JOURNAL OF VIROLOGY, Feb. 1975, p. 238-243

Vol. 15, No. 2
Printed in U.S.A.

Temporal Appearance of Bacteriophage T4-Modified Valyl tRNA Synthetase in *Escherichia coli*[1]

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Received for publication 14 August 1974

Bacteriophage T4-induced modification of *Escherichia coli* valyl-tRNA synthetase (EC 6.1.1.9) requires: synthesis of a phage-gene specified τ factor, addition of the factor to host valyl-tRNA synthetase to produce a urea-stable enzyme, and interaction of the modified enzyme with tRNA to produce a more rapidly sedimenting valyl-tRNA synthetase activity on sucrose density gradients. This report demonstrates that the coincident, chloramphenicol-sensitive appearance of urea-stable and rapidly sedimenting valyl-tRNA synthetase activity are immediate early phage functions. It implies that once the τ factor is synthesized, further interactions are stoichiometric rather than catalytic. The potential for valyl-tRNA synthetase modification accumulates when *E. coli* is infected with T4 phage in the presence of chloramphenicol and is expressed during the resumption of protein synthesis whereas further RNA synthesis is inhibited by rifampicin. The modification phenomenon occurs similarly in several strains of *E. coli* and represents a novel virus-host interaction.

Shortly after bacteriophage T4 infects *Escherichia coli*, the heat stability, urea stability, and sedimentation rate of host valyl-tRNA synthetase (L-valine:tRNA ligase [AMP]; EC 6.1.1.9) are altered (8). A peptide factor τ, uniquely associated with the purified, modified enzyme and under the control of a phage gene, is necessary for these physical changes (8). The increased sedimentation rate of the modified enzyme, however, requires tRNA in addition to τ (9).

Although the physiological significance of the T4-induced modification of valyl-tRNA synthetase is unknown, accurate determinations of the onset and rate of modification are important to this goal. Though several approaches to the problem are given in the literature (1), variations in bacterial strains, media, temperature, and technique used to determine the extent of modification preclude consistent estimates of these important parameters. Additionally, the newly discovered tRNA effect on T4-modified valyl-tRNA synthetase suggests that the different criteria used to measure the rate of modification may have measured different configurations of the enzyme.

We, therefore, compared several methods used to judge the appearance of T4-modified valyl-tRNA synthetase activity. A comparison was also made of the appearance of the modified enzyme to the T4-induced enzymes dihydrofolate reductase (7,8-dihydrofolate:NADP$^+$ oxidoreductase EC 1.5.1.5), dCMP deaminase (EC 3.5.4.12), and deoxynucleotide kinase (EC 2.7.4.12). Finally, we used the metabolic inhibitors, chloramphenicol and rifampicin, to dissociate transcription from translation. Valyl-tRNA synthetase modification was compared to the known behavior of the above phage-induced enzymes during this uncoupling procedure.

The results indicate that the T4-induced alterations in the sedimentation velocity and urea stability of valyl-tRNA synthetase appear simultaneously. Compared with other phage-induced enzymes, modification resembles an immediate early T4 function since the potential for valyl-tRNA synthetase modification accumulates in the presence of chloramphenicol. (This paper was presented in part at the Annual Meeting of the American Society for Microbiology, 12 to 17 May 1974, Chicago, Ill.)

MATERIALS AND METHODS

Bacteria and bacteriophage. *E. coli* NP4 (a B strain), NP2 (a KB strain), and NP29 (a temperature-sensitive mutant derived from NP2), were obtained from F. C. Neidhardt (1). NP29 grows normally at 30 C but fails to grow at 40 C because of thermal inactivation of its valyl-tRNA synthetase (3). Bacteriophage T4Bc$^+$, which has no cofactor requirement for adsorption, was used in all experiments (1).

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Chemicals. All common chemicals were reagent grade. Adenosine 5'-triphosphate (ATP), glutathione, bovine serum albumin, 2'deoxycytidine 5'-monophosphoric acid (dCMP), reduced triphosphopyridine nucleotide ($NADPH^+$), 5-methyl DL-tryptophan, chloramphenicol, lysozyme, and rifampicin were purchased from Sigma. tRNA extracted from *E. coli* B was purchased from General Biochemicals. [*methyl*-^{3}H]thymidine 5'-monophosphate ammonium, specific activity 39 Ci/mmol in 50% ethanol, and L-[2,3-^{3}H]valine, specific activity 6 Ci/mmol in 0.01 N HCl, were purchased from Schwarz/Mann. AG-I-X8 ion exchange resin (200 to 400 mesh) was purchased from Bio-Rad Laboratories.

Media. Cells were grown in various media specifically indicated for each experiment. Casamino Acid media contained per liter: 7.1 g of Na_2HPO_4, 13.6 g of KH_2PO_4, 1 ml of 0.1 M $CaCl_2$, 1 ml of 1.0 M $MgSO_4$, 2 g of $(NH_4)_2SO_4$, and 10 g of Casamino Acids (Difco Certified). Glycerol-Casamino Acid medium was prepared according to Fraser and Jerrel (4) and contained per liter: 10.5 g of Na_2HPO_4, 4.5 g of KH_2PO_4, 3 ml of 0.1 M $CaCl_2$, 0.3 g of $MgSO_4$, 15 g of Casamino Acids (Difco Certified), 100 ml of 30% (wt/vol) glycerol, and 1 ml of a 1% solution of gelatin (Difco).

Preparation of cultures and cell-free extracts. Cultures in the appropriate medium (100 to 200 ml) were grown aerobically on a New Brunswick gyrotory water bath shaker at 37 C. At a cell density of 6×10^8 cells/ml, the cultures were infected at 37 C with bacteriophage $T4Bc^+$ at a multiplicity of five. At various times before and after infection, 10-ml portions were withdrawn and pipetted into centrifuge tubes, containing 1 ml of a chloramphenicol solution (1.1 mg/ml), precooled at −15 C in a salt-ice solution. After being centrifuged for 10 min at 9,880 × *g*, the pellets were resuspended in 2 ml of 6 mM potassium phosphate buffer, pH 7.2, containing 6 mM 2-mercaptoethanol. Cells in a 10-ml glass beaker immersed in ice water were disrupted by treating with a Branson Sonifier, using the standard 0.5-inch (1.27 cm) probe for three 10-s intervals at an output of 60 W. Cell debris was removed by centrifugation at 9,880 × *g* for 10 min at 4 C, and the supernatant was then 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. (7).

Enzyme assays. Valyl-tRNA synthetase activity before and after treatment with 4M urea was measured by determining the amount of tritiated valine attached to tRNA and has been fully described (8). After sucrose density gradient centrifugation, valyl-tRNA synthetase activity was measured by adding 0.45 ml of reaction mix directly to even numbered fractions (0.05 ml). Urea stability was analyzed by a 5-min incubation of odd numbered fractions (0.05 ml) with 0.05 ml of 8 M urea before subsequent addition of reaction mix. A comparison of the resulting counts per minute of the urea-treated and the corresponding untreated enzyme was termed percentage of urea stability. In cell-free extracts, modified valyl-tRNA synthetase has approximately one-half of the specific activity of the unmodified enzyme. Percentage of modification, an expression of the fraction of molecules modified (F_m), therefore, was determined using the formula: $F_m = 2A_s (2A_s + A_u)$, where A_s and A_u refer to the enzyme activities that are stable and unstable, respectively, to 4 M urea treatment.

Dihydrofolate reductase and dCMP deaminase were assayed according to Warner and Lewis (11). Deoxynucleotide kinase activity was determined using [^{3}H]dTMP as substrate according to Lembach et al. (5).

Separation of transcription and translation. The procedure employed is a modification of the methods described by Lembach et al. (5, 6). *E. coli* strain NP4 was grown in 100 ml of the glycerol-Casamino Acid medium of Fraser and Jerrel (4) at 30 C to a density of 10^9 cells/ml. After centrifugation at 9,880 × *g* for 10 min, the pellet was resuspended in 2.5 ml of an adsorption medium consisting of: 0.01 M potassium phosphate (pH 7.2), 0.4% NaCl, 0.4% KCl, 0.1% Casamino Acids, and 0.1% glucose. Chloramphenicol (275 μg in 0.25 ml) was added and the culture was allowed to incubate for 5 min at 30 C. The culture was infected with T4 phage at a multiplicity of infection of 5 by the addition of 3.0 ml of phage lysate, 0.9 ml of adsorption medium, and 0.35 ml of chloramphenicol solution (1,100 μg/ml). After incubation for 20 min at 30 C to allow accumulation of phage mRNA, the culture (7.0 ml) was divided into two equal portions. One portion received 0.5 ml of a rifampicin solution (400 μg/ml in 2% ethanol); the other received 0.5 ml of 2% ethanol. After 5 min of additional incubation at 30 C, each culture was added to 7 volumes (28 ml) of ice-cold salt solution consisting of 0.15 M NaCl and 0.01 M $MgCl_2$; 4.0 ml of the rifampicin solution was added to one portion, 2% ethanol to the other. After 2 min at 0 C to allow extraction and dilution of chloramphenicol from the cells, the cultures were centrifuged at 9,880 × *g* for 10 min. The pellets were resuspended in 4.3 ml of adsorption medium; 0.7 ml of either rifampicin or 2% ethanol were added to each culture. Each 5.0-ml suspension of cells was placed in a 30 C water bath and converted to protoplasts by adding in order: 2.5 ml of adsorption medium, 2.5 ml of 1.5 M sucrose, 0.35 ml of 30% bovine serum albumin, 0.75 ml of lysozyme (2 mg/ml in Tris-hydrochloride, pH 8.0), 0.75 ml of 4% EDTA (pH 8.0), and 0.65 ml of water. Protein synthesis was initiated by adding 4 volumes (50 ml) of prewarmed (30 C) medium consisting of: 0.1% Casamino Acids, 0.1% glucose, 0.04 M $MgSO_4$, 0.3 M sucrose, and either 7.4 ml of rifampicin solution or 2% ethanol. Samples (20 ml) were withdrawn at 1, 15, and 30 min after the resumption of protein synthesis, and cell-free extracts were prepared as previously described.

RESULTS

Appearance of urea-stable valyl-tRNA synthetase after T4 infection. Figure 1 illustrates the ability of valyl-tRNA synthetase in cell-free extracts prepared after T4 infection of *E. coli* strain NP4 to survive disruption by 4 M urea. The valyl-tRNA synthetase activity (5 to

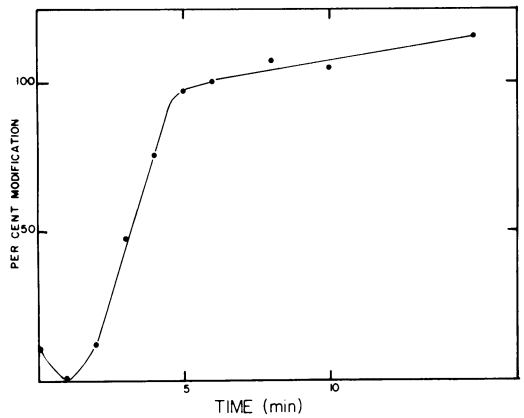

FIG. 1. *The appearance of phage-modified valyl-tRNA synthetase activity in E. coli after T4 phage infection. E. coli strain NP4 in balanced growth at 37 C was infected at a cell density of 2 × 10^8 cells/ml with T4 phage (multiplicity of infection of 5). Samples were withdrawn and urea-stable valyl-tRNA synthetase activity in cell-free extracts was converted to percentage of modification (●) as described in the text.*

10%), that appears to be stable to urea disruption at the time of adding phage, is due to activity that renatures during the 5-min assay period. In contrast, the phage-modified activity exhibits a constant rate of aminoacylation throughout the assay (8). The disappearance of this renaturable activity at 1 min after infection is unexplained but accounts for the occasional absence of such activity (see Fig. 4) when samples are taken slightly after the addition of phage.

Modification beginning at 1 to 2 min after infection is essentially complete 5 min after infection. The activity in excess of 100% modification is due to the activation of completely modified enzyme during 4 M urea treatment. Our results agree with those of Earhart and Neidhardt (2) who demonstrated the same time-course of appearance of a relatively thermostabile valyl-tRNA synthetase when the *E. coli* mutant NP29, possessing a thermolabile enzyme, was infected with T4 phage. As a component of urea stability of phage-modified valyl-tRNA synthetase is due to tRNA (9), the sensitivity of the rate of appearance of urea stable activity to chloramphenicol was measured. The appearance of modified activity could be arrested at stages intermediate to full conversion and confirms and extends the results of Chrispeels et al. (1) (Fig. 2).

Coincidence in the appearance of urea-stable and rapidly sedimenting valyl-tRNA synthetase. The above properties of the modification reaction indicate rapid expression of the appropriate phage gene(s). Chrispeels et al. (1), however, using *E. coli* strain NP-2 suggested a somewhat slower rate of modification as judged by conversion of the host valyl-tRNA synthetase to a more rapidly sedimenting form in sucrose density gradients. Since we have recently shown that the rapidly sedimenting property of modified valyl-tRNA synthetase requires the presence of tRNA, their results may have indicated a two-step mechanism for modification, i.e., addition of τ to host valyl-tRNA synthetase, followed by interaction with tRNA to produce the more rapidly sedimenting species. Consequently, we attempted to separate the appearance of urea stability from the appearance of the high-molecular-weight form of valyl-tRNA synthetase.

The emerging urea-stable activity is associated with the more heavily sedimenting form of valyl-tRNA synthetase (Fig. 3). In no case could we demonstrate a urea-stable light form of modified valyl-tRNA synthetase utilizing cell-free extracts. When the percentage of modification is measured by percentage of urea stability and the sucrose density gradient technique, near superimposable curves are obtained (Fig. 4).

Comparison of the rates of modification of valyl-tRNA synthetase in several strains of E. coli. To further judge the efficacy of the urea test as a measure of percentage of modification, we used three strains of *E. coli* in several media and at several growth temperatures. In general,

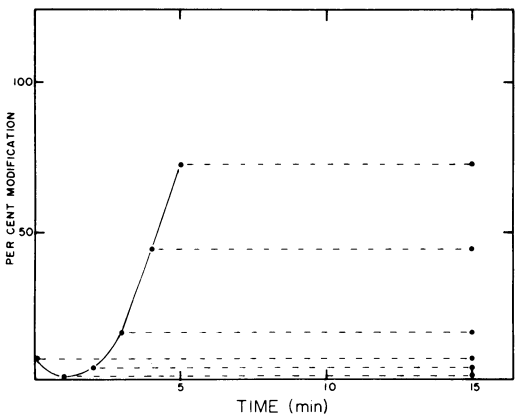

FIG. 2. *Sensitivity of bacteriophage T4 modification of valyl-tRNA synthetase of E. coli to chloramphenicol. E. coli strain NP4 was infected with bacteriophage T4 as indicated in Fig. 1. Samples were withdrawn, mixed with chloramphenicol, and incubated at 37 C until 15 min after infection. Percentage of modification (●) was judged by urea-resistant valyl-tRNA synthetase activity.*

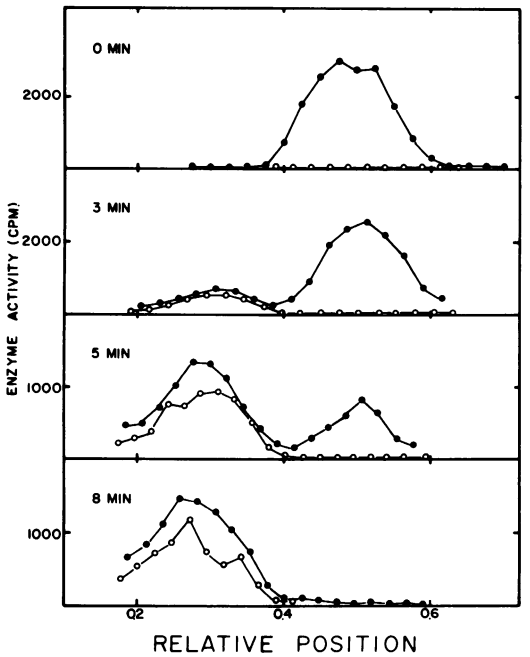

FIG. 3. *Sedimentation rate and urea stability of valyl-tRNA synthetase activity on sucrose density gradients after bacteriophage T4 infection of E. coli. Cell-free extracts prepared from bacteriophage T4-infected E. coli (see Fig. 1) were prepared from samples removed at 0, 3, 5, and 8 min after infection. Centrifugation was conducted at 4 C in a Beckman L2-65B centrifuge using the SW50.1 rotor. Extracts containing approximately 200 μg of protein in 0.2 ml were layered on 5 to 20% (wt/vol) linear sucrose gradients (5 ml) buffered with 6 mM potassium phosphate, pH 7.2, containing 6 mM 2-mercaptoethanol and sedimented for 18 h. Alternate 0.05-ml fractions collected from the bottom of each centrifuge tube were assayed for valyl-tRNA synthetase activity with (O) and without (●) treatment with 4 M urea. Activity is expressed as counts per minute of [^3H]valine attached to tRNA during a 5-min assay at 37 C. Graphs were normalized by plotting data relative to the total number of fractions collected so that position 0.0 is the bottom of the centrifuge tube.*

the rate of production of urea-stable valyl-tRNA synthetase was found to double upon a 10 C increase in growth temperature (results not shown). Media containing Casamino Acid supplements generally resulted in more rapid rates of modification than glucose minimal media. Figure 5 presents the results of one such experiment. At this lower temperature the rate of modification in strain NP4 was reduced. Although strain NP2 initially had a rate of modification similar to that of NP4, we were unable to achieve 100% modification in the observed time interval. This result is consistent with the work of Chrispeels et al. (1). For

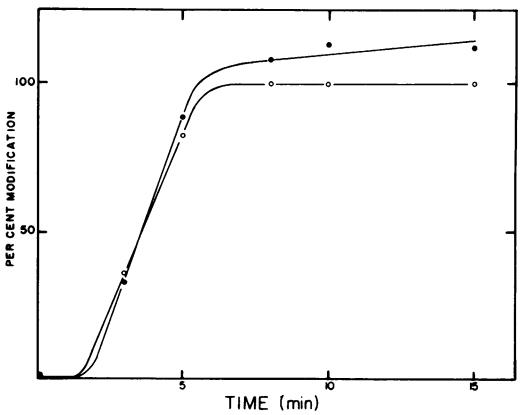

FIG. 4. *Comparison of the appearance of modified valyl-tRNA synthetase activity as judged by urea-resistant and rapidly sedimentating activity in cell-free extracts prepared from bacteriophage T4-infected E. coli. A culture of E. coli strain NP4 was grown and infected with bacteriophage T4 as described in Fig. 1. Portions were removed and analyzed for percentage of modification by the criterion of urea resistance (●) or rapid sedimentation (O) as described in Fig. 1 and 3.*

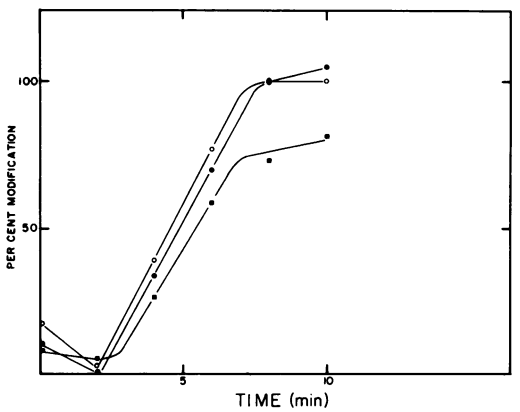

FIG. 5. *The appearance of modified valyl-tRNA synthetase activity in several strains of E. coli infected with bacteriophage T4. E. coli strains NP2, NP4, and NP29 growing at 30 C were infected with bacteriophage T4 as described in Fig. 1. Cell-free extracts prepared from samples removed at the indicated times were assayed for modified valyl-tRNA synthetase activity. Percentage of modification was determined in strains NP2 (■) and NP4 (●) by the criterion of urea resistance. Cell-free extracts of strain NP29 contain little aminoacylation activity for valine in vitro but acquire such activity during the course of viral growth. Thus, the aminoacylation activity present after 8 min of infection was arbitrarily set at 100% and percentage of modification in strain NP29 was normalized to this value (O).*

comparison, the emergence of significant valyl-tRNA synthetase activity in the mutant strain NP29 is presented. As phage-modified activity

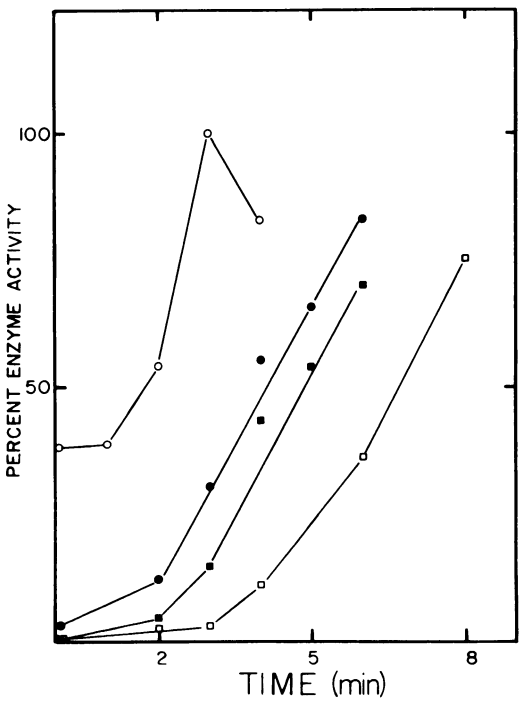

FIG. 6. *Appearance of modified valyl-tRNA synthetase activity relative to other T4-induced enzyme activities. E. coli strain NP4 was grown and infected with bacteriophage T4 as indicated in Fig. 1. Cell-free extracts were prepared from portions at the various times after infection and assayed for: phage-modified valyl-tRNA synthetase activity (●), dCMP deaminase activity (■), dihydrofolate reductase activity (○), and deoxynucleotide kinase activity (□). All activities are expressed as percentage of the maximum obtained during the course of the experiment. Kinetic data is presented for only the first 8 min of viral development.*

in NP29 represents 90 to 95% of the valyl-tRNA synthetase activity that can be detected, the activity after 8 min of infection was arbitrarily set at 100%. The results are, therefore, consistent with those from the parent strain NP2 but formally resemble the NP4 data, for the fortuitous reason that modification is essentially complete by 8 min in NP4.

Comparison of the appearance of modified valyl-tRNA synthetase with other phage-induced enzymes. Figure 6 compares the appearance of phage-modified valyl-tRNA synthetase activity with appearances of the T4-induced enzymes: dihydrofolate reductase, dCMP deaminase, and deoxynucleotide kinase. Because the appearance of modified enzyme was more nearly like dihydrofolate reductase, the appearance of modified enzyme was tentatively classified as an immediate early phage function.

Accumulation of the potential for valyl-tRNA synthetase modification in the absence of protein synthesis. To further characterize the regulation of valyl-tRNA synthetase modification, we used the technique of Lembach et al. (5, 6) to separate transcription from translation. Cells were infected with T4 phage in the presence of chloramphenicol, allowed to accumulate mRNA for 20 min, and sampled for the activities of several phage enzymes after the restoration of protein synthesis in the presence or absence of rifampicin. As illustrated in Fig. 7, the potential for modification of valyl-tRNA synthetase resembles dihydrofolate reductase-

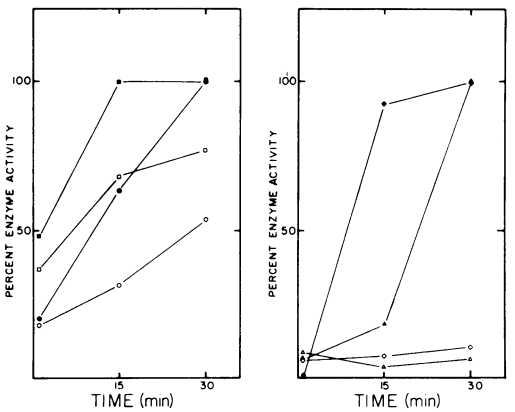

FIG. 7. *Expression of phage-induced, enzyme-forming capacity after T4 infection of E. coli in the presence of chloramphenicol. E. coli strain NP4 was grown on glycerol-Casamino Acid medium at 30 C, centrifuged, and resuspended in adsorption medium that contained chloramphenicol (100 μg/ml). After 5 min, bacteriophage T4 was added at a multiplicity of infection of 5 and the infection was allowed to proceed for 20 min. Rifampicin was then added to one-half the culture (100 μg/ml); the remainder served as a control. Each culture was then diluted into 7 volumes of ice-cold salt solution with or without rifampicin (100 μg/ml). After 2 min to allow extraction and diluted of the chloramphenicol, each culture was centrifuged and pellets were resuspended in adsorption medium with or without rifampicin (100 μg/ml). Each culture was converted to protoplasts and protein synthesis was initiated by the addition of 4 volumes of prewarmed medium with and without rifampicin. Cell-free extracts were prepared from portions taken from the control (closed symbols) and rifampicin-treated (open symbols) cultures at 1, 15, and 30 min after the restoration of protein synthesis. Samples were assayed for phage-modified valyl-tRNA synthetase activity (●, ○); dihydrofolate reductase activity (■, □); dCMP deaminase activity (◆, ◇); and deoxynucleotide kinase activity (▲, △). All activities are expressed as percentage of maximal activity obtained during the 30-min incubation in the control cultures which lacked rifampicin.*

forming potential in that both accumulate during phage infection and growth in the presence of chloramphenicol. In contrast, dCMP deaminase and deoxynucleotide kinase-forming potential do not accumulate during this period. Thus, modification resembles an immediate early phage function.

DISCUSSION

The coincident appearance of urea-stable and rapidly sedimenting valyl-tRNA synthetase activity after bacteriophage T4 infection of *E. coli* suggests that the chloramphenicol-sensitive synthesis of the τ peptide, a product of the T4 *vs* gene, rapidly increases the affinity of the enzyme for tRNA. This result is substantiated by T4 *vs* gene mutants, in which urea stability is correlated with rapidly sedimenting valyl-tRNA synthetase activity (W. H. McClain, G. L. Marchin, F. C. Neidhardt, K. V. Chace, M. L. Rementer, and D. H. Hall, Virology, in press). In vitro experiments confirm the expectation that purified τ added to cell-free extracts from uninfected *E. coli* produced both urea-stable and heavily sedimenting activities (1). Additionally, tRNA removal by DEAE-cellulose chromatography of cell-free extracts prepared from infected bacteria result in a valyl-synthetase activity which is urea stable but with the sedimentation rate of unmodified enzyme. Whether additional modifications of the molecule occur is unknown.

The immediate early nature of modification is verified by comparison with known phage enzymes and the accumulation of modification potential in the presence of chloramphenicol. The inability to completely convert valyl-tRNA synthetase to the modified form in *E. coli* strain NP2 is probably due to the poor adsorption of T4 phage by this strain.

We have utilized this new information about the kinetics of appearance of phage-modified valyl-tRNA synthetase to prepare purified, modified valyl-tRNA synthetase with radioactive label in the τ peptide. Antisera to the various forms of valyl-tRNA synthetase are being prepared so that a radioimmune assay for the τ peptide can be developed. We hope to use such an assay to analyze T4 mutants deficient in their ability to produce urea-stable and heavily sedimenting valyl-tRNA synthetase activity. Because such mutants grow reasonably well on laboratory strains of *E. coli*, it is important to quantitate the degree of the genetic lesion to ascertain whether the *vs* gene is dispensible for T4 bacteriophage.

ACKNOWLEDGMENTS

The material in this paper is part of a thesis submitted by U. R. M. to the Kansas State University in partial fulfillment of the requirements for the M.S. degree. This work was supported by Public Health Service grant AI 10858-01 from the National Institute of Allergy and Infectious Diseases, by National Institutes of Health Biomedical Sciences Support grant FR 7036, and by the Kansas Agricultural Research Experiment Station.

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