Effect of Elevated Temperature on Extended Enzyme Synthesis Induced by Bacteriophage T4 *Amber* Mutants Unable to Synthesize Deoxyribonucleic Acid

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The extended synthesis of early enzymes by the deoxyribonucleic acid-negative *amber* mutants of bacteriophage T4 after infection of the nonpermissive host *Escherichia coli* B was prevented by incubating the infected cells at 44 C. This effect did not occur if the incubation temperature was 43 C or less or if the cells were grown and infected in broth rather than minimal medium (C medium). Once early enzyme synthesis had ceased at 44 C, lowering the incubation temperature to 37 C did not occasion resumption of synthesis. Experiments with chloramphenicol at 44 C indicated that increased degradation of early enzymes is an unlikely explanation for the effect. Examination of pulse-labeled ribonucleic acid and polysomes made at 37 and 44 C in infected cells revealed some differences, but at present there is no obvious way in which these differences may be related to the effect on enzyme formation. There was no discernible difference between the ribosomal ribonucleic acid and ribosomes at the two temperatures, nor was there a difference in the cell-free amino acid-incorporating systems isolated from cells infected at the two temperatures as judged by polyuridylic stimulation of phenylalanine incorporation. Incubation of cells infected with T4*am*N82 at 44 C with protein synthesis blocked by 5-methyltryptophan for 15 min did not prevent the typical pattern of enzyme synthesis at 44 C when the block was reversed by excess L-tryptophan. The relation of this and other observations relative to the effect at 44 C on the synthesis of early enzymes is discussed.

The regulation of the synthesis of early enzymes induced by bacteriophage and by animal viruses is an intriguing and as yet not fully understood area of virology. In the case of phages T2 and T4, early enzyme synthesis (it being understood that the term "early enzymes" as used in this paper includes a group of enzymes, which, although appearing early in the replicative cycle, do not all first appear simultaneously) generally ceases about 15 min after infection at 37 C. However, in certain instances such as infection by either ultraviolet-irradiated phage (4, 5, 12) or *amber* (*am*) mutant bacteriophage unable to code for a critical early function (22), normal enzyme shut-off fails to occur and these enzymes are made for an extended period. It has been postulated (5, 12, 20, 23) that phage deoxyribonucleic acid (DNA) synthesis is requisite for stoppage of early enzyme formation; however, Mathews and Kessin (14) have reported

that certain temperature-sensitive mutants of T4 which do not synthesize DNA when grown at 44 C will nevertheless cease making early enzymes at the expected time.

This paper reports another instance of early enzyme shut-off in the absence of DNA synthesis, in this case employing DNA-negative (DO) *am* mutants of bacteriophage T4. Data bearing on the nature and possible site of this effect are reported.

MATERIALS AND METHODS

Bacteriophage, bacterial strains, and media. The DO *amber* mutants used were: *am*N122 [gene 42, deoxycytidine monophosphate (dCMP) hydroxymethylase], *am*N101 (gene 43, DNA polymerase), and *am*N82 (gene 44, unknown function). T4D was used as the wild-type or *am*⁺ strain.

Stocks of *am* mutants were grown in broth [10 g of tryptone (Difco), 5 g of yeast extract (Difco), 10 g of NaCl, and 1 g of D-glucose per liter] pre-

vously inoculated with the permissive strain of *Escherichia coli* S26R1E.

For most experiments, the nonpermissive host was *E. coli* B. The ribonuclease I-deficient strain D10 (6) was used as a nonpermissive host for the in vitro amino acid incorporation experiments.

In most of the experiments, except where noted otherwise, the cells were grown and infected in C medium (3) containing 2% D-glucose. Before infection, L-tryptophan was added to the medium at a final concentration of 25 μ g/ml to facilitate bacteriophage adsorption.

Induction and preparation of bacteriophage-specific enzymes. *E. coli* was grown at 37 C with forced aeration to a concentration of 2×10^8 to 3×10^8 cells/ml. The cells were pelleted at $4,000 \times g$ for 5 min and resuspended in 1 to 2 ml of adsorption buffer (8). The concentrated suspension of cells was divided in half and added to cultures of C medium (prewarmed to 37 or 44 C) at a final concentration of 4×10^8 to 6×10^8 cells/ml. The cells were infected within 1 min of addition to the prewarmed medium by using the appropriate bacteriophage strain at a multiplicity of infection of 4 to 8.

At the indicated times, samples were removed and mixed with an equal volume of crushed C medium ice. All subsequent operations were at 0 to 4 C. After sedimenting the cooled cells at $10,000 \times g$ for 10 min, the pellets were resuspended in 1.6 ml of potassium phosphate buffer (0.05 M, pH 7.4) and disrupted by sonic vibration with a Bronwill Biosonik II ultrasonic probe. The disrupted cells were centrifuged for 20 min at $10,000 \times g$, and the supernatant fractions were frozen at -85°C until assayed for enzymatic activity.

Enzyme assays. dCMP hydroxymethylase activity was determined by the method of Pizer and Cohen (18) by employing Dowex 50-H⁺ columns (8 by 1 cm) to separate the product 5-hydroxymethyl-2'-deoxycytidylate from 2'-deoxycytidylate. One unit of dCMP hydroxymethylase activity is defined as the incorporation of 0.01 μ mole of formaldehyde into 5-hydroxymethyl-2'-deoxycytidylate in 20 min at 37 C.

The procedure of Goulian, Lucas, and Kornberg (7) was used to assay DNA polymerase activity with ¹⁴C-deoxyadenosine triphosphate as the labeled nucleotide precursor. The incorporation of 10 nmoles of total nucleotide into acid-insoluble product in 30 min at 37 C is defined as one unit of activity.

Deoxycytidine pyrophosphatase activity was assayed spectrophotometrically by measuring dCMP formation at 280 nm. The dCMP formed was isolated with a column (4.5 by 50 mm) of Biorad AG-1-X8, 200 to 400 mesh resin and ammonium formate as described by Warner and Barnes (21). A unit of deoxycytidine pyrophosphatase activity is defined as the formation of 1 μ mole of dCMP per hr.

Protein synthesis. ¹⁴C-labeled L-leucine (0.13 μ Ci per ml of culture medium, 1.0 μ Ci per 0.13 μ mole) was used to monitor protein synthesis. At the appropriate times, duplicate 0.2-ml samples were removed and added to 0.8 ml of 6.25% trichloroacetic acid. The samples were heated at 90 C for 15 min in loosely

closed screw-top tubes. After chilling the samples in ice, they were filtered on membrane filters (0.45- μ m porosity) (Millipore Corp.), washed with three 10-ml portions of 5% trichloroacetic acid, and counted.

Pulse-labeled RNA. *E. coli* B was grown in C medium to a density of 3×10^8 cells/ml. The cells were centrifuged, divided in half, and resuspended in C medium at either 37 or 44 C. The cells were infected with the appropriate bacteriophage. At the times indicated, samples were removed from the flask and added to tubes containing sufficient ¹⁴C-uracil for a final concentration of 0.34 μ mole/ml and with a specific activity of 7.1 μ Ci/ μ mole. The incubation was terminated after 1 or 3 min by addition of ice-cold trichloroacetic acid. The cold acid-precipitable radioactivity was measured by collecting the filtrate on a membrane filter of 0.45- μ m pore size.

Pulse-chase procedure. This procedure was patterned after that described by Mathews (13). *E. coli* B was grown in C medium containing 20 μ g of uracil per ml to repress pyrimidine synthesis after infection (12). After attaining a density of 2×10^8 to 3×10^8 cells/ml, the cells were sedimented, divided in half, resuspended at 37 or 44 C in C medium containing 1 μ g of uracil per ml, and immediately infected. At the times indicated, ¹⁴C-uracil was added (55 μ Ci/ μ mole final specific activity) to each flask. Three minutes later, the pulses were terminated by a chase of ¹²C-uracil sufficient to establish a final concentration in the cultures of 200 μ g of uracil per ml. Samples were removed at the indicated times and assayed for cold acid-precipitable radioactivity by filtration through membrane filters (Millipore Corp.) having a pore size of 0.45 μ m.

Isolation and analysis of pulse-labeled RNA. Infected cells at 37 or 44 C were labeled with either ¹⁴C-uracil (7.9 μ M, 42.4 mCi/mmole) or ³H-uracil (57 nM, 6 Ci/mmole) for 2 min. Labeling was terminated by adding the infected cells to an equal volume of C medium ice. The cells were sedimented and washed once by centrifugation, and the RNA was isolated and analyzed on a 5 to 20% sucrose gradient by the method of Asano (1).

Isolation of ribosomal RNA, ribosomes, and polysomes. *E. coli* B was grown and infected at 37 or 44 C as described for the enzyme induction studies. The procedure for isolation of ribosomal RNA (rRNA) was similar to that described by Nakada and Marquisee (15). At the appropriate time after infection, the cells were chilled by mixing with C medium ice, sedimented, and washed twice with a solution of 5×10^{-3} M tris(hydroxymethyl)amino-methane (Tris), 10^{-4} M magnesium acetate, pH 7.2. The cell pellet after the second wash was frozen at -85°C and then homogenized in a glass homogenizer with 2 volumes of Alcoa alumina. Tris-magnesium acetate solution (described above) was added to the mixture and centrifuged at $18,800 \times g$ for 20 min. The resulting supernatant fluid was centrifuged for a second time at $18,000 \times g$ for 20 min. Sodium dodecyl sulfate was added to the supernatant fluid from the second centrifugation at a final concentration of 5 mg/ml. The sample was shaken for 15 min at room temperature. A portion of the sample was

layered on a 24-ml, 5 to 20% linear sucrose gradient and centrifuged for 14 hr at 24,000 rev/min and 15 C in a Spinco SW25 rotor; then fractions were collected and analyzed for absorbancy at 260 nm.

The initial steps in the isolation of ribosomes were the same as for those employed for rRNA through the two 20-min centrifugations at $18,800 \times g$, with the exception that the solution added after homogenization with alumina contained 10^{-2} M $MgCl_2$ and 10^{-2} M Tris (pH 7.4). The supernatant fluid from the second $18,800 \times g$ centrifugation was centrifuged at 39,000 rev/min for 2 hr in a Spinco 40 rotor at 4 C. The pellet was resuspended in 0.5 ml of a 10^{-4} M $MgCl_2$, 10 mM Tris (pH 7.4) solution and layered on a 30-ml linear 10 to 30% sucrose gradient for centrifugation in a Spinco SW25 rotor (24,000 rev/min, 16 hr, 4 C). Fractions were collected and analyzed at 260 nm.

Polysomes were isolated and analyzed by the method of Ron, Kohler, and Davis (19), with the exception that chloramphenicol was added immediately before chilling the culture by adding C medium ice and cooling the mixture in a dry ice-acetone bath. The cells were grown and infected as for the enzyme induction studies.

In vitro amino acid incorporation. The in vitro amino acid-incorporating system was isolated and assayed as described by Nirenberg (17). Polyuridylic acid (poly U; Miles Laboratories, Inc.) was used to stimulate incorporation of phenylalanine. In these studies, the *E. coli* mutant, D10, deficient in ribonuclease I was used. The reaction mixture contained, in a final volume of 0.25 ml: 100 mM Tris, pH 7.8; 14 mM $MgAc$; 50 mM KCl; 1 mM adenosine triphosphate; 0.2 mM guanosine triphosphate; 6 mM 2-mercaptoethanol; 7.4 mM sodium phosphoenolpyruvate; 0.2 mM unlabeled amino acid mixture; 0.2 mM ^{14}C -phenylalanine (4 mCi/mmol).

RESULTS

Enzyme induction. Infection of the nonpermissive host, *E. coli* B, by DO *am* mutants of bacteriophage T4 at 37 C resulted, as originally reported (23), in prolonged and increased synthesis of the bacteriophage-induced early enzymes (Fig. 1, 2). This phenomena for *amN82* is shown with dCMP hydroxymethylase and DNA polymerase (Fig. 1) and for *amN122* with deoxycytidine pyrophosphatase (Fig. 2). However, infection of the nonpermissive host with either mutant at 44 C resulted in apparent shut-off of enzyme formation at the normal time of approximately 15 min after infection (Fig. 1, 2). The effect at 44 C was also discernible when protein synthesis was measured by incorporation of ^{14}C -leucine into hot acid-insoluble radioactivity when *amN82*, *amN122*, or *amN101* was used, although the difference for *amN101* was not as striking as with the other mutants (Fig. 3). ^{14}C -leucine incorporation was affected similarly by elevated temperature in *T4am*⁺-infected cells.

FIG. 1. Induction by *T4amN82* of hydroxymethylase and DNA polymerase at 37 and 44 C. *E. coli* B, grown at 37 C, was infected at 37 or 44 C, and samples were removed for assay at the indicated times. Units of hydroxymethylase per milligram of protein at 37 C (●) or 44 C (○); units of DNA polymerase at 37 C (▲) or 44 C (△).

FIG. 2. Induction by *T4amN122* of deoxycytidine pyrophosphatase (dCTPase) at 37 and 44 C. *E. coli* B, grown at 37 C, was infected at 37 and 44 C, and samples were removed for assay at the indicated times. Units of dCTPase per milligram of protein at 37 C (●) or 44 C (○).

FIG. 3. Protein synthesis in cells infected at 37 or 44 C. *E. coli* B, grown at 37 C, was infected by either (A) T4amN82, (B) T4amN122, or (C) T4amN101 at 37 C (●) or 44 C (○). ¹⁴C-leucine was added at the initiation of infection. Samples, removed at the indicated times, were processed and measured for radioactivity in protein.

This effect of increased temperature on early enzyme synthesis was demonstrable at 44 C; however, at 43 C the same pattern of abnormally extended enzyme synthesis occurred as at 37 C (Fig. 4). Thus, there was a very sharp transition from one condition to the other within the range of one degree. This effect was consistently observed and, because the transition range was so narrow, special care was taken to maintain and monitor the temperature as accurately as possible. (A Warner-Chilcott shaker water bath was used and the temperature, which varied by less than ± 0.5 C, was monitored with a total immersion thermometer subdivided into one-tenth degree divisions with a range from 25 to 55 C.) Early enzyme synthesis will not resume by incubating the infected cells at 37 C after an initial 15-min incubation at 44 C. Interestingly, if the cells were grown and infected in broth rather than C medium, the effect on enzyme synthesis at 44 C was prevented (Fig. 4). (The appearance of hydroxymethylase activity in broth at 37 C was the same as at 44 C in broth.)

A possible explanation for the temperature effect is that the shut-off of enzyme activity at 15 to 20 min is only apparent and results from increased degradation of the enzymes at 44 C, balancing the unabated synthesis of new enzyme rather than actual cessation of enzyme synthesis. To test this hypothesis, *E. coli* B was infected with amN82 at 44 C, and at 15 min postinfection chloramphenicol (30 μ g/ml final concentration) was added to block further new enzyme synthesis. (Prior experiments showed that this concentration of chloramphenicol almost immediately blocks further incorporation of labeled ¹⁴C-leucine into protein.) If the shut-off resulted from increased degradation or inactivation of the enzymes already made, the blocking of any further enzyme synthesis would

FIG. 4. Induction by T4amN82 of hydroxymethylase in C medium at 37 and 43 C and in broth at 44 C. *E. coli* B was either grown at 37 C in C medium and infected in C medium at 37 C (●) or 43 C (○), or grown at 37 C in broth and infected in broth at 44 C (▲). Samples were removed for assay at the indicated times.

unmask this effect. This was not observed (Fig. 5). Enzyme activity (dCMP hydroxymethylase) remained relatively unchanged for at least 15 min after chloramphenicol addition, thus demon-

FIG. 5. Hydroxymethylase levels in *T4amN82*-infected cells at 44°C after addition of chloramphenicol. *E. coli B* was grown at 37°C and infected at 44°C. At 15 min postinfection, chloramphenicol (CM) was added to a final concentration of 30 µg/ml. Samples were removed for assay at the indicated times before and after addition of CM.

strating that the levelling off of enzyme activity at 44°C is not due to enhanced degradation of early enzymes but rather cessation of synthesis. During the 15-min block with chloramphenicol, the level of enzyme activity decreased by 10%, whereas at 30 min after infection the enzyme level at 44°C was about 30% that at 37°C (Fig. 1).

Incubation of cells infected with *amN82* for 15 min at 44°C with protein synthesis blocked did not prevent the typical pattern of enzyme synthesis at 44°C when the block was reversed (Fig. 6). In these experiments, 5-methyltryptophan was added at the time of infection at 44 or 37°C and as expected there was no enzyme synthesis. Addition of L-tryptophan in 50-fold molar excess, relative to 5-methyltryptophan, reversed the block of protein synthesis, and at both temperatures the time course of enzyme synthesis developed as if from the initiation of infection. Sekiguchi and Cohen (20) obtained a similar result when, at 37°C, *T6r⁺* was used to infect the polyauxotrophic mutant *E. coli THU* which requires histidine for growth. Addition of histidine to cells infected for 6 min in the absence of histidine resulted in the normal pattern of hydroxymethylase induction. Recently, Lembach and Buchanan (11), using T4-infected *E. coli B* at 30°C in conjunction with 5-methyltryptophan and rifamycin, found a similar effect, also concluding that the messenger RNA

(mRNA) formed in the presence of 5-methyltryptophan is sufficient to direct the synthesis of control levels of hydroxymethylase. An implication of these experiments is that some biochemical sequence initiated by infection must follow its course before the shut-off of early enzymes occurs at 44°C. In addition, general deterioration of the infected cells at 44°C would not seem responsible for the effect on enzyme formation. In this regard, uninfected cells incubated for 15 min at 44°C and then infected at 37 or 44°C showed essentially the same pattern of enzyme induction as cells grown entirely at 37°C before infection.

The experiments that follow seek to ascertain the specific locus of action of the elevated temperature.

Pulse-labeled RNA. The possibility that viral mRNA synthesis is altered at 44°C was examined. Cells infected at 37 or 44°C with *amN82*, *amN122*, or *amN101* were pulse-labeled for 3-min periods beginning 2 min after infection. The pattern of pulse-labeled RNA synthesis at 44°C differed from that at 37°C in two respects (Fig. 7A-C). First, whereas the rate of synthesis at 37°C was highest from 5 to 8 min (*amN82*) or approximately equal to the rate from 2 to 5 min

FIG. 6. Effect of 5-methyltryptophan (5MT) on hydroxymethylase induction by *T4amN82* at 37 and 44°C. *E. coli B*, grown at 37°C, was infected at 37°C (●) or 44°C (○). One minute before infection, sufficient 5-MT was added for a final concentration of 10 µg/ml. At 15 min postinfection, the block of enzyme synthesis was reversed by 50-fold molar excess of L-tryptophan. Samples were removed as indicated for assay.

FIG. 7. Pulse-labeling of RNA in infected cells for 3-min periods at 37 or 44 C. *E. coli* B, grown at 37 C, was infected by either (A) T4amN82, (B) T4amN122, or (C) T4amN101 at 37 C (●) or 44 C (○). The infected cells were pulse-labeled as indicated for 3-min periods with ¹⁴C-uracil.

(*amN122* and *amN101*) and then declined thereafter, the rate of synthesis at 44 C was highest in the first period measured, 2 to 5 min, and then declined at the same rate as at 37 C. In the particular experiment shown for *amN82*, the maximum rate in the 2- to 5-min period at 44 C was somewhat higher than the maximum rate at 37 C for the 5- to 8-min period. This was generally found and, in part, may reflect the divisions of the time chosen for measurement. In other experiments with *amN82* (Fig. 8), shorter, 1-min periods of labeling were used. The maximum rate at 37 C was found from 6 to 7 min postinfection and from 4 to 5 min postinfection at 44 C. Second, at both temperatures, the rate of synthesis reached a plateau and at about 20 min the rate of synthesis at 44 C was about one-third to one-half that at 37 C. It is not readily apparent how these differences could account for the shut-off of early enzyme synthesis at 44 C, when at 20 min the rate of enzyme formation and protein synthesis at 44 C is slight, whereas at 37 C it is proceeding at a maximal rate.

The preceding experiments on pulse-labeled RNA were performed under conditions in which exogenous uracil was added only at the time of the pulse and then only in trace amounts. Mathews (12) has reported that growing *E. coli* B in the presence of uracil makes the cells dependent upon exogenously added uracil after infection. Pulse-labeled RNA was therefore

measured under this condition, and a pattern similar to that depicted in Fig. 7 was found.

A completely different result was obtained when the pulse-labeling experiment was performed with broth instead of C medium. (Exogenous ¹⁴C-uracil was in the broth as in the previous experiment with C medium.) At both temperatures, the pulse-labeled RNA was highest from 1 to 2 min and declined thereafter. There was no difference in the apparent rate of labeling at the later times. It should be recalled that cells grown and infected in broth show the same enzyme induction pattern at 44 as at 37 C.

A pulse-chase experiment revealed no significant difference in stability between the pulse-labeled RNA formed at 37 or 44 C at 5 or 20 min after infection (Fig. 9). In these experiments, the *E. coli* B had been grown in C medium containing 20 μg of uracil to repress de novo pyrimidine synthesis (12). After infection, the cells were washed and incubated at 37 and 44 C, respectively, in C medium containing 1 μg of uracil per ml. The pulse of labeled uracil (55 μCi/μmole final specific activity) was added at 2 or 17 min postinfection to both cultures, and 3 min later a 100-fold excess of unlabeled uracil was added as the chase. The samples taken 2 min after the addition of unlabeled uracil at 22 min postinfection showed a slight further rise in cold acid-precipitable radioactivity for the infected cells at either temperature. This was

FIG. 8. Pulse-labeling of RNA in T4amN82-infected cells for 1-min periods at 37 and 44 C. *E. coli* B, grown at 37 C, was infected at 37 C (●) or 44 C (○) and pulse-labeled with ¹⁴C-uracil as indicated for 1-min periods.

FIG. 9. Pulse-chase experiment with T4amN82-infected cells at 37 and 44 C. *E. coli* B, grown at 37 C was infected at 37 C (●) or 44 C (○) and pulsed with ¹⁴C-uracil from 2 to 5 min or 17 to 20 min. A chase of ¹²C-uracil was added at either 5 or 20 min, and samples were removed as indicated for measurement of radioactivity.

observed in other experiments of this type at 20 min postinfection (but as may be observed in Fig. 9 not at 5 min postinfection) and may reflect, as suggested, a preferential utilization of uridylic acid derived from degradation of RNA (10, 16).

Examination of the pulse-labeled RNA synthesized at 37 and 44 C at 20 to 22 min postinfection by sucrose gradient sedimentation shows that there was a somewhat greater preponderance of higher-sedimentation labeled material at 37 than at 44 C, although there is no evidence of any extensive fragmentation of the 44 C pulse-labeled RNA (Fig. 10). The same results obtained if the labels were reversed vis-à-vis the incubation temperature, thus eliminating the possibility that the relative difference in pulse-labeling patterns at the two temperatures was a result of the difference in specific activity of the ³H- and ¹⁴C-uracil. The significance of the slight difference in sedimentation pattern is not known at present.

rRNA, ribosomes, and polysomes. There was no observable alteration in quantity or sedimentation pattern of the rRNA or ribosomes from amN82-infected cells at 37 or 44 C after 20 min of infection (Fig. 11A and B).

Sucrose gradient profiles of the polysomes formed at the two temperatures were also attempted. If the cells were grown and infected in

FIG. 10. Sedimentation profile of pulse-labeled RNA synthesized at 37 or 44 C from 20 to 22 min postinfection with T4amN82. Infected cells at 37 C (●) or 44 C (○) were pulsed between 20 and 22 min with ³H-uracil or ¹⁴C-uracil, respectively. The labeled cells were combined for extraction of RNA which was sedimented at 24,000 rev/min in an SW25.1 rotor for 14 hr in a 5 to 20% sucrose gradient. For purposes of comparison, the radioactivity in each fraction is expressed as per cent of the total counts per minute recovered from the gradient.

FIG. 11. Sedimentation profiles of rRNA and ribosomes from *E. coli B* infected with T4amN82 at 37 or 44 C. The rRNA of ribosomes was isolated from the infected cells 20 min postinfection at 37 C (●) or 44 C (○). The rRNA was sedimented at 24,000 rev/min in an SW25.1 rotor for 14 hr in a 5 to 20% sucrose gradient. For the ribosomes, a 30-ml 10 to 30% sucrose gradient was employed and centrifugation was for 16 hr at 24,000 rev/min in an SW25.1 rotor. (A) rRNA, (B) ribosomes.

broth, excellent profiles were obtained as judged by high yield of polysomes comparable to previously reported results. There was no difference in sedimentation profile at 22 min for the two temperatures. This was not unexpected since, as reported above, growth and infection in broth prevented the effect at 44 C on enzyme synthesis. Cells grown and infected in C medium and extracted in the same manner as the broth-maintained cells consistently showed poor yields of polysomes with the preponderance of material appearing in the 70S or lower area of the gradient (Fig. 12A). However, at 22 min, there was a proportionately greater amount of polysomes at 37 than at 44 C, albeit that at both temperatures the absolute amount was small (Fig. 12B). This may reflect the apparent difference in pulse-labeled RNA at this time.

In vitro amino acid incorporation. Equal

amounts (as judged by protein concentration) of ribosomes and S-100 fraction extracted from amN82-infected cells incubated for 20 min at 37 and 44 C showed equal ability to incorporate ^{14}C -phenylalanine in response to poly U at 37 C (Fig. 13).

Effect of elevated temperature on wild-type bacteriophage. There was no difference in the pattern of formation of hydroxymethylase activity in C medium at 37 and 44 C for the *am*⁺ strain tested, T4D. Incorporation of ^{14}C -leucine into hot trichloroacetic acid-insoluble counts was decreased at 44 C as compared to incorporation at 37 C in a pattern similar to that depicted for *am*N101 in Fig. 3C. The burst size was markedly depressed at 44 C in C medium. At 37 C, the burst size was 11 plaque-forming units per cell, whereas at 44 C it was only about 0.4. In broth, the burst size was decreased at 44 C but not as much as in C medium. The burst sizes in broth were 146 and 31 plaque-forming units per cell at 37 and 44 C, respectively.

Reversibility. As stated previously, early enzyme synthesis will not resume by incubating the infected cells at 37 C after a previous 15 min of incubation at 44 C. The 44 C incubation temperature had a variable effect on uninfected *E. coli B*. In some experiments, the higher temperature had little or no effect for as long as 100 min on either growth (measured turbidimetrically and by colony formation on solid medium) or DNA, RNA, and protein synthesis (deter-

FIG. 12. Sedimentation profiles of polysomes from *E. coli B* 20 min after infection with T4amN82 at 37 or 44 C. Cells infected at 37 C (●) or 44 C (○) were extracted after 22 min to obtain polysomes which were analyzed by sedimentation in a 15 to 30% sucrose gradient in an SW25.1 rotor for 2.5 hr at 24,000 rev/min. For purposes of comparison, the absorbance at 260 nm for each fraction is expressed as per cent of the total absorbance at 260 nm recovered from the gradient. Part B is a depiction of the first portion of the gradient shown in part A but with the ordinate scale expanded.

FIG. 13. *In vitro* amino acid incorporation by extracts of cells infected by T4amN82 for 20 min at 37 or 44 C. *E. coli* mutant D10 was infected at 37 or 44 C for 20 min. Ribosomes and S-100 fraction were isolated and tested for phenylalanine incorporation. Symbols: ●—●, 37 C extract; ●---●, 37 C extract plus poly U; ○—○, 44 C extract; ○---○, 44 C extract plus poly U. Equivalent amounts of extract were compared as judged by protein concentration.

mined as described above) In other experiments, cell replication as well as DNA, RNA, and protein synthesis were significantly decreased and in some instances stopped completely. In these latter instances, the inhibition was reversible, with control growth rate (at 37 C) returning approximately 20 min after changing the incubation temperature to 37 C. Growth of *E. coli* B, may, as for infected cells, be very sensitive to inhibition with slight change in temperature at the critical level. For infected cells, the critical range occurred between 43 and 44 C. The uninfected cell may be somewhat less sensitive to inhibition by elevated temperature, with the critical range occurring at 44 C and higher.

DISCUSSION

The DO T4 mutants which have abnormally extended early enzyme synthesis offer an opportunity to examine the problem of regulation of

virally induced enzymes. Experiments by Bose and Warren (2) with amN122-infected cells indicate that the continued synthesis of early enzymes, as represented by thymidylate synthetase, requires continued synthesis of mRNA, in that actinomycin D causes an immediate decline and eventual cessation of enzyme formation with a half-life of decay of enzyme-forming ability equal to 2 to 4 min.

Experiments reported by Warner and Hobbs (22), Mathews (13), and in this paper indicate that pulse-labeled RNA synthesis, presumably mRNA, in DO-negative mutants decreases markedly after about the first 8 min of infection even though at this time early enzyme synthesis is proceeding maximally.

Kasai and Bautz (9) have studied synthesis of rII mRNA by wild-type T4 and amN82 by a method which employs deletion mutants and DNA-RNA hybridization. In the wild-type phage, rII mRNA, expressed as per cent total RNA, increases until 8 to 10 min and then declines, whereas in the amN82-infected cells rII mRNA synthesis continues to increase towards an asymptote at about 20 min postinfection.

When, as described in this report, early enzyme synthesis by amN82 shuts off at about 20 min if the infected cells are incubated at 44 C, the pulse-labeled RNA synthesis appears to be 20 to 50% of similarly infected cells at 37 C, depending upon the conditions employed (Fig. 7, 8). After 20 min, there is still maximal early enzyme synthesis at 37 C. It appears that, as in the case of wild-type bacteriophage infection, early mRNA is made at 44 C and 20 min after infection but ceases to be translated. The sucrose gradient, sedimentation profile of pulse-labeled RNA at 44 C and 20 min postinfection reveals a slight decrease in material with higher sedimentation values compared to the pulse-labeled RNA at 37 C and 20 min postinfection (Fig. 10). In spite of this apparent small difference in distribution, these results rule out the possibility that, although measurable pulse-labeled RNA is made at 44 C, it is extensively fragmented and thus untranslatable. Similarly, pulse-chase experiments (Fig. 9) mitigate against the possibility that the pulse-labeled RNA made at 44 C is more rapidly degraded and thus less is available for translation.

It is possible that the difference in pulse-labeled RNA synthesis at 20 min postinfection is significant if most of the RNA made at 44 C and 20 min postinfection is not concerned with early enzyme synthesis. This apparent difference at 20 min postinfection then could involve complete cessation of the synthesis of mRNA coding for early enzymes. Whether this is so

cannot be ascertained at present, and although it is not deemed likely the possibility still remains that not all of the RNA made at 44 C and 20 min is early RNA. The patterns of pulse-labeled RNA at the two temperatures also differ in the first 10 min after infection. Comparison of the patterns of pulse-labeled RNA at 44 and 37 C shows that the peak pulse-labeling times differ (for *amN82* 4 to 5 min at 44 C and 6 to 7 min at 37 C), and there is a somewhat higher maximal rate of labeling at 44 C. The differences in pulse-labeled RNA synthesis occur within the first 10 min after infection. The loss of reversibility also occurs within that period at 44 C.

Examination of ribosomes and rRNA at 44 C shows no apparent change relative to quantity or sedimentation characteristics (Fig. 11A and B). No difference exists in response to poly U stimulation of phenylalanine incorporation in an in vitro system prepared from *amN82*-infected cells 20 min after infection at 37 or 44 C (Fig. 13). Experiments are now in progress to determine whether there is any difference in amino acid incorporation in vitro with natural mRNA, ribosomes, and S-100 fraction from cells infected at the two temperatures. Preliminary results indicate no major difference with these substituents in the various possible combinations, but final judgment on this matter requires more experiments.

The evidence accumulated thus far does not allow a clear judgment as to the locus of the temperature effect. The only clear difference found was in the pattern of pulse-labeled RNA, although other related experiments (pulse-chase, sedimentation profile) lessen the attractiveness of transcription as the site of action. On the other hand, in vitro experiments dealing with translational processes thus far show no difference. The effect of 5-methyltryptophan is intriguing but it is premature to say that this indicates involvement of a protein factor. In general, one might speculate that some sort of factor(s) is involved (this might be a particular mRNA or protein) and that this factor which is essential for continued early enzyme synthesis is more labile at 44 than at 37 C. Conversely, it may be argued that such a factor when formed terminates early enzyme synthesis and is formed more rapidly at 44 than 37 C. With regard to the former possibility, it might be reiterated that the inhibition at 44 C is reversible when it occurs in the uninfected *E. coli* but not in the infected cells. A hypothesis relating to a critical translational factor could account for this difference between uninfected and infected cells in that the higher temperature would cause the loss or accelerated loss of a factor which is

coded for only by the bacterial genome. Thus, the *E. coli* cells can resume growth upon return to a more favorable temperature as they can restore the level of the depleted factor, whereas in the infected cells, in which host-directed synthesis is abolished, this cannot occur. Although further speculation and schemes are possible, they should be deferred until more data are available.

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