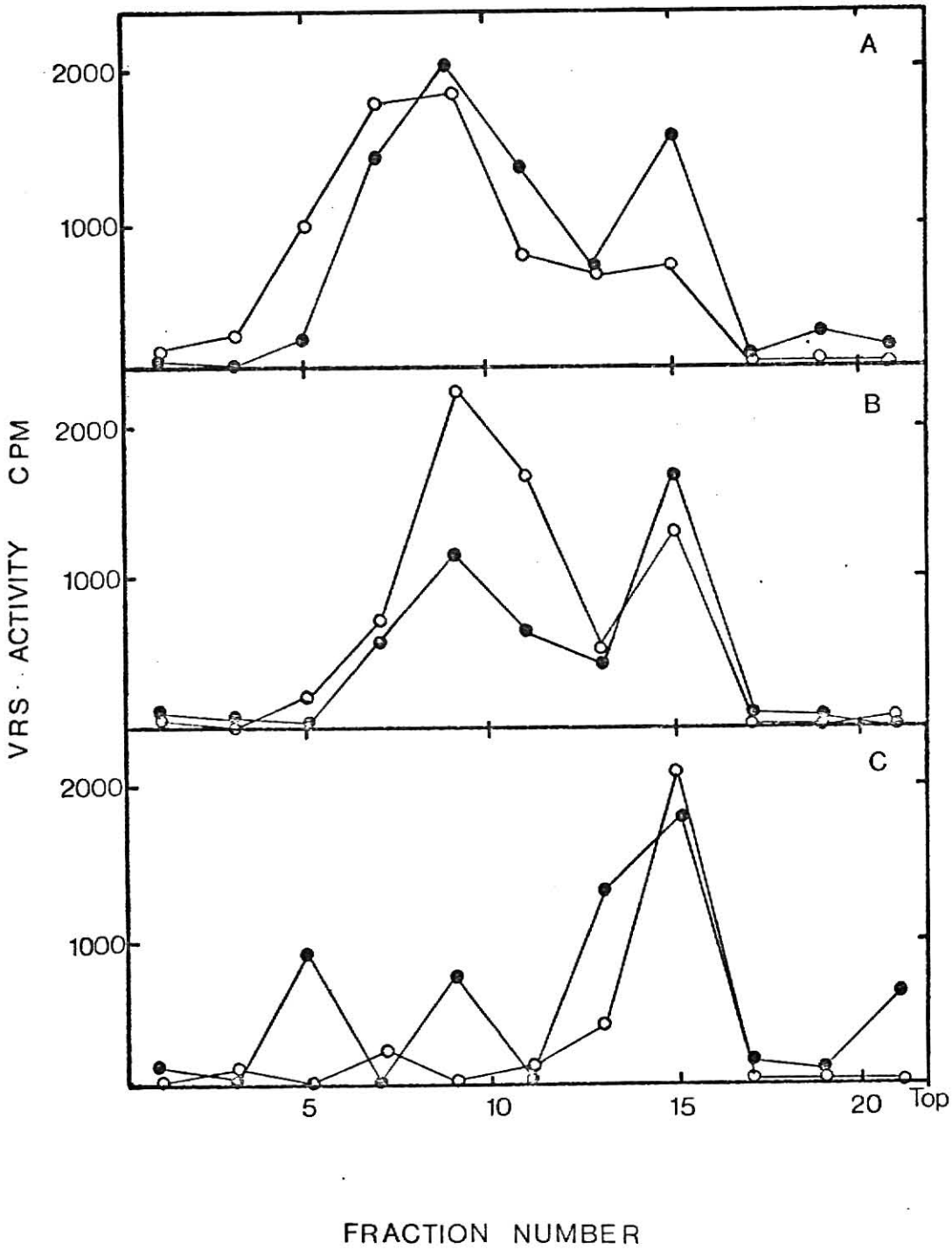
High titer lysates of the phage strains $T_{4Bc}$+, vs1, vs2, and vs2RF01 were prepared on both E. coli B and Shigella dysenteriae. Titers

of approximately 1 x $10^{11}$ pfu/ml were obtained for all the phages on both strains. Cell-free extracts of the four phage-infected and uninfected cultures of NP4 and Sh-15 were made using the appropriate lysates. Urea stability tests showed that modification of valyl-tRNA synthetase aminoacylation activity occurred *in vivo* in the Sh-15, although the enzyme was less stable to urea denaturation than the enzyme modified in the *E. coli* host (Table I). Besides all the $vs^-$ mutants being able to grow on the *Shigella* strain, the valyl-tRNA synthetase activities displayed similar urea stability and, presumably, modification, in the proper relative order.

The same cell-free extracts also were layered on glycerol density gradients, centrifuged for 16 hours, and fractionated for enzyme analysis. In general, similar profiles of the aminoacylation activity of the heavy and light forms of valyl-tRNA synthetase were obtained for each virus on the two bacterial strains (Figure 2). This suggests that the results of the tRNA interaction with the enzyme of the infected Sh-15 were similar to those of the infected NP4 enzyme. The reason for the small peaks in the heavy region of the *vs2*-infected *E. coli* gradient profile is unknown.

An attempt was made to identify a mutant of *E. coli* in which the growth of *vs2* was specifically impaired. If such a conditional mutant (the mutant would grow wild-type $T_4$ normally) could be isolated, modification of the valyl-tRNA synthetase would be demonstrated to have a physiological significance to the $T_4$ bacteriophage; and an analysis of the bactrial mutant might define what that significance was.

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) at a concentration of 100 µg/ml, a concentration shown to produce a high yield of mutants (36), was incubated at 30°C with NP4 cells in logrithimic phase that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VRS · ACTIVITY CPM
5
10
15
20
Top
FRACTION NUMBER

Figure 2. Glycerol density gradient sedimentation of the cell-free extracts of phage-infected NP4 and Sh-15 cells.

Glycerol density gradients, 32% to 8% w/v, were prepared, and 0.2 ml of the extracts of $T_4$-, vs1-, and vs2-infected cells from each strain, NP4 and Sh-15, were layered on top of the 4.8 ml gradients. After centrifugation at 40,000 rpm for 16 hours, the gradients were collected from the bottom in 10 drop fractions. Every other fraction was assayed for valyl-tRNA synthetase activity. Panel A is $T_4$-infected Sh-15 ( ● ) and NP4 ( ○ ) cells; Panel B is vs1-infected Sh-15 ( ● ) and NP4 ( ○ ) cells; and Panel C is vs2-infected Sh-15 ( ● ) and NP4 ( ○ ) cells.

| Phage added | NP4 cell-free extracts | | | | Sh-15 cell-free extracts | | | |
|---|---|---|---|---|---|---|---|---|
| | urea-treated (%) | control (%) | urea-stability (%)[a] | (%)[b] | urea-treated (%) | control (%) | urea-stability (%)[a] | (%)[b] |
| $T_{4\underline{vs}}^{+}$ | 1117 | 1277 | 87.4 | 100 | 452 | 1420 | 31.8 | 100 |
| <u>vs</u>1 | 295 | 1216 | 24.2 | 27.7 | 96 | 964 | 10.0 | 31.5 |
| <u>vs</u>2 | 134 | 1375 | 9.7 | 11.1 | 45 | 1063 | 4.2 | 13.2 |
| <u>vs</u>2RF01 | 739 | 1094 | 67.5 | 77.2 | 299 | 1216 | 24.5 | 77.0 |

a. Relative to activity prior to urea addition
b. Relative to $T_{4\underline{vs}}^{+}$ activity

Table 1. Urea stability of valyl-tRNA synthetase (VRS) in extracts of phage-infected S. dysenteriae (Sh-15) and E. coli (NP4) cells.

The crude extracts of phage-infected NP4 and Sh-15 cells were each diluted 1/3 in standard buffer, and 0.15 ml of the diluted sample was added to a urea and buffer tube. The urea tube contained 4 M urea, 100 µg BSA, and 0.05 ml of buffer to total 0.5 ml after the addition of the extract. The buffer tube had an equivalent volume of buffer to replace the urea. The tubes were incubated at 37°C for 5 minutes, and then 0.1 ml samples were assayed for aminoacylation activity. The activity of the buffer tube was considered 100% for each extract. $T_4$-, vs1-, vs2-, and vs2RF01-induced VRS were tested for each strain, NP4 and Sh-15.

were resuspended in Tris-maleic buffer. Treatment of cells in buffer instead of broth seems to prevent the killing effect of MNNG (36). Isolated colonies were grown from the suspension of treated cells at 30°C. Each colony was transferred to a separate tube containing Benzer broth and incubated at 30°C. Two pour plates of $T_4$ and vs2 were prepared, each containing the Benzer broth culture. One plate of each virus was grown at 30°C and at 40°C to test for temperature sensitive mutants. Approximately 300 isolated colonies were processed. Table II shows the isolates with atypical plaque morphology for $T_4$ and/or vs2 that were found. None of the isolates had different plaque morphology at the two temperatures. More importantly, none of the tested colonies was discovered to be specifically incapable of vs2 propagation. Although this was a small search, it does show that mutagenesis of E. coli cells can cause $T_4$ and vs2 to develop different plaque morphologies on the same isolate.

The possibility of tau's envolvement as a regulatory element in the rate of macromolecular synthesis during $T_4$ development seems highly feasible because of its interaction with valyl-tRNA synthetase. Aminoacyl-tRNA synthetases have been implicated to have a role in the regulation of transcription (12) and translation (13). The rates of protein synthesis and RNA synthesis were measured by the incorporation of $^3$H-arginine and $^3$H-uridine, respectively, in infected and uninfected cells. NP4 was incubated in minimal medium at 30°C until the cells reached mid-logrithimic growth. The cells were centrifuged and then resuspended in the same medium at one-tenth the volume. The phage were added and the suspension kept at 0°C for five minutes to allow for good adsorption of the phage. The infected cells then were placed

Table II. The atypical phenotypes of plaques formed on mutagenized E. coli cells by $T_4$ and vs2 phages.

NP4 cells in logrithimic growth were filtered and resuspended in Tris-maleic buffer at the original volume. MNNG (100 µg/ml) was incubated with the suspension for 1 hour at 30°C. One milliliter of this mixture was filtered and resuspended in 10 ml of M9 minimal medium without glucose, dilution plated, and grown at 30°C The isolated colonies were transferred to Benzer broth tubes and incubated at 30°C. Two pour plates of each colony and $T_4$ and vs2 phages were prepared, and one plate of each virus was grown at both 30°C and 40°C. The plaques that differed from the normal morphology are listed.

| Isolate number | $T_{4\underline{vs}}$+ plaques* | <u>vs</u>2 plaques* |
|---|---|---|
| 1 | 3x normal size | 3x normal size |
| 2 | 2x normal size | normal size |
| 3 | normal size | 0.5x normal size |
| 4 | normal size | 0.5x normal size |
| 5 | 2x normal size | 2x normal size |
| 6 | 2x normal size | normal size |
| 7 | 1.5x normal size | 1.5x normal size |
| 8 | 2x normal size | normal size |
| 9 | 3x normal size | 2x normal size |
| 10 | 2x normal size | normal size |

* The plaque morphology was the same at 30°C and 40°C.

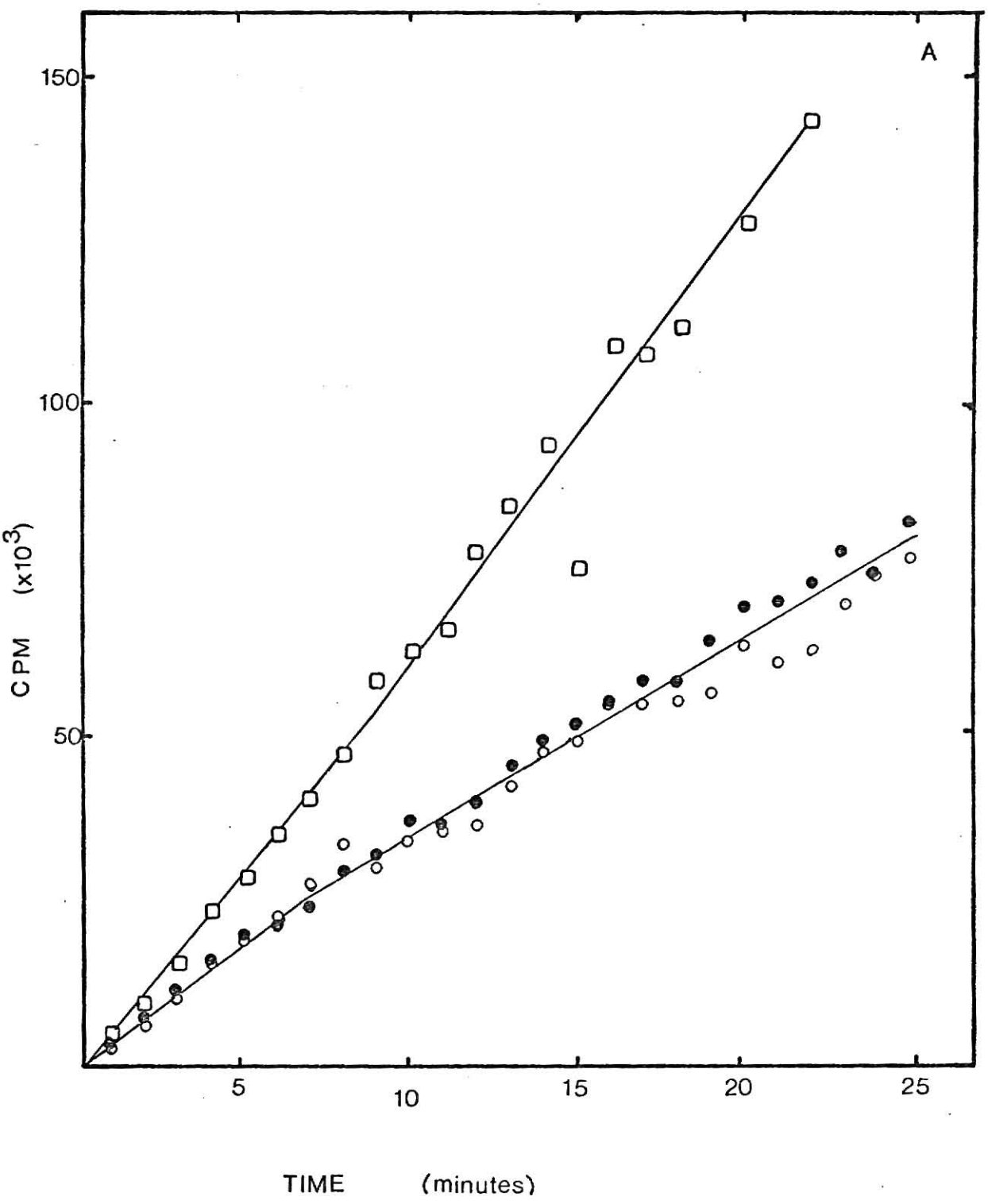
in flasks at 30°C that contained the radioactive label and enough medium to bring the cells up to their original concentration. Every minute for 25 minutes after introduction to the label, samples were removed, placed in cold 5% trichloroacetic acid, and filtered. The rate of protein synthesis in infected and uninfected E. coli is illustrated in Figure 3.

Several points are worthy of emphasis. First, uninfected cells incorporate the isotope at an exponential rate as expected. Second, all four infected cultures on the other hand incorporate the isotope at linear rates which are approximately 50% of the uninfected rate. This result agrees with measurements of Gausing et al. (38) who showed the rate of polypeptide chain elongation as 8 amino acids/second at 37°C in $T_4$-infected E. coli in contrast to uninfected cells at 15-18 amino acids/second. Finally, all four infected cultures appear to synthesize protein at the same rate irrespective of the capability to modify E. coli valyl-tRNA synthetase.

Rates of RNA synthesis have been shown by Bremer and Yuan to vary in uninfected cells, 55 nucleotides/second at 37°C (39), as compared with $T_4$-infected cells, 28 nucleotides/second (40). The net rate of RNA synthesis in NP4 is depicted in Figure 4. As expected the accumulation of RNA in uninfected cells increases exponentially, representing presumably synthesis of stable species of rRNA and tRNA. Net RNA synthesis in the virus infected cells, however, reaches a plateau at a time when the specific activity of the $^3$H-uridine in the intracellular pools becomes constant. The plateau presumably represents the steady state concentration of phage mRNA and tRNA. Nevertheless, neither the rate nor the steady

Figure 3. The incorporation of $^{3}$H-arginine into TCA precipitable material by uninfected and phage-infected cells.

NP4 cells grown to a density of 2 x $10^{8}$ cells/ml were spun down and resuspended in MOPS minimal medium plus tryptophan at 2 x $10^{9}$ cells/ml. One milliliter of the culture was put in 5 separate tubes, phage was added at a multiplicity of 5 to four of the tubes, and all the tubes were kept at $0^{\circ}$C for 5 minutes. The infected cultures were then added to a flask containing 9 ml of MOPS medium plus tryptophan (50 µg/ml), 2 µg/ml arginine, and 25 µCi $^{3}$H-arginine in a $30^{\circ}$C shaking water bath. Every minute for 25 minutes of growth, 0.2 ml samples were removed and put in 2.5 ml of cold 5% TCA. These samples were filtered and counted for the following cultures: uninfected cells ( □ ), $T_4^-$ infected cells ( ● ), and vs1-infected cells ( ○ ), Panel A; and vs2-infected cells ( ▲ ) and vs2RF01-infected cells ( △ ), Panel B.


A
150
100
50
CPM (x10^3)
5
10
15
20
25
TIME (minutes)

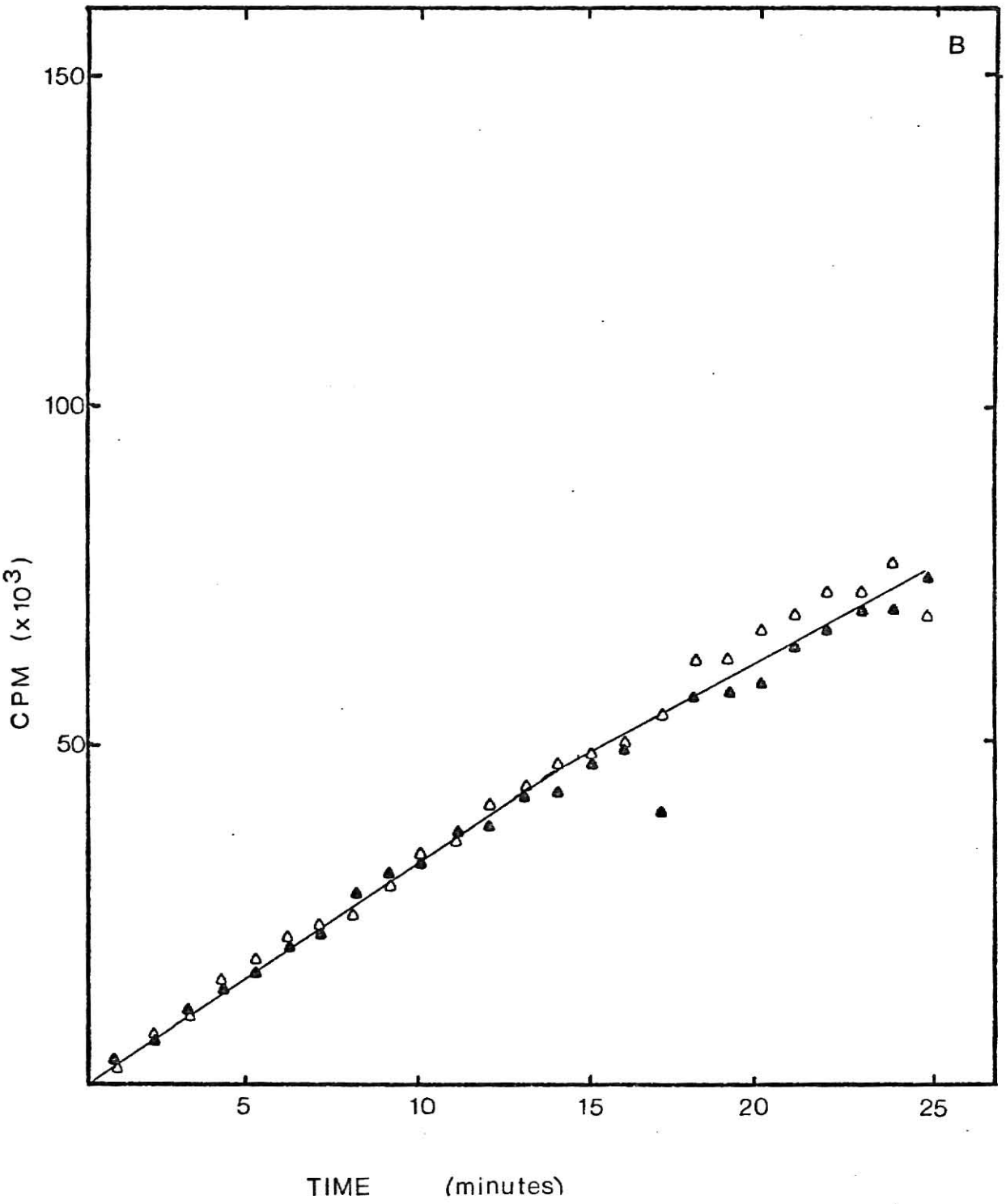
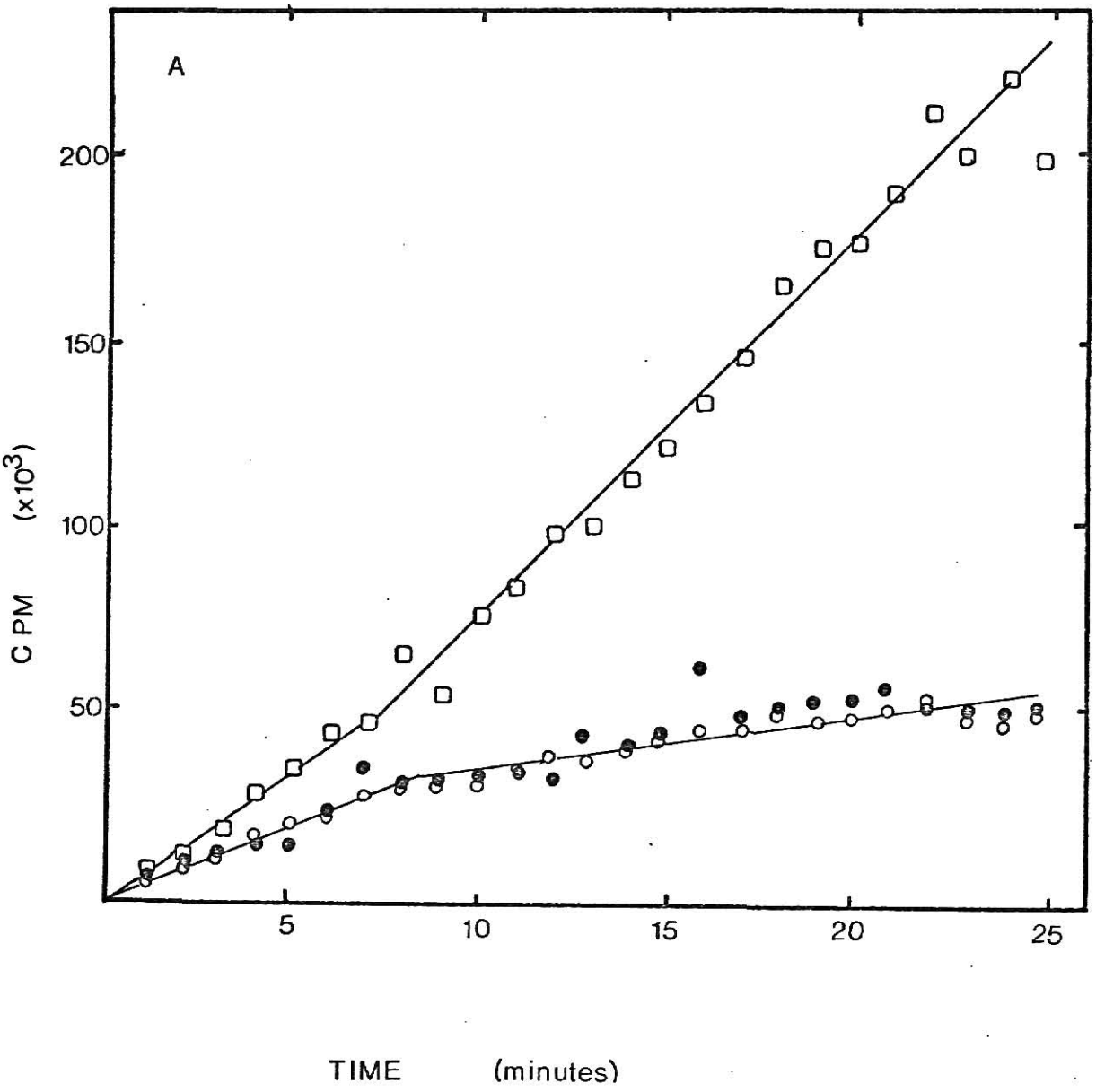
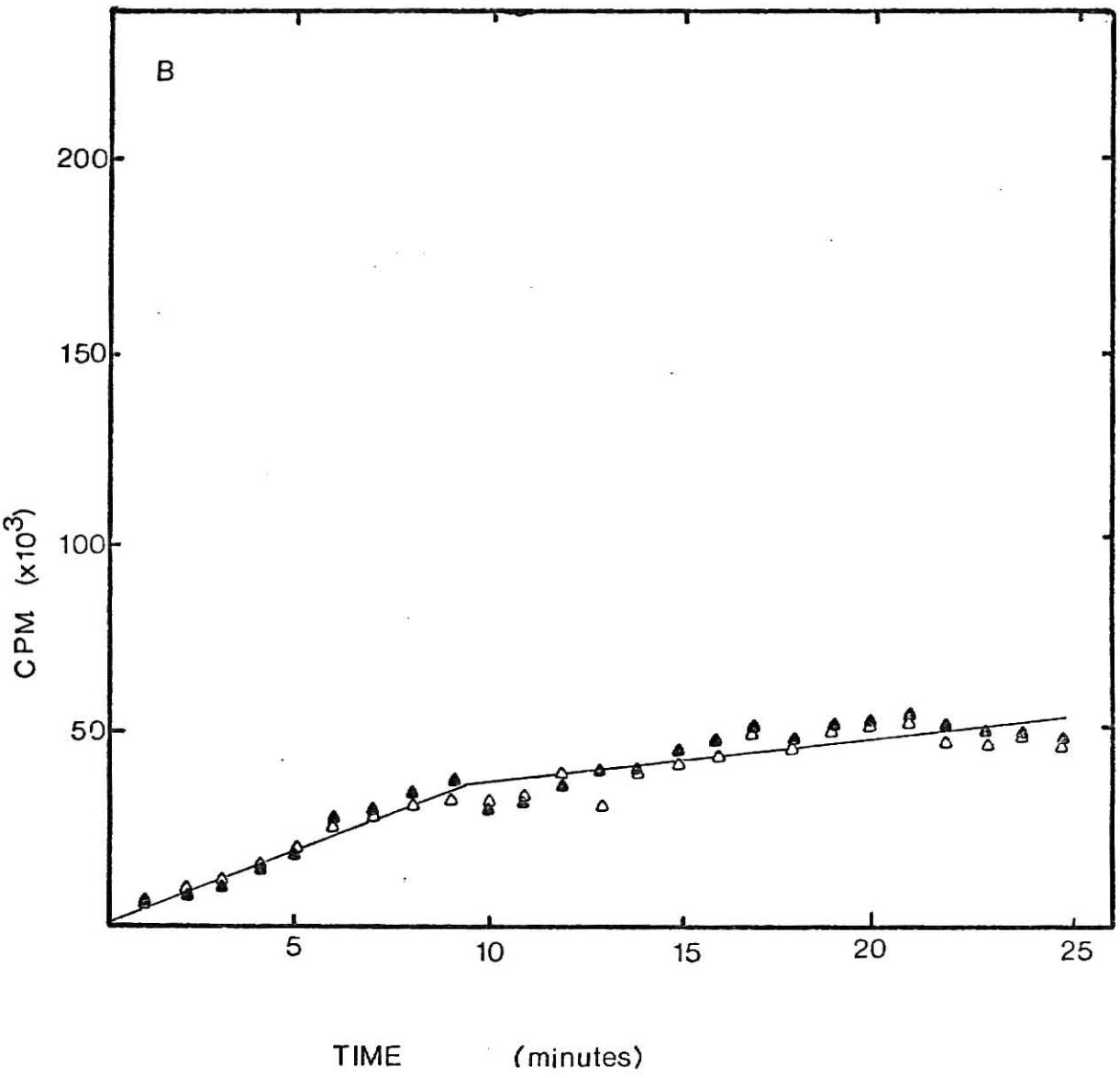

B
150
100
50
CPM (x10^3)
5
10
15
20
25
TIME (minutes)

Figure 4. The incorporation of $^{3}$H-uridine into TCA precipitable material by uninfected and phage-infected cells.

The infected cultures were prepared as in Figure 3, and then added to flasks containing 20 µg uridine, 25 µCi $^{3}$H-uridine, and 9 ml of MOPS medium plus tryptophan (50 µg/ml). Samples were removed and processed as in Figure 3 for the following cultures: Uninfected cells ( □ ), $T_4$-infected cells ( ● ), and vs1-infected cells ( ○ ), Panel A; and vs2-infected cells ( ▲ ) and vs2RF01-infected cells ( △ ), Panel B.


A
200
150
100
50
C P M (x10³)
5
10
15
20
25
TIME (minutes)


B
200
150
100
50
CPM (x10^3)
5
10
15
20
25
TIME (minutes)

state level of RNA's is different among the $T_4$ phage-infected cells tested.

Proteolytic inactivation of enzyme activity and molecular weight analysis of the resulting products can help describe their tertiary structure (16, 17, 18). In our laboratory previous work has shown the trypsin treatment of purified preparations of the unmodified and $T_4$ modified forms of valyl-tRNA synthetase to give different results (27). The unmodified form was more stable to trypsin inactivation than the modified form. The tau peptide was believed to confer upon the enzyme a conformational change that increased the sensitivity of the enzyme to proteolysis. The active site for tRNA on the modified enzyme also has a higher affinity for tRNA, presumably because of a structural alteration in the enzyme molecule induced by the tau peptide (27). The addition of tRNA to purified valyl-tRNA synthetase in the modified and unmodified forms indicated that tRNA gives a substantial increased stability against trypsin inactivation only to the modified form (27). (The other substrates, l-valine and ATP, had no protective effect against trypsin.) The modified enzyme loses over 40% of its activity under one set of conditions of trypsinolysis, but with the addition of E. coli B tRNA almost no aminoacylation activity is lost. The unmodified enzyme remains unchanged by tRNA addition (27).

Only the $T_4$-modified valyl-tRNA synthetase and the unmodified form had been subjected to trypsinolysis. We also wanted to analyze the effect of trypsin on the enzyme in vs1-, vs2-, and vs2RF01-infected cells. Since it has never been proven effectively that a partial tau peptide, created by the amber vs mutation in the vs2 phage, does not

interact with the valyl-tRNA synthetase, we did not wish to risk the removal of a possible fragment of *tau* that might be less tightly bound to the enzyme, by subjecting the enzyme to the multiple step purification procedure. The cell-free extracts of uninfected cells and $T_4$-infected cells were placed, therefore, only on a DEAE cellulose column primarily to remove endogenous tRNA (28). Trypsin treatment was then performed to see if results similar to those with the highly purified enzymes could be obtained. The partially purified enzymes from $T_4$-infected and uninfected *E. coli* B did give the expected effect (Figure 5).

The cell-free extracts of *vs*1-, *vs*2-, *vs*2RF01-infected cells were also subjected to the one step purification procedure. The pools of all five enzymes after DEAE cellulose chromatography were tested for their urea stability (Table 3). Prior to this time only cell-free extracts of *vs*1-, *vs*2-, *vs*2RF01-infected cells have been analyzed for the denaturation effect of urea on their valyl-tRNA synthetases' activity, which gave typical data as shown in Table III. Interestingly, the *vs*1-induced enzyme after chromatography gave a slightly lower than normal percentage of urea stable activity, but with tRNA added to the urea tube containing the *vs*1 enzyme the percent was raised to its expected level.

The partially purified enzymes were treated with equal amounts of trypsin at 37°C and assayed for their percentage surviving amino-acylation activity at 5, 10, 30, and 60 minutes after trypsin addition. The zero time, before addition of trypsin, was considered as 100% activity of the enzyme for comparison of the activities at the later times (Figure 6). Trypsin treatment had an intermediate effect on the

Figure 5. The effect of trypsin on normal and T4-specified valyl-tRNA synthetase (VRS) in the presence or absence of bulk tRNA from E. coli B.

Partially purified VRS from uninfected and $T_4$-infected cells was treated with 45 µg of trypsin in the presence or absence of tRNA. The reaction mix contained in a volume of 1.5 ml the following: 6 mM potassium phosphate (pH 7.1), 6 mM 2-mercaptoethanol. 70 µg normal VRS or 105 µg $T_4$-specified VRS. Before addition of the trypsin, 100 µl tRNA (5 mg/ml) or an equivalent volume of buffer was added and the control samples were withdrawn. Trypsin was then added in a volume of 45 µl and incubation at 37°C began. The following samples were withdrawn at intervals and assayed for aminoacylation activity: normal VRS activity ( ○ ), normal VRS plus tRNA ( ● ), $T_4$-specified VRS activity ( △ ), and $T_4$-specific VRS plus tRNA ( ▲ ).

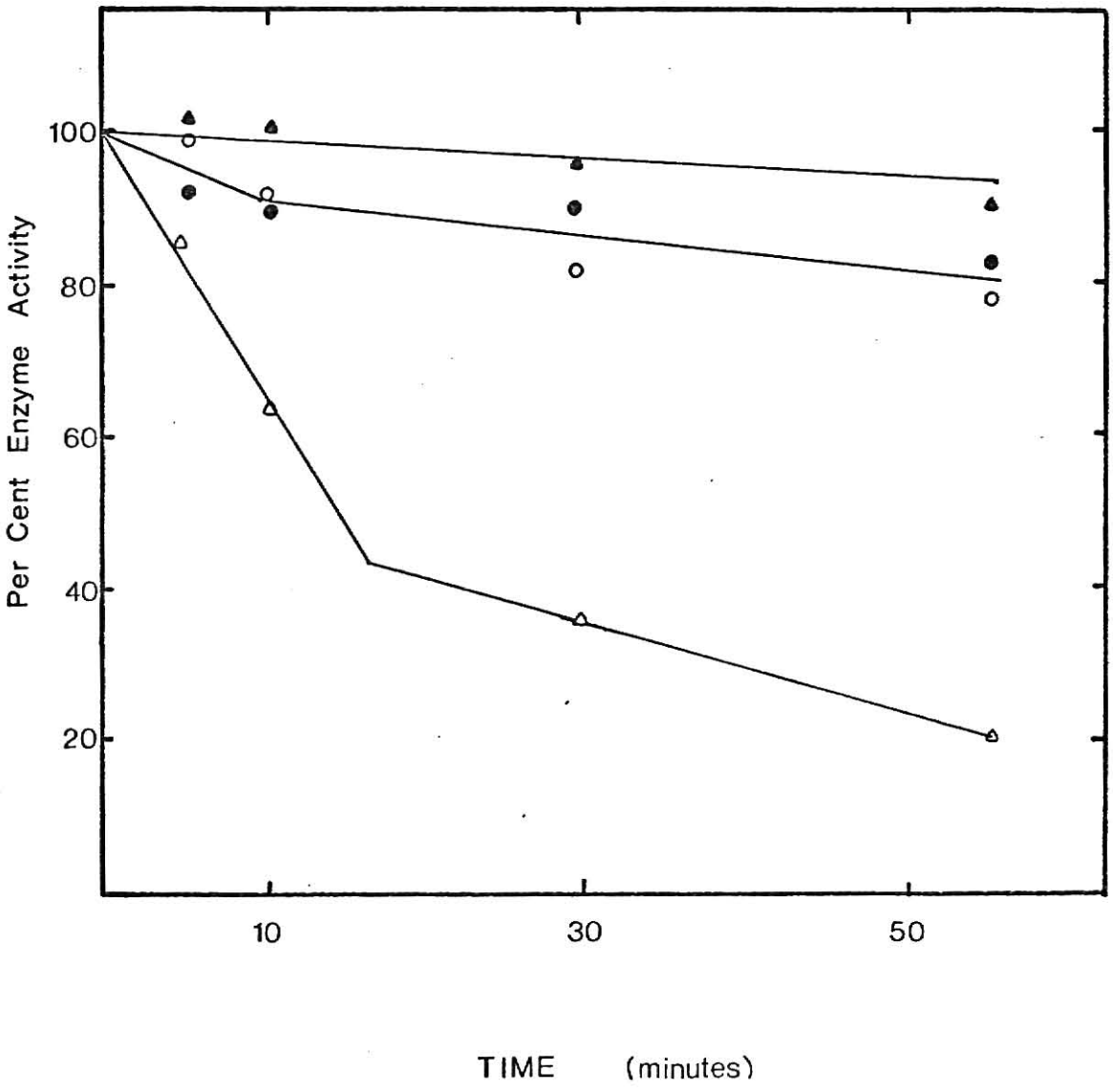

Per Cent Enzyme Activity
100
80
60
40
20
10
30
50
TIME (minutes)

Table III. The urea stability of crude and partially purified valyl-tRNA synthetase (VRS) from uninfected and phage-infected cells.

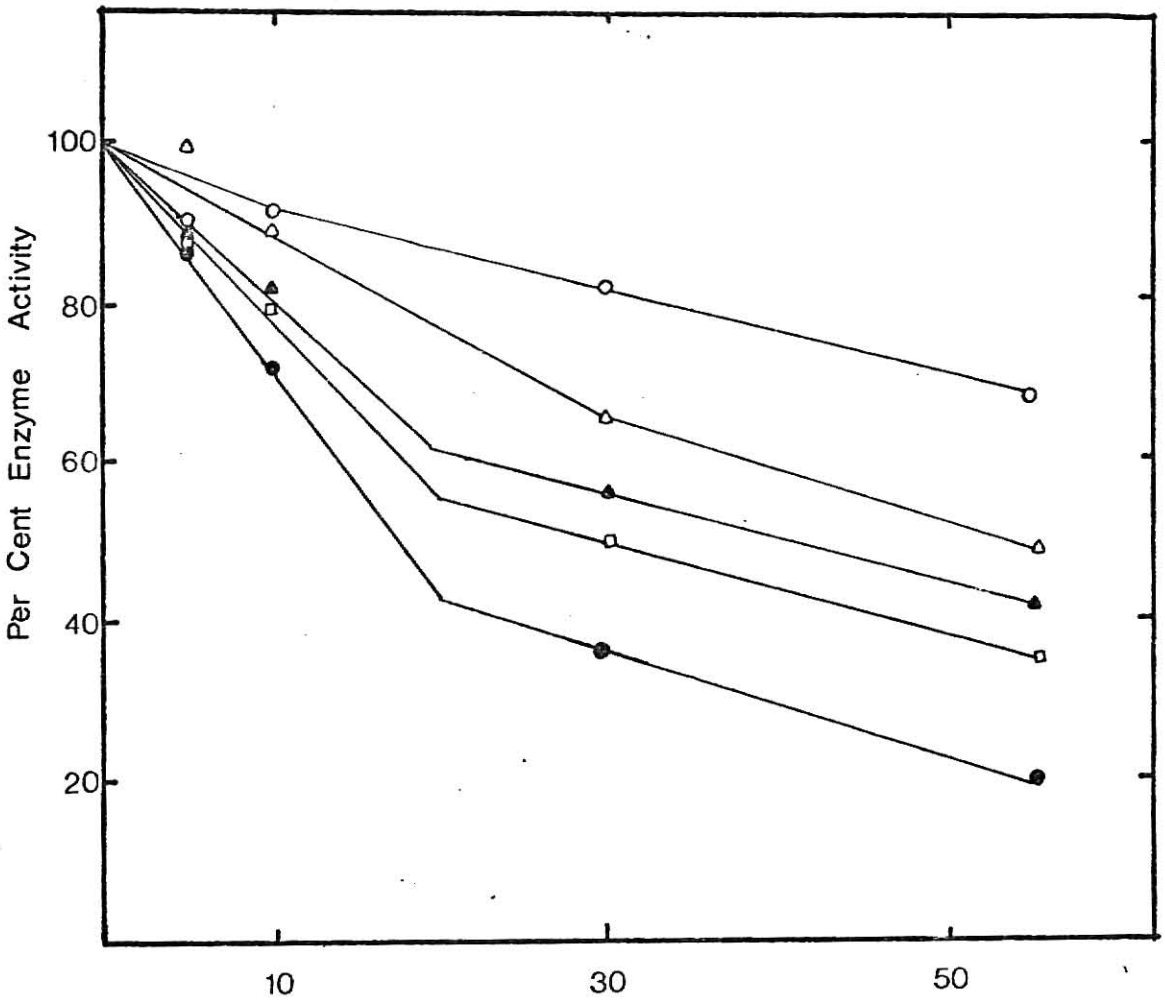
Urea tubes with a volume of 0.45 ml contained 4 M urea, standard buffer, and 100 µg BSA. The buffer tubes had an equivalent volume of buffer to replace the urea. The partially purified enzymes were prepared as follows: normal VRS was diluted 1/5; $T_4$-specific VRS, 1/3; vs1-specific VRS, undiluted; vs2-specific VRS, 1/2, and vs2RF01-specific VRS, 2/3; and then 50 µl of each sample was added to the urea and buffer tubes for incubation at 37°C for 5 minutes. Samples of 0.1 ml were assayed for aminoacylation activity. The vs1-specific VRS was also tested in the presence of 100 µl bulk tRNA form E. coli B (5 mg/ml), which replaced an equivalent volume of buffer in the urea and buffer tubes.

The urea and buffer tubes were prepared for the cell-free extracts as explained above, except with a volume of 0.35 ml. The cell-free extracts were diluted 1/3 and 0.15 ml was then added to the urea and buffer tubes. The urea stability assay was then continued as described for the partially purified enzymes.

| Partially purified VRS | urea-treated (cpm) | control (cpm) | urea-stability (%) | Cell-free extracts | urea-treated (cpm) | control (cpm) | urea-stability (%) |
|---|---|---|---|---|---|---|---|
| Normal VRS | 88 | 1563 | 5.6 | uninfected cells | 92 | 1703 | 5.4 |
| $T_4$-specific VRS | 1083 | 1053 | 102.8 | $T_4$-infected cells | 1703 | 1681 | 101.3 |
| vs1-specific VRS, no tRNA | 154 | 1102 | 13.9 | vs1-infected cells | 547 | 2003 | 27.3 |
| with tRNA | 278 | 1001 | 27.7 | | | | |
| vs2-specific VRS | 119 | 1008 | 11.8 | vs2-infected cells | 146 | 1787 | 8.2 |
| vs2RF01-specific VRS | 812 | 991 | 81.9 | vs2RF01-infected cells | 1625 | 1995 | 81.4 |

Figure 6. The effect of trypsin on normal and the phage-specified valyl-tRNA synthetase (VRS).

Partially purified VRS from uninfected and all four phage-infected cells was treated with 45 µg of trypsin. The reaction mix contained in a volume of 1.5 ml, the following: 6 mM potassium phosphate (pH 7.1); 6 mM 2-mercaptoethanol; and 70 µg normal VRS ( ○ ), 105 µg $T_4$-specific VRS ( ● ), 195 µg vs1-specific VRS ( □ ), 220 µg vs2-specific VRS ( ▲ ), or 150 µg vs2RF01-specific VRS ( △ ). The control samples were withdrawn and then trypsin was added in a volume of 45 µl. After incubation at 37°C, the samples of 0.1 ml were withdrawn at intervals and assayed for aminoacylation activity.


Per Cent Enzyme Activity
100
80
60
40
20
10
30
50
TIME (minutes)

mutant phage induced enzymes compared to the modified and unmodified enzymes. This showed that the vs2-induced form of valyl-tRNA synthetase differs from the normal enzyme, implying that the conformation of the enzyme had been changed after vs2 infection.

Each enzyme preparation also was investigated for trypsin inactivation in the presence of tRNA. Equal quantities of tRNA and trypsin were added to each partially purified enzyme and incubated at 37°C for 5, 10, 30, and 60 minutes. The zero time activity was based on the enzyme plus tRNA before the addition of trypsin. The graphs of each phage infected and the uninfected preparations include trypsin treatment with and without tRNA, and additional quantities of trypsin incubated with each enzyme (Figures 7a, b, c, d, e). The tRNA protected all of the phage induced enzymes yet not the unmodified form. This demonstrated that infection of E. coli with the mutant phage, including vs2, adds a factor to the existing host valyl-tRNA synthetase that allows the interaction of tRNA with the enzyme, which resulted in a conformational change that decreased the sensitivity of the enzyme to trypsin attack.

Although the physiological significance of modification remains a mystery, the effect of infection by vs mutants of $T_4$ on the host valyl-tRNA synthetase is more clearly defined. The fact that the vs2 phage has always been considered as lacking the tau interaction with the host enzyme (which is required for modification) and the above evidence now states that such an interaction does exist, may have hindered the search for the function of tau.

Figure 7. The effect of trypsin at different concentrations or in the presence or absence of bulk tRNA from E. coli B on normal and phage-specified valyl-tRNA synthetase (VRS).

Partially purified VRS from uninfected and all four phage-infected cells was treated with 150 µg trypsin ( ▲ ), 45 µg trypsin, and 15 µg trypsin ( △ ). The treatment with 45 µg trypsin was performed as described in Figure 6, in the presence ( ● ) or absence ( ○ ) of 100 µl tRNA (5 mg/ml). The 150 µg and 15 µg trypsin treatments were carried out in a reaction mix that contained the following in a volume of 1.5 ml: standard buffer and 70 µg normal VRS, 105 µg $T_4$-specific VRS, 155 µg vs1-specific VRS, 130 µg vs2-specific VRS, and 150 µg vs2RF01-specific VRS. The control samples were withdrawn and then trypsin added in a volume of 15 µl. After incubation at 37°C, the following samples of 0.1 ml were withdrawn at intervals and assayed for aminoacylation activity: normal VRS (Fig. 7a), $T_4$-specific VRS (Fig. 7b), vs1-specific VRS (Fig. 7c), vs2-specific VRS (Fig. 7d), and vs2RF01-specific VRS (Fig. 7e).

Figure 7a

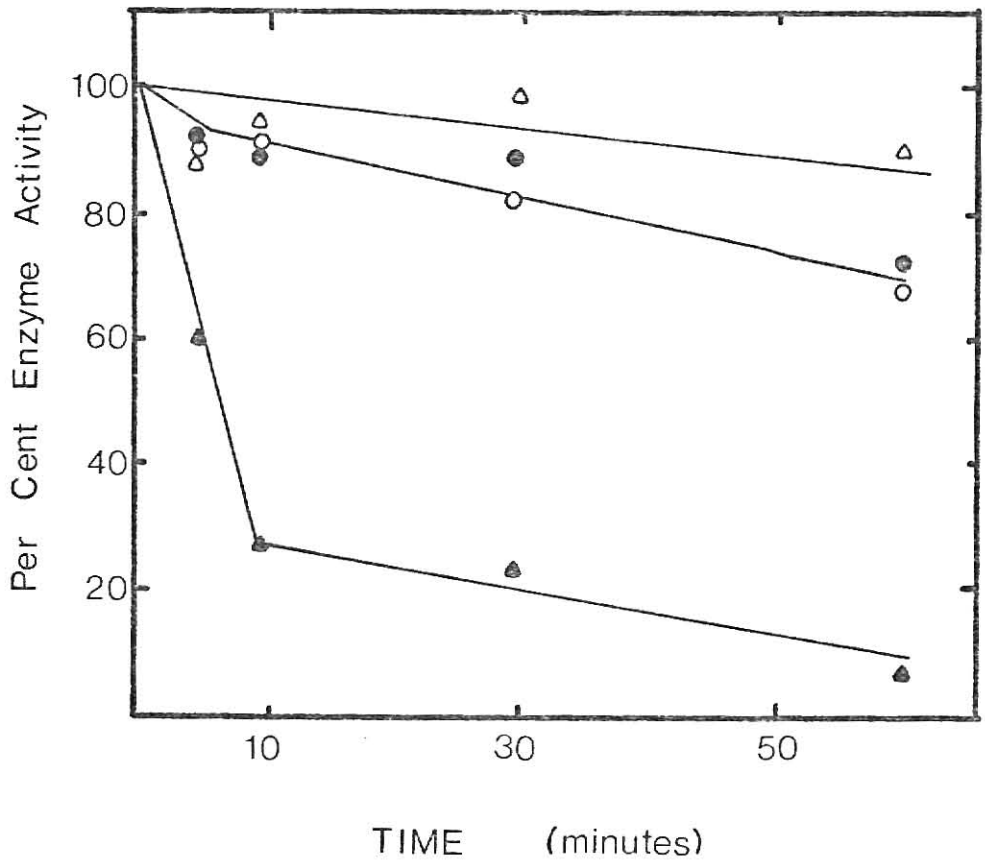



Figure 7b

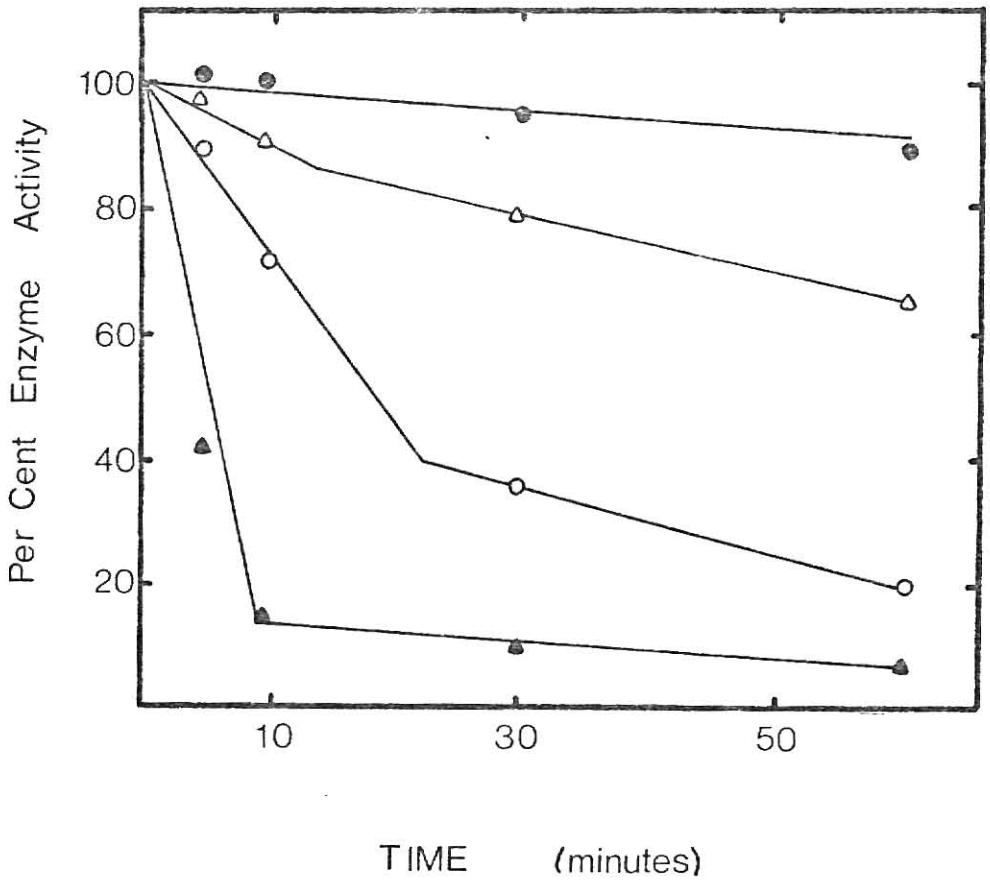


Figure 7c

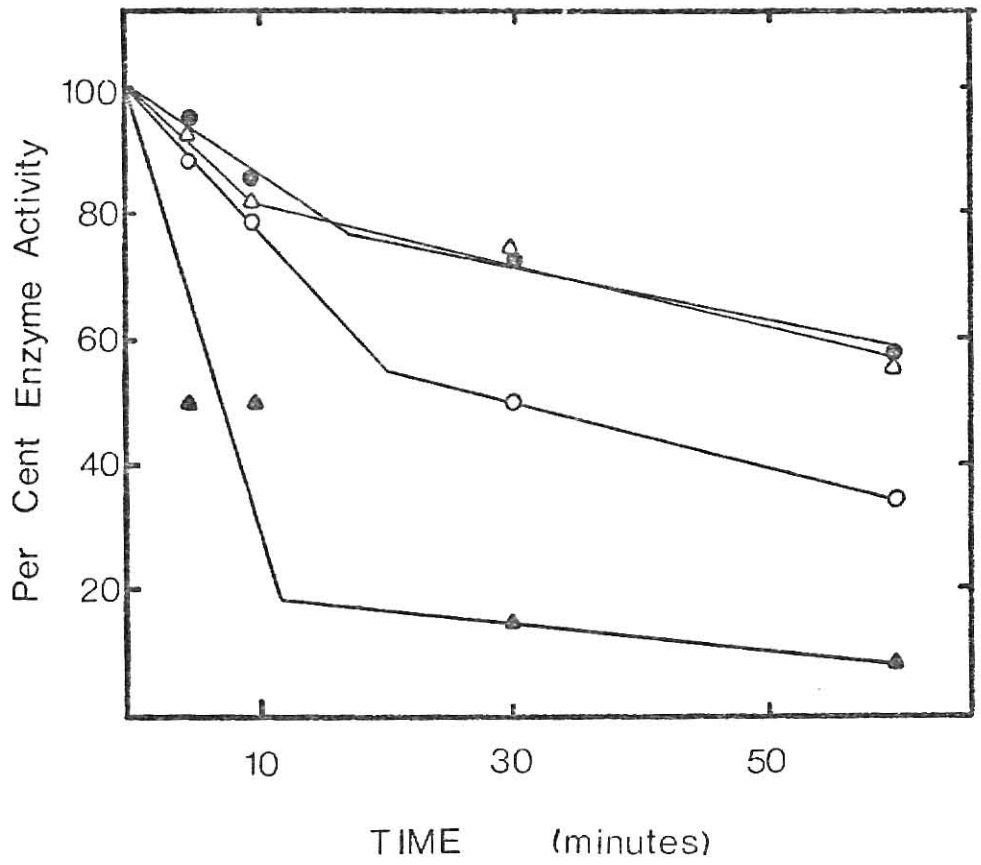



Figure 7d

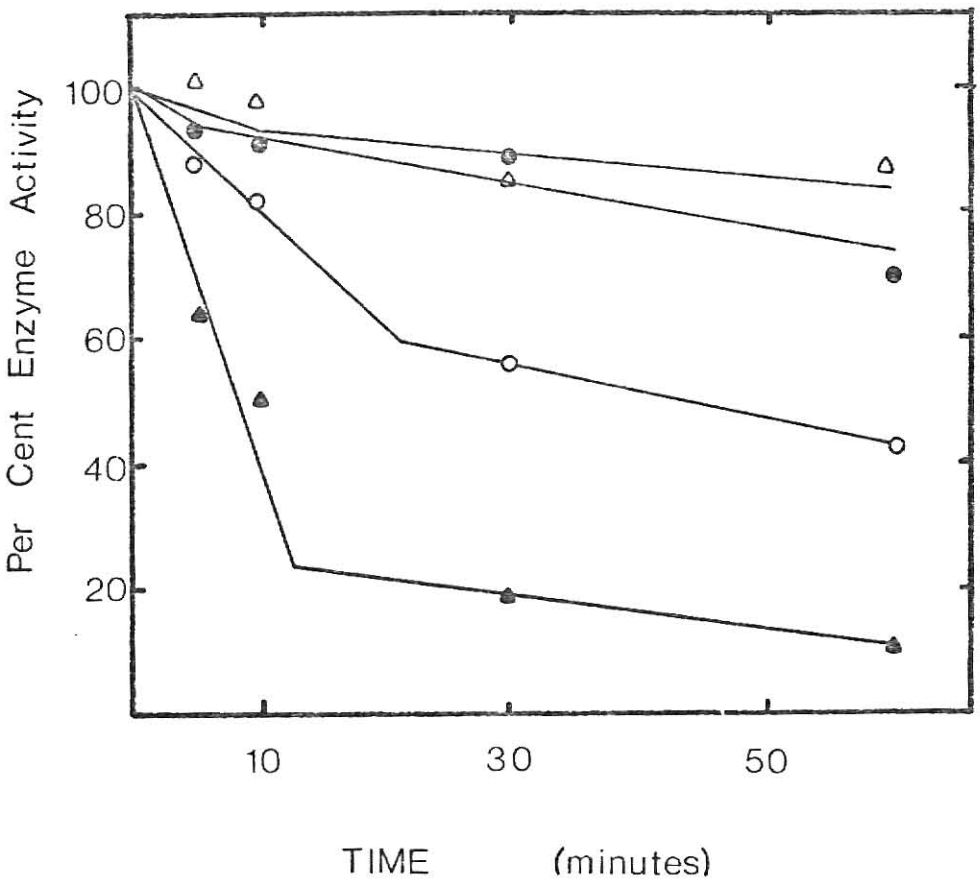
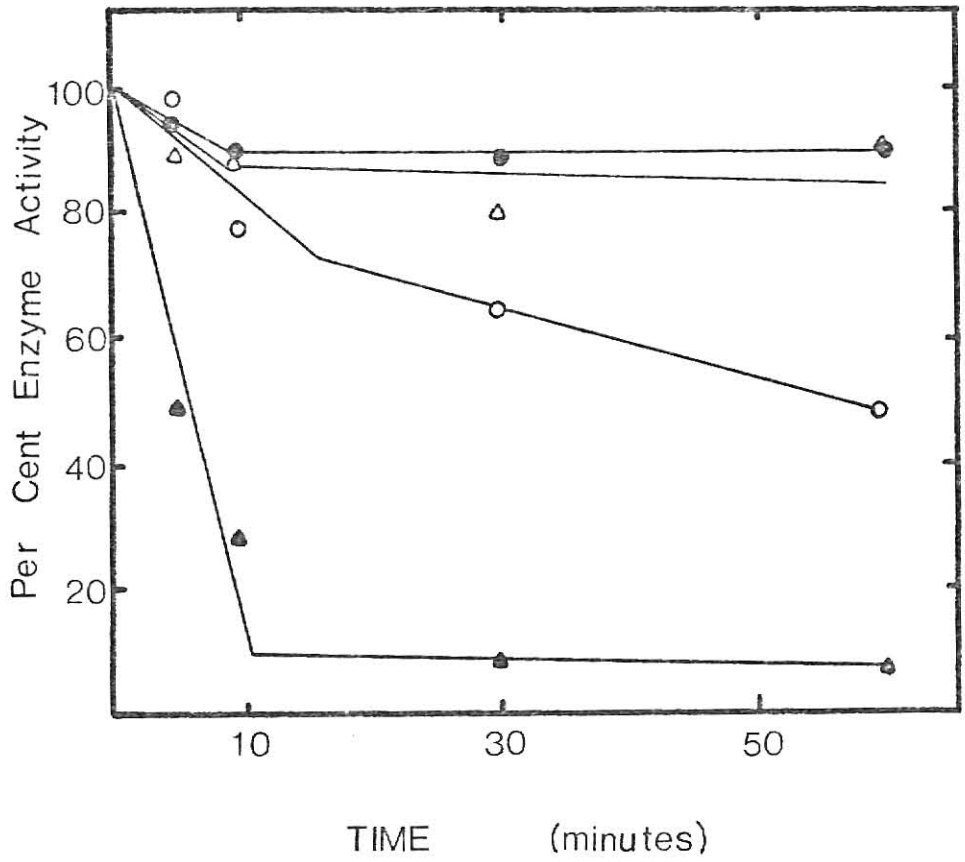


Figure 7e



# DISCUSSION

The question of why the bacteriophage $T_4$ modifies the E. coli valyl-tRNA synthetase has been raised many times. The physiological significance has remained elusive, though much research has been directed toward this problem. Mutations are extremely useful tools in biological research and, therefore, analysis of mutants that were less efficient or believed to be lacking in their ability to modify the host enzyme was undertaken in this study to aid in the search for the function.

After the infection of E. coli with the $T_4$ bacteriophage, a small peptide, tau, of approximately 10,000 molecular weight (23) is synthesized as an immediate-early gene product (21). This peptide interacts with the host valyl-tRNA synthetase (21) and increases its affinity for tRNA (28). The modified enzyme differs from the unmodified host enzyme in its stability to heat, stability to denaturation by urea, ability to charge yeast tRNA, electrophoretic mobility, sedimentation rate in sucrose (or glycerol) gradients, and molecular size (25). The specific activity in vitro also is lower after modification by approximately 50% (25, 28). Although so many biochemical characteristics vary, the physiological function of the tau peptide in cell metabolism remains unknown.

A mutant of the $T_4$ bacteriophage, vs1, has been isolated (26). Urea denaturation of its valyl-tRNA synthetase aminoacylation activity showed it to have an intermediate stability as compared with the $T_4$-modification and unmodified enzymes. It contains one-half the fast sedimenting species of the modified enzyme in sucrose gradients and thus has a high affinity for tRNA (26). It is unable, though, to fully

repair the temperature sensitive valyl-tRNA synthetase of the E. coli strain NP-29 (26).

Another $T_4$ mutant, vs2, showed no urea stable valyl-tRNA synthetase activity resembling the host unmodified enzyme (26). On sucrose density gradients the valyl-tRNA synthetase activity consisted almost entirely of the light form of the enzyme (26). It apparently has no greater affinity for tRNA than does the unmodified enzyme (27). The temperature sensitive enzyme of NP-29 was not suppressed by infection with vs2 (26).

A spontaneous revertant of vs2, vs2RF01, has been isolated by its ability to repair the NP-29 valyl-tRNA synthetase at nonpermissive temperatures. This phage was able to modify the host enzyme as shown by urea stability (Table III) and its fast sedimenting species in glycerol gradients, implying its high affinity for tRNA (Figure 1). The vs2RF01 phage is believed to "correct" the amber codon in the vs2 phage, making vs2 and vs2RF01 an isogenic pair.

In the initial description of the vs1 and vs2 mutants, it was noted that both mutants had normal burst sizes on E. coli B, produced normal amounts of dCMP deaminase and produced phage at the same rate as $T_{4vs}+$ (26). These studies were extended to a wide number of physiological parameters with the result that not one physiological parameter, of those studied seemed to differ between $T_{4vs}+$ and vs2 (30). In short, vs2 has been the main tool of research designed to unravel the physiological significance of valyl-tRNA synthetase modification.

With the above information and the firm belief that cell metabolism is too exact to continually produce a peptide with no purpose and which causes several noticable changes in the properties of an

enzyme is involved crucially in protein synthesis, the search for a function for tau was extended to a different host, Shigella dysenteriae. Host synthesis arrest by $T_4$ is incomplete in this species (4). Shigella dysenteriae infected with $T_4$ is unable to stop host synthesis by the method in E. coli that requires protein synthesis (4, 5). This system does, however, use another method that is dependent upon the multiplicity of infection (4) and may occur through the action of a phage coat protein (5). Since $T_4$ modified the Shigella dysentariae valyl-tRNA synthetase, this suggests that tau does not play a major role in host synthesis arrest. If the tau peptide was unable to interact with the enzyme of S. dysenteriae, it might have been considered feasible that the modification in the E. coli host allowed only the synthesis of $T_4$ proteins by the action of the new enzyme activity in some unknown regulatory mechanism. In S. dysenteriae, though, both host and $T_4$ proteins could then be produced by the unmodified enzyme. The data obtained does not totally rule out tau having a function in host synthesis arrest, because the site of action of the modified enzyme might not be present in the S. dysenteriae cell.

The modified valyl-tRNA synthetase of S. dysenteriae is less stable to urea denaturation than modified enzyme of E. coli (Table I). This suggests that tau interacts with the S. dysenteriae enzyme creating a conformation that is less urea stable than is the conformation developed from its binding with E. coli enzyme. The mutant phage vs1, vs2, and vs2RF01 grew quite well on the S. dysenteriae strain. The modified valyl-tRNA synthetase also was less stable to urea denaturation, but relative to each other the mutants had the

correct order of stability (Table I). This suggests that tau may be unnecessary for $T_4$ development in S. dysenteriae or that the mutants make a gene product that is sufficient in its ability to carry out its in vivo function. The $T_4$ and vs1 enzymes apparently do interact with tRNA, an effect of modification that was demonstrated by the glycerol gradients having the heavy species of the enzyme.

MNNG appears to be a potent chemical mutagen (36). Heavy mutagenesis with such a powerful agent would be likely to create the desired mutant if such a mutant could exist. There is a high probability when MNNG is used that every cell will be mutated at more than site (36). So our use of MNNG on NP4 cells to search for a conditionally permissive mutant, one that would grow $T_4$ but not vs2 at permissive or nonpermissive temperatures, seemed to be a useful method for our investigation. It is unknown why the plaque morphology on the isolates infected with $T_4$ and vs2 differed, because the analysis of these mutants would have proven difficult and most likely unprofitable; however, it is important to note that a difference was created by the MNNG treatment. The desired mutant probably was not found because the mutant search was too small. It also is possible that the desired mutant could not be isolated because the vs gene product was dispensible for $T_4$ phage production or that the vs2 phage was able to synthesize enough of the tau peptide to fulfill its putative, yet unknown function.

Aminoacyl-tRNA synthetases have been implicated to have a role in the regulation of transcription (12) and translation (13). The analysis of the rate of protein and RNA synthesis by the incorporation of radioactive components specific to each process might indicate

if any change occurs in either synthesis rate of cells infected with the mutant phages compared to the $T_4$ phage. The $T_4$ modified valyl-tRNA synthetase is known to have a lower specific activity *in vitro*, by about 50%, than the unmodified form (25, 28). The rate of protein synthesis (38) and RNA synthesis (39, 40) of cells infected with $T_4$ phage also is known to be substantially lower than uninfected cells. Any correlation between these two known occurrences might become evident by the use of $T_4$ phage having mutant *tau*'s. Since all of the mutants, *vs*1, *vs*2, and *vs*2RF01, showed the same rate of synthesis, the lower specific activity of the modified enzyme is not directly related to the lower protein or RNA synthesis rates, or all the mutants are able to modify the enzyme with enough efficiency for the enzyme to be able to perform its regulatory function. Again, the analysis of these results depends upon whether or not the *vs*2 phage synthesizes a *tau* peptide fragment that is able to satisfy its function.

Any information that can be deduced from this research, therefore, is dependent upon knowing whether or not the *vs*2 phage is deficient or not in making an effective *tau* peptide. Preliminary investigations (Müller and Davis, unpublished observations) on the composition of valyl-tRNA synthetase from *vs*2-infected cells suggested the possibility that a *tau*-like peptide is associated with the enzyme after infection. The *vs*2 phage has an amber mutation at some unknown locus within the *vs* gene. The mutation may be distal enough from the origin of the gene to enable the production of a fragment that can bind specifically to the valyl-tRNA synthetase. This is not evident from urea stability experiments, possibly because the conformational change by a *tau* peptide fragment

is not great enough to confer upon the enzyme stability to 4 M urea denaturation. A tau interaction with the host enzyme of vs2-infected cells also is not obvious by glycerol density gradient sedimentation data, which shows almost a total lack of a heavy sedimenting species. (The heavy sedimenting species is composed of the modified enzyme with tRNA bound (41).) This may result from the tau peptide fragment being unable to bind as tightly to the enzyme as the wild-type can, and is pulled off the enzyme by the forces present during centrifugation.

Trypsinolysis of modified and unmodified valyl-tRNA synthetases of E. coli has lead to speculations about their structures. The unmodified enzyme is believed to consist of 2 globular sections covalently linked by a small peptide bridge with several strong non-covalent bonds forming a trough between them (16, 17). The binding of tau strongly suggests that the molecule undergoes a conformational change which is without apparent effect on the binding sites for ATP and valine, but renders a site directly or indirectly involved in the binding of tRNA more susceptible to proteolytic digestion (27). The ability of the modified enzyme to bind tRNA appears simultaneously with the formation of a urea stable enzyme, the binding of tau, as immediate-early gene functions (41). It appears likely that the tau may bind to a locus removed from the active site for tRNA and cocomitantly shifts the configuration of the tRNA site to higher substrate affinity and sensitivity to proteolytic clipping (27). When tRNA binds to the modified enzyme, its susceptibility to proteolytic digestion is lessened (27).

The valyl-tRNA synthetase aminoacylation activity from wild-type $T_4$, vs1, vs2, and vs2RF01 infected cells and uninfected cells was tested for susceptibility to trypsin inactivation. The vs1, vs2RF01, and, most importantly, the vs2 forms of the enzyme showed greater tendency to be inactivated by the trypsin than the unmodified form, although to a lesser extent than the $T_4$ modified enzyme. This suggests that all the phage code for a tau factor that interacts at a specific site on the enzyme and increases the susceptibility of one or more regions of the enzyme to trypsin. Since the susceptibility is lower than with the wild-type modified enzyme, the vs gene mutant phages may produce a tau peptide or fragment, in the case of vs2, that causes a less drastic conformational change than the tau of the normal or wild-type phage.

A more conclusive indication that a tau-like peptide associates with the valyl-tRNA synthetase in vs2-infected cells was demonstrated by the tRNA addition during trypsinolysis. This enzyme developed a significant decrease in sensitivity to trypsin digestion when tRNA was added. The unmodified enzyme showed no change in susceptibility to trypsin because of the presence of tRNA. The enzymes modified by the vs1 and vs2RF01 phages also showed a similar decrease in sensitivity. None of the vs mutants were able to render the enzyme as stable to trypsin clipping by the addition of tRNA to the reaction mixture as was the wild-type $T_4$ phage. The different conformational change created by the interaction of a mutant tau with the enzymes caused tRNA to bind so that more areas of the enzyme were vulnerable to trypsin attacks. This could have been caused by a less efficient association of the

tRNA with the enzymes modified by the mutants or a slightly different mode of tRNA binding created by the altered conformation of the enzyme modified with the partial or missense tau peptides.

The analysis of the vs gene mutants, vs1, vs2, and vs2RF01, has lead to the following conclusions. All three have tau peptides that differ from the wild-type and from each other as shown by different urea stabilities, glycerol gradient profiles, and trypsin inactivation susceptibilities. They are also able to increase the affinity of the valyl-tRNA synthetase for tRNA binding, one known effect of modification. They are able to synthesize protein and RNA at the same rate as the wild-type phage infected cells. If a regulatory function is performed by the interaction of tau to the enzyme, the mutant phages are also able to do this. The fact that vs2 may make a tau-like peptide that associates with the enzyme and increases its affinity for tRNA could explain why no mutant of E. coli was isolated that would be nonpermissive for phage development if an effective tau was not synthesized (as previously believed to be the case for vs2). This also may explain why no change in the synthesis rates of protein and RNA was produced by the infection of NP4 cells with vs2 phage. It does not guarantee, though, that such a mutant could exist or that a change in the synthesis rates would be caused by infection with a phage having a vs gene deletion. It also remains unknown whether the vs2 tau fragment was able to bind with the valyl-tRNA synthetase of Shigella dysenteriae.

A physiological significance for modification is indicated by the fact that a small peptide is coded for in the $T_4$ bacteriophage genome that is produced as an immediate early gene function (25) which is able

to interact specifically after infection with a host enzyme of crucial importance to protein synthesis and of possible regulatory significance in transcription (12) and/or translation (13). After modification the *in vitro* specific activity is decreased approximately 50% (25, 28), and several obvious changes like stability to urea denaturation (24), higher affinity for tRNA (28), and greater sensitivity to proteolysis (27) occur, which in some manner could give the modified enzyme a regulatory significance. The work done so far in searching for the physiological significance has proven unprofitable, probably because it was assumed the *vs*2 phage was unable to modify valyl-tRNA synthetase. Several modes of action in which modification might act *in vivo* as a regulatory mechanism were tested, based upon the premise that *vs*2 leaves the host enzyme unmodified (30). From the information obtained in this study, we now know this premise to be questionable, and a new line of research must be developed. Mutants of $T_4$ now are available to our laboratory (which we received too late for the work reported here) that have deletions through the *vs* gene (42). The deletions are quite large, though, involving several genes, and so results obtained with them may not reflect an effect created solely because of the absence of *tau*. These mutants grow poorly on *E. coli*, and also overproduce a few proteins, including dihydrofolate reductase, presumably because of an altered translational regulatory mechanism. The use of *vs*2 infected cells of NP-29, which has a temperature sensitive lesion in the valyl-tRNA synthetase molecule, is presently being investigated in our laboratory to see if a doubly mutated system might yield more information. Clearly, a new approach to finding the significance of modification of valyl-tRNA synthetase must be undertaken.

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# PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF MODIFICATION OF *ESCHERICHIA COLI* VALYL-tRNA SYNTHETASE BY *vs* MUTANTS OF THE BACTERIOPHAGE $T_4$.

by

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ABSTRACT

During growth on Escherichia coli, bacteriophage $T_4$ modifies the host valyl-tRNA synthetase by producing a small peptide, tau, that binds to the enzyme and enhances its tRNA binding capacity. $T_4$ mutants have been isolated that are deficient in the modification of E. coli valyl-tRNA synthetase. These mutants, vs1, vs2, and vs2RF01, are believed to be a missense, an amber, and a revertant of the amber mutation, respectively, in the vs gene of $T_4$ that codes for tau. The function of tau and modification is unknown. This research was directed toward the further characterization of the mutant phage and the determination of the function of modification.

In contrast to $T_4$ infected E. coli, host macromolecular synthesis in Shigella dysenteriae continues after $T_4$ infection. All $T_4$ phage which were examined ($vs^+$ and $vs^-$) were able to grow efficiently on S. dysenteriae and to produce altered valyl-tRNA synthetases. Modification of valyl-tRNA synthetase, therefore, does not appear to be crucial for the arrest of host macromolecular synthesis.

The rates of protein and RNA synthesis in the $T_{4vs}^+$ and $T_{4vs}^-$ infected E. coli cells were identical, indicating that modification may not be involved in the regulation of protein or RNA synthesis rates.

Trypsin inactivation of aminoacylation activity of the partially purified valyl-tRNA synthetases from $T_4$-, vs1-, vs2-, vs2RF01-infected and uninfected E. coli cells in the presence or absence of tRNA demonstrated that the $T_4$ vs2 genome must be distal from the vs gene

origin to produce a functional tau fragment that can associate with the host enzyme and allow its interaction with tRNA. Although the physiological significance of tau has not been discovered, it is evident that since vs2 alters the host enzyme, it should not be considered an absolute negative control in further studies to determine the function of modification.