*IN VIVO STABILITY OF PHAGE MESSENGER RIBONUCLEIC ACID**

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Infection of *Escherichia coli* with T-even bacteriophages leads to the synthesis of a variety of phage-related proteins.[1] Division of these proteins into at least two classes, early and late, is based on the temporal sequence in their synthesis. The early proteins include enzymes which are involved in the synthesis of some nucleotides and their polymerization into viral DNA. The structural proteins and lysozyme appear late in the development phase.

There is an apparent dependence of the formation of late proteins on the synthesis of viral DNA.[2, 3] Furthermore, the sequence of phage-induced protein synthesis can be interrupted by at least two procedures: (1) infection with UV-irradiated phage,[4, 5] and (2) infection of nonpermissive *E. coli* (su^-) with early amber mutants of phage.[2] The results of such infections are the continued formation of many early enzymes and the absence of synthesis of viral DNA and of late proteins. The mechanisms controlling the temporal sequence of synthesis of viral proteins and the level at which these mechanisms are operative in the infected bacteria are matters of considerable speculation.[2, 6, 7] Brief treatment with EDTA[8] alters the permeability properties, e.g., sensitivity to AM, of *E. coli* cells without impairing their ability to produce β-galactosidase[9] or T4 phage-induced enzymes[10, 11] and phage.[12] It is generally assumed that the primary consequence of AM is the inhibition of transcription which leads to the cessation of protein synthesis. We have utilized EDTA-treated *E. coli* cells to study the kinetics of decline in phage-induced enzyme-forming capacity after the addition of AM to bacteria infected with T4, $T4^{uv}$, and with *am*N122.

Materials and Methods.—Bacteria: *E. coli* R2, a variant of B, and CR63 were obtained from the laboratory of Dr. G. R. Greenberg of the University of Michigan, and W3350 from Dr. Charles Radding of Yale University. CR34 $thr^-leu^-B_1^-ura^-thy^-$ and 58-161 $met^-B_1^-thy^-$ were provided by Dr. Ronald Rolfe and Dr. Norman Melechen of this department. CR63 and CR34 are permissive (su^+) and W3350 and 58-161 nonpermissive (su^-) hosts for the suppressible amber mutants.

Bacteriophages: T4D and the amber mutant N122 (gene 42, hydroxymethylase defective) were supplied by Dr. R. S. Edgar of the California Institute of Technology. The wild-type and the mutant phage were propagated on R2 and CR63, respectively. Exponentially growing cultures (2×10^8 cells/ml) were infected at a multiplicity of 0.1 and aeration was continued at 37° for 4 hr. Sterile chloroform was added and the lysates were left at 22–23° overnight. Phage were purified by differential centrifugation, washed, and resuspended in PDF. Titers were checked on permissive as well as nonpermissive indicator bacteria by the agar-layer technique.[13]

Growth medium: The modified Fraser and Jerrel medium employed throughout this investigation has been described.[11]

UV irradiation: Phage were suspended in PDF at a concentration of 10^{11} plaque-forming units/ml and irradiated in thin layers with a 15-watt G.E. "Germicidal" lamp at a distance of 30 cm for 2 min. During irradiation the suspension was mixed with a magnetic stirrer. The titer dropped to *ca.* 10^6 PFU/ml.

Sensitization of bacteria to AM and infection: Exponentially growing cells were washed at room temperature with 0.12 *M* Tris-acetate buffer, pH 8.0, and treated with 1 mM EDTA according to Leive.[9] It was found that *E. coli* B and its derivatives were difficult to sensitize and rapidly be-

came refractory to AM. Therefore, the K12 strains CR34 (permissive), W3350 and 58-161 (non-permissive) were used routinely for all experiments with AM. Immediately after the termination of EDTA treatment by dilution with prewarmed growth medium, the cells were infected in the presence of L-tryptophan (50 μg/ml). With wild-type and amber mutants, the multiplicity of infection was 5–10; with $T4^{uv}$, 2 phage/bacterium were used to minimize multiplicity reactivation. The flasks were wrapped in aluminum foil to prevent photoinactivation of AM and also photoreactivation when $T4^{uv}$ was used. The temperature of incubation during infection in all experiments was 37°.

Pulse-labeling of RNA in infected bacteria: Synthesis of mRNA by the infected bacteria, as measured by the incorporation of uridine-5-H^3 into acid-insoluble product, was determined by the filter-paper disk technique.[11, 14] Within 1–2 min after the addition of AM, about 90–99% inhibition of uridine-5-H^3 incorporation was obtained.

Enzyme assays: Preparation of cell-free extracts and assay of dTMP synthetase activity were done as described earlier.[11] In some experiments dTMP synthetase was assayed by the H^3-release method.[15] Lyophilized *E. coli* cells were used in the assay of lysozyme.[16] The unit of lysozyme activity was defined as a decrease in A_{600} of 1 per minute.

Deoxyuridylic acid was obtained from California Corporation for Biochemical Research. Vitamin-free acid-hydrolyzed casein was purchased from Nutritional Biochemicals. All other chemicals were from Sigma Chemical Co. Actinomycin D was generously provided by Merck Sharpe & Dohme Research Laboratory. Uridine-5-H^3 (sp. act. 14.3 c/mM) was obtained from Nuclear-Chicago, Des Plaines, Ill. dUMP-5-H^3 (sp. act. 10.0 c/mmole) was purchased from Schwarz BioResearch.

Results.—Productive infection: We have previously shown that addition of AM immediately after infection with $T4^{uv}$ causes complete and irreversible inhibition of synthesis of early enzymes.[11] AM addition at later times allows protein synthesis for about five minutes, although at a slow rate, possibly reflecting the degradation of mRNA formed prior to AM addition.[17] Similar results have been obtained with TS synthesis after productive infection with wild-type T4 (Fig. 1). There is an immediate inhibition of the rate of enzyme synthesis after AM treatment. The effect of AM on lysozyme synthesis is also shown here. If AM is added 3 minutes after infection, no lysozyme activity appears in the ensuing 27 minutes. Addition of AM 12 minutes after infection results in the inhibition of

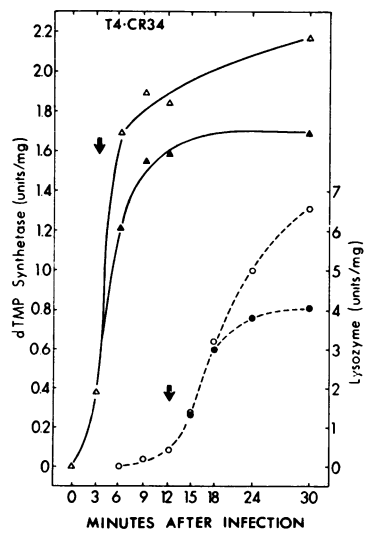

FIG. 1.—Kinetics of synthesis of phage-induced enzymes in T4-infected *E. coli*. Twelve hundred ml of sensitized *E. coli* CR34 cells were infected with T4D. At 3 min and 12 min after infection, 120-ml cells were placed in a flask containing 2.4 mg of AM. Incubation was continued in a water bath shaker at 37°. Aliquots were removed at indicated times and cellfree extracts prepared as described in ref. 11. *Open triangles:* TS, untreated control; *solid triangles:* TS, AM-treated; *open circles:* lysozyme, untreated control; *solid circles:* lysozyme, AM-treated. The arrows in the figures indicate time of addition of AM.

synthesis of lysozyme, confirming the observations of Protass and Korn[10] in a similar system.

Enzyme synthesis in CR34 infected with *am* N122 shows similar kinetics of inhibition with AM. Thus, AM added at the time of infection completely inhibits TS and lysozyme during the following 30-minute period (Fig. 2). It should be noted that in this system also no lysozyme was made when AM was added at the time of infection or four minutes later.

The rate of synthesis of early enzymes shows the characteristic arrest around 10–12 minutes after infection of CR34 with *am* N122 (Fig. 2). Lysozyme activity is detectable at this time, suggesting a possible relationship between the arrest of early enzyme synthesis and the initiation of late protein formation. AM, added at 12 minutes, causes a premature arrest of TS synthesis (1.20 units/mg protein instead of 1.38), showing that functional thymidylate synthetase mRNA is formed and *translated* into active protein even while late functions are similarly expressed. These results support the argument that there may not be a total arrest of synthesis of early enzymes in productive infections[18] provided all cells were synchronized with respect to infection and phage-induced functions. In wild-type T4-infected bacteria (Fig. 1) and in *am* N122-infected CR34 (Fig. 2), lysozyme synthesis levels off at 17–18 minutes if AM is added at 12 minutes. If viral DNA synthesis plays a role in the arrest of early enzyme synthesis and the initiation of late proteins, then concurrent formation of TS and lysozyme at 12 minutes (Figs. 1 and 2) suggests that synthesis of DNA proceeds to a critical level before control mechanism(s) becomes operative.

Unproductive infection: Earlier work has already shown that in the $T4^{uv}$ infection, mRNA for early enzymes (at least for dTMP synthetase) must be formed continuously.[11] The data in Figure 3 show the gradual cessation of TS synthesis after the addition of AM at ten minutes to $T4^{uv}$-infected CR34 cells. Sampling

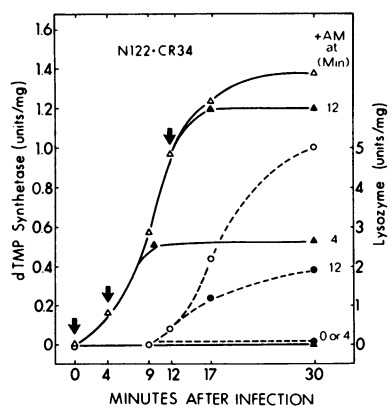

FIG. 2.—Kinetics of enzyme synthesis in T4 *am* N122-infected *E. coli* CR34. The infected cells were treated with AM at 0, 4, and 12 min after infection as described in the legend to Fig. 1. All other symbols are similar to those in Fig. 1.

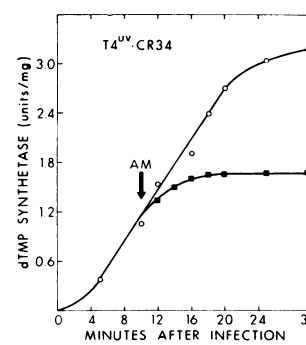

FIG. 3.—Kinetics of synthesis of phage-induced dTMP synthetase in *E. coli* CR34 infected with UV-irradiated T4. At 10 min after infection, 210 ml of infected cells were placed in a flask containing 4.2 mg of AM. Samples were removed as before for enzymatic assays. *Open circles:* TS, untreated; *solid squares:* TS, AM-treated.

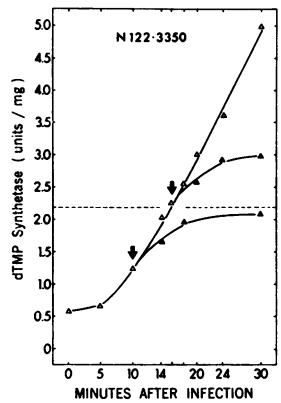

FIG. 4.—Kinetics of synthesis of phage-induced dTMP synthetase in T4 *am* N122-*E. coli* W3350. At 10 and 16 min after infection, 90-ml cells were placed in flasks containing 1.8 mg of AM and the incubation was continued. The horizontal line indicates the level at which dTMP synthetase is normally shut off when this cell strain is infected with T4, wild type.

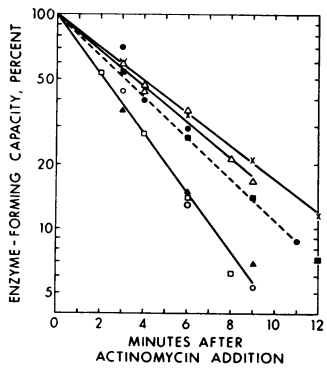

FIG. 5.—Exponential decay of enzyme-forming capacity of phage-infected *E. coli* after actinomycin treatment. The data from Figs. 1–4 and from additional experiments are replotted on semilog scale. Enzyme-forming capacity at time t = final yield of enzyme (at 30 min)–enzyme present at time t. One hundred percent represents the difference between enzyme at 30 min and that at the time of addition of AM. Thymidylate synthetase: *open circles*, T4-infected CR34; *open squares*, $T4^{uv}$-infected CR34; *solid triangles*, *am*N122-infected CR34; *open triangles*, N122-infected W3550; *solid squares*, T4-infected W3350; *crosses*, T4-infected 58-161. Lysozyme: *solid circles, stippled line*, T4-infected CR34.

at frequent intervals in this and other experiments reported here allows us to estimate the rate of decay of TS-forming capacity of the infected cells after AM treatment.

Infection of a nonpermissive host with *am* N122 leads to extended synthesis of several early enzymes.[2] Also, neither phage DNA nor late proteins are formed.[19] The kinetics of inhibition of synthesis of TS following the addition of AM to *am* N122-infected W3350 (Fig. 4) resembles that obtained with wild-type and irradiated phage infections. The data show that addition of AM at 10 minutes or at 16 minutes causes a gradual arrest of synthesis of TS, the final level of enzyme apparently reflecting the enzyme-forming capacity of the infected cells at the time of AM treatment.

The AM-induced decline in enzyme-forming capacity of phage-infected *E. coli* has been projected on the semilog plot in Figure 5. Such plots have been used to estimate the half life of decay of β-galactosidase-forming capacity of *E. coli* after removal of inducer[20] and also after treatment with AM.[21] The immediate inhibition of synthesis of TS and lysozyme by AM as well as the exponential nature of the decline in TS-and lysozyme-forming capacities of various phage-infected systems

is evident. Since the decline is exponential, we estimate that at 37° and under the conditions of the experiments the half life of the capacity to form TS is about two minutes in T4, $T4^{uv}$, and N122-infected *E. coli* CR34. In N122-infected W3350, however, the data from several experiments suggest that the longer half life of TS-forming capacity may be a characteristic of the nonsuppressible system. In view of this apparent discrepancy in the rates of decline of TS-forming capacities in phage-infected CR34 (su^+) and W3350 (su^-), we examined the kinetics of inhibition by AM in T4-infected W3350 and in another su^- strain, 58-161. The results depicted in Figure 5 indicate that AM-induced decline in phage-induced TS synthesis is slower in su^- hosts. Experiments are now in progress to study in detail the differential rates of enzyme synthesis in various su^+ and su^- hosts and the effect of protein synthesis on the rate of decline of enzyme-forming capacities in phage-infected bacteria after AM addition. The capacity to synthesize lysozyme decays with a half life of about 3.2 minutes, indicating the instability of lysozyme mRNA.

Discussion.—Our data show that in unproductive infection, such as infection with UV-irradiated T4, or infection of a nonpermissive host with an amber mutant, defective in an early function, the early mRNA not only continues to be synthesized throughout the infection period but is also functional at all times. Since we measured enzyme-forming capacity of T4-infected *E. coli* instead of complementarity of pulse-labeled RNA with T4 DNA, we can estimate the functional capacity of the transcribed material after its synthesis was halted with AM. There is good evidence for the assumption that AM inhibits RNA synthesis and not translation.[21] When AM is added to a culture the final level of enzyme activity attained probably reflects the amount of functional mRNA at the time of addition of the antibiotic (and the conversion of inactive precursor into active enzyme[22]). The capacity of phage-infected cells to synthesize TS and lysozyme decreases and finally stops after the addition of AM. The decay of the enzyme-forming capacity of AM-treated infected cells is exponential in the systems studied (Fig. 5). The half life of the decay of phage-induced TS-forming capacity is two to four minutes under the conditions of these experiments. This is especially clear in the systems where the formation of TS is protracted, i.e., infection with $T4^{uv}$ or of nonpermissive host with *am* N122. Unlike the usual induced enzyme synthesis, e.g., formation of β-galactosidase, where natural arrest of enzyme synthesis can be effected by the removal of inducer, formation of phage-induced enzymes cannot be arrested to provide an independent measure of the decay of enzyme-forming capacity. The rate of synthesis of most early enzymes, however, undergoes a drastic reduction with time in productive infections. Several studies[6, 23] have established that early mRNA continues to be synthesized even when the formation of early enzymes is greatly reduced, i.e., at late time. The data represented in Figure 5 show that the rate of decay of TS-forming capacity of T4-infected and N122-infected permissive bacteria after treatment with AM is identical to that seen in the unproductive infections with UV-irradiated phage. The formation of TS in the untreated infected bacteria is arrested around 9–12 minutes under our experimental conditions (Fig. 1). It would seem, therefore, that the gradual arrest of formation of TS by AM treatment in the productive systems should augment the natural arrest mechanism. The decay of TS-forming capacity by AM-treated su^+ host shown

in Figures 1 and 2 has a half life of about two minutes (Fig. 5) uncorrected for the natural arrest of TS formation. This suggests that either AM inhibits the implementation of the natural arrest mechanism and therefore the observed decay is unaffected by the autogenous control mechanism, or the TS-forming capacity in productive infection has a longer half life.

When protracted synthesis of early enzymes is obtained, one can measure the decay of enzyme-forming capacity due to inhibition of transcription by AM without the superimposition of the natural arrest mechanism. The kinetics of inhibition of TS synthesis in N122-infected·W3350 (Fig. 4) indicate that the TS-forming capacity declines with a half life of about 3.5 minutes after AM treatment at 10 and 16 minutes. Furthermore, the final level of TS when AM was added at 16 minutes is about 1.5 times greater than that with AM at 10 minutes. These results show mRNA coding for TS is synthesized continuously in the amber·nonpermissive system, confirming our previous data with $T4^{uv}$-infected cells.[11]

A half life of about three minutes for the mRNA specifying the T*2-induced early enzymes has been estimated.[24] In this system the nonglucosylated viral genome of the infecting phage underwent degradation in the nonpermissive host, *E. coli*, thus limiting the extent of transcription under unproductive conditions.

We can only speculate regarding the host-dependent alteration in the rate of decline of TS-forming capacity in T4-infected su^+ and su^- *E. coli*. There is a small but definite decrease in the rate of synthesis of TS in T4-infected W3350 and 58-161 relative to that in infected CR34. It is probable that the rate of degradation of TS-mRNA is also slower in the su^- hosts. This hypothesis is supported by the results of Hattman, Revel, and Luria,[24] who observed 5-methyltryptophan-induced protection of T2 mRNA. Chloramphenicol, another inhibitor of protein synthesis, is also reported to decrease the rate of decay of mRNA in *Bacillus subtilis*.[25]

The addition of AM at various times after infection inhibits the synthesis of TS within five to six minutes under our experimental conditions, viz., EDTA-treated cells at 37°. These results differ from those of Guthrie and Buchanan,[26] who observed that the synthesis of early enzymes by "protoplasts" of T4-infected *E. coli* was abruptly inhibited by AM only after 20 minutes. They postulated that (1) synthesis of early phage mRNA was not continuous, (2) the natural arrest of early enzyme formation resulted from a limited synthesis of mRNA, and (3) AM addition simulated the natural arrest mechanism in shutting off mRNA synthesis. In all of our experiments with sensitized *E. coli* cells, RNA synthesis stops within one to two minutes after the addition of AM,[11] and the capacity to synthesize enzymes declines immediately and *exponentially*. Inhibition of transcription may or may not be the arrest mechanism operative in productive infection.

Summary.—*E. coli*, sensitized to actinomycin D by EDTA treatment, was used to study the *in vivo* stability of T4 messenger RNA. The kinetics of formation of phage-induced thymidylate synthetase and lysozyme in the absence of mRNA synthesis was studied after infection of su^+ and su^- hosts with wild-type T4, UV-irradiated T4, and an early *amber* mutant N122. Addition of actinomycin to infected cells causes an immediate decline and, ultimately, cessation in the ability to produce enzymes. This decrease is exponential and the half life of decay in the ability to form thymidylate synthetase and lysozyme is two to four minutes at 37°. The extended synthesis of thymidylate synthetase in bacteria infected with UV-

irradiated T4 or in nonpermissive infection with *am* N122 requires continued synthesis of mRNA.

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[8] Abbreviations used: EDTA, ethylenediamine tetraacetic acid; AM, actinomycin D; TS, thymidylate synthetase; $T4^{uv}$, ultraviolet-irradiated T4; mRNA, messenger RNA; PDF, phage dilution fluid (gelatin, 0.001%; NaCl, 0.59%; $MgCl_2$, 1 mM and Tris-HCl buffer, pH 7.4, 10 mM).

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