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# In Vivo Stability of Bacteriophage T4 Messenger Ribonucleic Acid

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ABSTRACT

COHEN, PAUL S. (St. Jude Children's Research Hospital, Memphis, Tenn.), AND HERBERT L. ENNIS. In vivo stability of bacteriophage T4 messenger ribonucleic acid. J. Bacteriol. 92:1345–1350. 1966.—A mutant of *Escherichia coli* B, defective in its transport and concentration of $K^+$, synthesizes ribonucleic acid (RNA) without the simultaneous synthesis of protein when depleted of this cation. The mutant was used to study the in vivo stability of phage T4 messenger RNA (mRNA) in the presence and absence of $K^+$. Experiments were performed in which the turnover of phage T4 mRNA was determined in infected cells continuously synthesizing RNA and in cells in which RNA synthesis was inhibited by actinomycin D. Phage mRNA was found to be more stable in the absence of $K^+$ than in the presence of either the cation or chloramphenicol.

*Escherichia coli* B 207 is a mutant of *E. coli* B which is defective in its transport and concentration of $K^+$ from the growth medium (9). Mutant cells depleted of $K^+$ do not synthesize protein, but continue to synthesize ribonucleic acid (RNA) at about 50% the normal rate (4). This mutant was used to examine the role of $K^+$ in deoxyribonucleic acid (DNA), RNA, and protein synthesis in cells infected with phage T4 (3). The requirement of $K^+$ for DNA synthesis is independent of the necessity for the cation for protein synthesis. In contrast, RNA synthesis continues in the absence of $K^+$. The finding that RNA synthesis continues in the absence of protein synthesis in $K^+$-depleted infected cells is similar to the results obtained with the use of chloramphenicol (1, 11) or amino acid starvation (13) to inhibit protein synthesis.

T-even phage messenger RNA (mRNA) turns over rapidly in infected cells incubated in a rich medium containing $K^+$ (13, 17). The mRNA synthesized in the presence of chloramphenicol turns over less rapidly than that in uninhibited cultures (1, 13). In the present investigation, the kinetics of T4 mRNA synthesis and turnover during inhibition of protein synthesis by $K^+$ depletion have been studied. A preliminary report of some of these findings appeared previously (Cohen and Ennis, Bacteriol. Proc., p. 125, 1966).

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## MATERIALS AND METHODS

*Bacteria and bacteriophage.* *E. coli* B 207, a mutant of *E. coli* B which cannot concentrate $K^+$ normally from the growth medium (9), and wild-type bacteriophage T4 were used in the experiments reported.

*Media and growth conditions.* These have been described previously (3). In medium lacking $K^+$, the $K^+$ phosphates were replaced by an equimolar concentration of $Na^+$ phosphates.

*Infection of E. coli B 207.* Log-phase cells growing at 37 C were infected with phage T4 at a multiplicity of 5 in a $K^+$-free medium (3). $K^+$ was added 5 min later, and the cultures were incubated for an additional 10 min. Cells were then washed free from $K^+$ by use of membrane filters (3; Millipore Filter Corp., Bedford, Mass.), and $K^+$ (33 mM), $Na^+$ (33 mM), or chloramphenicol (100 μg/ml) and $K^+$ (33 mM) were added to the cultures, depending on the experiment.

*Chemicals.* Uracil-2-$C^{14}$ (30 mc/mmole) was purchased from New England Nuclear Corp., Boston, Mass. Chloramphenicol was a gift from Parke, Davis & Co., Detroit, Mich. Macaloid was a product of the Inerto Co., San Francisco, Calif., and was purified according to the method of Stanley (Ph.D. Thesis, Univ. Wisconsin, Madison, 1963).

*Measurement of incorporation of radioactivity into RNA and DNA.* These techniques have been described previously (3).

*Sensitization of cells to actinomycin D and measurement of decay of T4 mRNA.* The method is a variation of that used by Lieve (8). *E. coli* B 207 inoculated at a concentration of $1.5 \times 10^8$ cells per milliliter (15 ml) was grown in a 250-ml flask at 37 C with shaking to $5 \times 10^8$ cells per milliliter. The cells were then infected

with five phage particles per cell and incubated for an additional 10 min. The contents of each flask were filtered through a membrane filter (pore size, 0.45 $\mu$; diameter, 47 mm; Millipore Filter Corp.), and washed first with 15 ml of $K^+$-free medium and then with 15 ml of 0.12 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (*p*H 8). The cells on the filter were resuspended in 5 ml of Tris-HCl buffer (*p*H 8). Ethylenediaminetetraacetate (EDTA) was added ($4 \times 10^{-4}$ M), and the cells were shaken at 37 C for 3 min.

Samples (1.2 ml) of the EDTA-treated cell suspension were added to 12 ml of growth medium lacking $K^+$. $C^{14}$-uracil was added (0.2 $\mu$c/ml, 0.75 $\mu$g/ml), and the cell suspension was shaken for 5 min at 37 C. Then nonradioactive uracil (1 mg/ml), actinomycin D (115 $\mu$g/ml), and $K^+$ (33 mM) or $Na^+$ (33 mM) or chloramphenicol (100 $\mu$g/ml) and $K^+$, according to the experiment, were added, and samples were taken at intervals for determination of radioactivity in RNA.

*Extraction of RNA and sucrose density gradient analysis.* Infected cells were collected by centrifugation at 3,200 $\times$ *g* for 10 min in a Servall RC2 centrifuge at 4 C and resuspended in $10^{-2}$ M sodium acetate buffer (*p*H 5.1). Macaloid was added to a final concentration of 0.1% to inhibit ribonuclease activity, and the cells were broken in a French pressure cell at 8,000 psi. Macaloid was again added to a concentration of 0.1%, and the RNA was extracted by the hot-phenol method (12). The RNA was dissolved (approximately 1 mg/ml) in $10^{-2}$ M acetate buffer (*p*H 5.1) containing $5 \times 10^{-2}$ M NaCl and $10^{-4}$ M magnesium acetate. A 1-ml amount was layered on a 25-ml 5 to 20% (w/v) linear sucrose density gradient containing the same buffer, and centrifuged in the SW-25.1 rotor of a Spinco model L-2 centrifuge at 25,000 rev/min for 18 hr at 3 C. Samples were collected and processed as previously described (5).

## RESULTS

*Type of RNA synthesized in the absence of $K^+$.* Shortly after infection of *E. coli* B with T-even phages, host RNA synthesis stops and only phage mRNA is made (2, 6, 10, 17). To study the stability of phage mRNA in the absence of $K^+$, it is important to show that phage mRNA is also synthesized in the absence of $K^+$ under the experimental conditions employed. Cells treated with chloramphenicol 5 min after infection only synthesize phage mRNA (11). A similar experiment was therefore performed with *E. coli* B 207 infected with T4. One culture (A) was infected in the absence of $K^+$ for 5 min and was then allowed to incorporate $C^{14}$-uracil for 20 min in the absence of $K^+$. The cells were collected by centrifugation, and RNA was extracted and subjected to sucrose density gradient analysis. A second culture (B) was incubated in the presence of $K^+$ for 10 min after infection in the absence of $K^+$. The cells were filtered and washed free from $K^+$, and were resuspended in $K^+$-free medium. $Na^+$ (33 mM) was added to the culture, which was then allowed to incorporate $C^{14}$-uracil for 20 min. The cells were collected by centrifugation, and RNA was extracted and analyzed by sucrose density gradient centrifugation. The results of this experiment are illustrated in Fig. 1.

When cells were infected in the absence of $K^+$ and pulsed with $C^{14}$-uracil for 20 min in the absence of $K^+$, a considerable amount of the RNA synthesized sedimented with 23*S* and 16*S* ribosomal RNA (Fig. 1A). (Since actual corrected sedimentation velocities were not obtained, *S* values were used only as a convenient label for the main classes of RNA as described by others.) However, if similarly infected cells were incubated for 10 min in a $K^+$-containing medium, before pulsing with $C^{14}$-uracil for 20 min in a $K^+$-free medium, essentially all the RNA synthesized subsequently in the absence of $K^+$ sedimented between 16*S* and 4*S*, which corresponds to the mRNA region (Fig. 1B). These results showed that, under the experimental conditions outlined in the legend to Fig. 1B, host ribosomal RNA synthesis stopped and mRNA synthesis continued.

It has been shown that all *E. coli* RNA synthesis stops almost immediately after infection with T4 (11). Since *E. coli* B 207 ribosomal RNA synthesis stopped during the 10-min incubation of infected cells in $K^+$-containing medium (Fig. 1A and B), it is reasonable to assume that all host mRNA synthesis stopped. The mRNA synthesized in the absence of $K^+$ must therefore be T4 mRNA (Fig. 1B).

*RNA synthesis in the absence of $K^+$.* T-even phage mRNA turns over less rapidly in the presence of chloramphenicol than in its absence (1, 11, 13). To test whether $K^+$ depletion, which also inhibits protein synthesis, results in a similar effect, the following experiment was performed. Infected cells were incubated in the presence of $K^+$ for 10 min, at which time host RNA synthesis had stopped (*see* Fig. 1). The cells were filtered, washed free from $K^+$, resuspended in fresh $K^+$-free medium, and divided into three equal portions. $K^+$ (33 mM) was added to the first culture, $K^+$ (33 mM) and chloramphenicol (100 $\mu$g/ml) were added to the second culture, and $Na^+$ (33 mM) was added to the third culture. $C^{14}$-uracil was added to each of the cultures, and the kinetics of incorporation of the isotope into RNA were followed.

Figure 2 shows that the incorporation of $C^{14}$-uracil into RNA in the uninhibited culture and the chloramphenicol-inhibited culture was the same as that previously observed (1). During normal infection, $C^{14}$-uracil was incorporated into RNA linearly for about 10 to 20 min, and then the rate of incorporation markedly de-

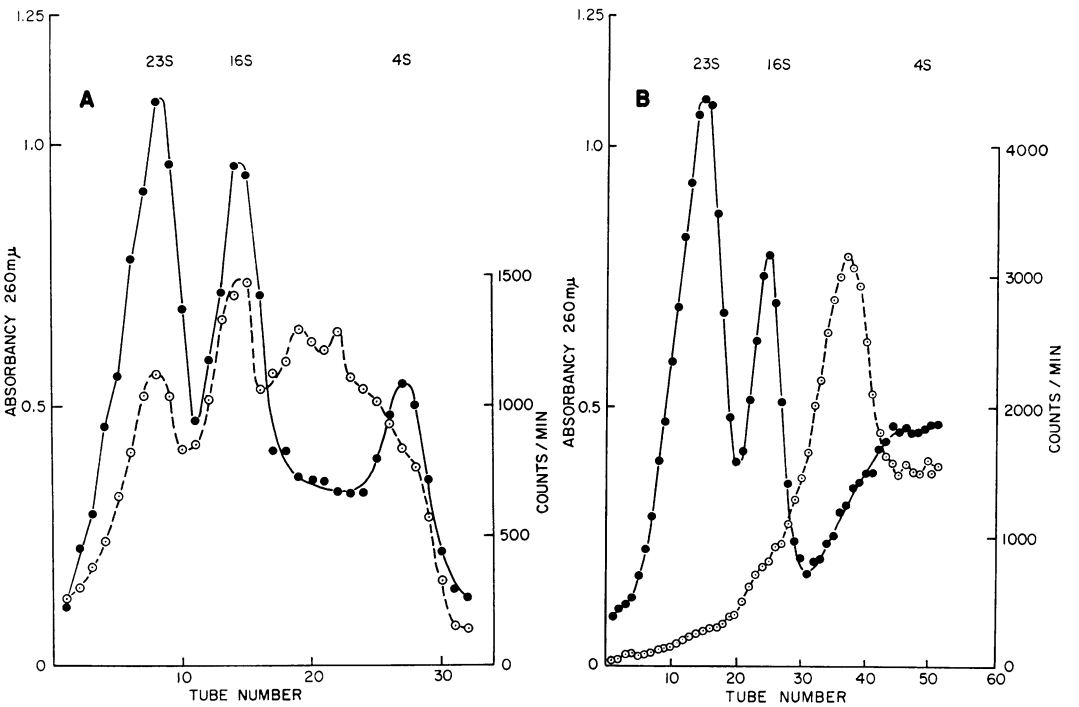


FIG. 1. *Sedimentation in sucrose density gradients of RNA synthesized in the absence of $K^+$. (A) A suspension of Escherichia coli B 207 was infected with phage T4 in the absence of $K^+$ at a multiplicity of 5. After 5 min of adsorption, $C^{14}$-uracil (0.5 μc/ml, 1.87 μg/ml) was added, and the cells were incubated for an additional 20 min. The cells were collected by centrifugation, mixed with whole unlabeled cells to provide an optical density marker, and the RNA was extracted. The purified RNA was subjected to sucrose density gradient analysis. Symbols: ●, absorbancy at 260 mμ; ○, counts per minute. (B) A suspension of cells was infected as in (A) but was incubated in a $K^+$-containing medium (33 mM) for 10 min after the 5-min adsorption period in the absence of $K^+$. The cells were then filtered, washed, and resuspended in $K^+$-free medium. $C^{14}$-uracil (0.5 μc/ml, 1.87 μg/ml) was added, and the culture was incubated for 20 min. Further manipulations were carried out as in (A). Symbols same as in (A).*

creased. In the presence of chloramphenicol, RNA synthesis continued linearly throughout the duration of the experiment, and the final total amount of radioactivity incorporated was about three times that of the control value. RNA synthesis in T4-infected cells incubated in the absence of $K^+$ was similar to that observed in the chloramphenicol-inhibited culture, but proceeded at a slower rate.

*Turnover of RNA in the absence of $K^+$.* T-even phage mRNA turns over rapidly in normally infected cells incubated in a rich medium, but turnover is reduced in the presence of chloramphenicol (1, 13, 17). The finding illustrated in Fig. 2 is compatible with the idea that $K^+$ depletion of infected cells also results in increased stabilization of T4 mRNA. To test this possibility further, cells were infected for 10 min in growth medium (containing $K^+$). Host RNA synthesis stopped under these conditions. The cells were then filtered and washed free from $K^+$, and resuspended in $K^+$-free medium. The culture was then split into three equal portions, and each was pulsed for 5 min with $C^{14}$-uracil. At 5 min, nonradioactive uracil (500 μg/ml) and $K^+$ (33 mM) were added to one culture; nonradioactive uracil (500 μg/ml), $K^+$ (33 mM), and chloramphenicol (100 μg/ml) were added to a second culture; and nonradioactive uracil (500 μg/ml) and $Na^+$ (33 mM) were added to the third culture. Samples of all three cultures were taken at intervals, and the amount of radioactivity in RNA was determined. Figure 3 shows the results of this "pulse-chase" type of experiment. As shown previously (1, 13, 17), T4 mRNA turns over rapidly in normally infected cells, losing approximately 50% of its trichloroacetic acid-precipitable counts in 30 min. In agreement with previous work (1, 13), chloramphenicol inhibited turnover of T4 mRNA,

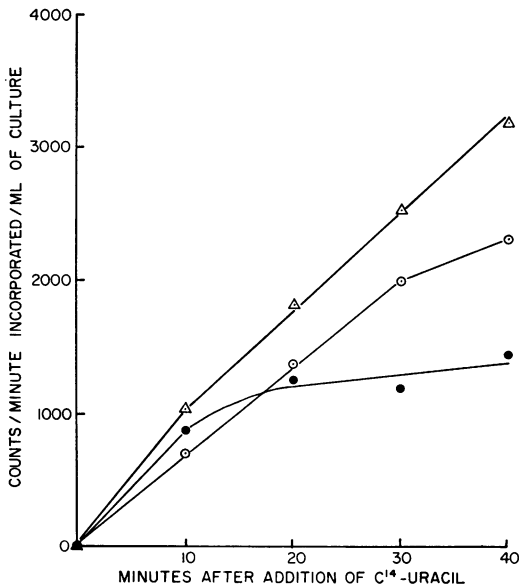


FIG. 2. *RNA synthesis in the absence of $K^+$ and presence of chloramphenicol. A suspension of Escherichia coli B 207 was infected with phage T4 in the absence of $K^+$ at a multiplicity of 5. After 5 min of adsorption, $K^+$ (33 mM) was added, and the cells were incubated for an additional 10 min. The cells were filtered, washed free from $K^+$, resuspended in $K^+$-free medium, and divided into three equal portions. $K^+$ (33 mM) was added to the first culture (●); $K^+$ (33 mM) and chloramphenicol (100 μg/ml) were added to the second culture (△); and $Na^+$ (33 mM) was added to the third culture (○). After 5 min of incubation at 37 C, $C^{14}$-uracil (0.25 μc/ml, 0.94 μg/ml) was added to each culture, and the incorporation of radioactivity into RNA was determined at the indicated times, as described in Materials and Methods.*

which lost only 10% of its trichloroacetic acid-precipitable counts in 30 min. In contrast, instead of a decay of mRNA, there was an increase in the number of counts incorporated into T4 mRNA in the culture which was incubated in the absence of $K^+$. These data suggested that T4 mRNA was most stable in infected cells incubated in the absence of $K^+$.

Turnover of phage mRNA in the presence of $K^+$ was also studied in infected cells in which DNA synthesis was inhibited by more than 90% by fluorodeoxyuridine (10 μg/ml). Turnover of RNA was unaffected during inhibition of DNA synthesis.

*Decay of RNA in the absence of RNA synthesis.* During a pulse-chase experiment such as has been described in the preceding section, RNA synthesis can obscure the true rate of RNA turnover. To overcome this difficulty, *E. coli* B 207 was made permeable to actinomycin D by treatment with EDTA. This antibiotic was used to inhibit RNA synthesis during a subsequent chase with nonradioactive uracil.

A relatively high concentration of actinomycin D (115 μg/ml) was required to inhibit RNA synthesis by greater than 95% in infected cells. This concentration of actinomycin inhibited RNA synthesis by greater than 95% in either the absence or presence of $K^+$, and had little effect on DNA synthesis during normal infection. Higher concentrations of actinomycin caused lysis of some of the cells.

The level of actinomycin D necessary to achieve maximal inhibition of RNA synthesis was much higher than the amount reported by Lieve (8). Perhaps there is a strain difference in sensitivity to actinomycin D.

In the pulse-chase experiment in which actinomycin D was used (Fig. 4), 75% of the T4 mRNA decayed in 6 min after the addition of actinomycin D to cells incubated in growth medium con-

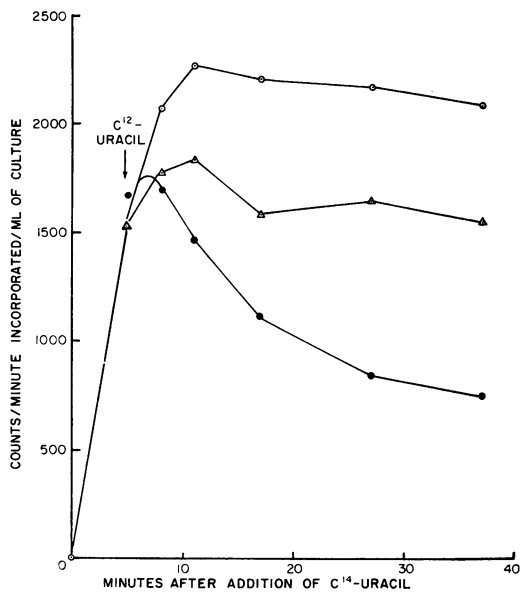


FIG. 3. *T4 mRNA turnover. A suspension of Escherichia coli B 207 was infected with phage T4 at a multiplicity of 5, washed, and resuspended in three equal portions as described in the legend to Fig. 2. $C^{14}$-uracil (0.25 μc/ml, 0.94 μg/ml) was added, and each culture was incubated for 5 min. The following additions were made to each of the three cultures: (●), nonradioactive uracil (500 μg/ml) and $K^+$ (33 mM); (△), nonradioactive uracil (500 μg/ml), chloramphenicol (100 μg/ml), and $K^+$ (33 mM); and (○), nonradioactive uracil (500 μg/ml) and $Na^+$ (33 mM). Samples were taken at the indicated times, and the radioactivity in the RNA was determined as described in Materials and Methods.*

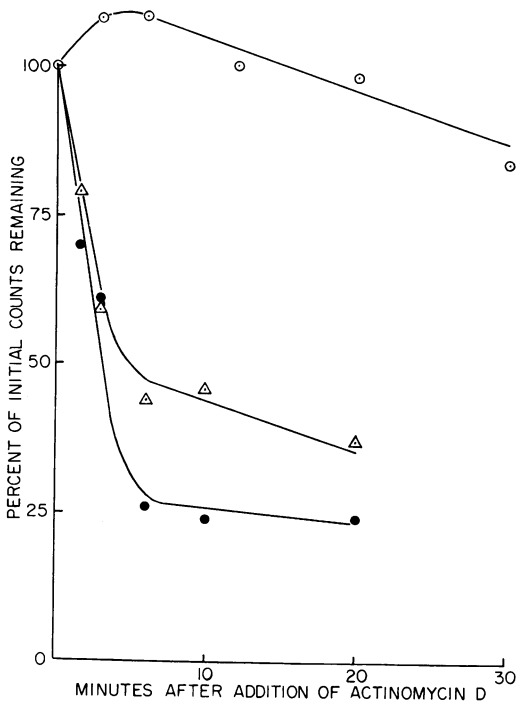


FIG. 4. *T4 mRNA decay in the presence of actinomycin D. A suspension of Escherichia coli B 207 was infected with phage T4 at a multiplicity of 5, made permeable to actinomycin D (see Materials and Methods), and resuspended in $K^+$-free medium. Three equal portions were pulsed with $C^{14}$-uracil (0.20 μc/ml, 0.75 μg/ml) for 5 min, after which the following additions were made: (●), nonradioactive uracil (1 mg/ml), actinomycin D (115 μg/ml), and $K^+$ (33 mM); (△), nonradioactive uracil (1 mg/ml), actinomycin D (115 μg/ml), chloramphenicol (100 μg/ml), and $K^+$ (33 mM); and (○), nonradioactive uracil (1 mg/ml), actinomycin D (115 μg/ml), and $Na^+$ (33 mM). Samples were taken at the indicated times, and the radioactivity in the RNA was determined as described in Materials and Methods.*

taining $K^+$, whereas, in the same period, only 55% of the RNA decayed in the chloroamphenicol-inhibited culture. In contrast, T4 mRNA decayed in cells incubated in the absence of $K^+$ at a rate of less than 10% of that observed in either of the other two cultures.

In another experiment, infected cells were incubated in the absence of $K^+$ for 15 min after the pulse and actinomycin addition. $K^+$ (33 mM) was then added back to one culture and $Na^+$ (33 mM) to another. RNA decayed rapidly in the culture to which $K^+$ was added, whereas the decay in the absence of $K^+$ continued at its initial slow rate. These results showed that T4 mRNA made in vivo in the absence of $K^+$ was susceptible to decay even upon delayed addition of $K^+$, and that the RNA did not become stable during prolonged incubation in the absence of $K^+$.

It might be argued that the T4 mRNA synthesized during the 5-min pulse in the absence of $K^+$ was in some way different in its stability than T4 RNA synthesized during a 5-min pulse in the presence of $K^+$ or chloramphenicol (and $K^+$). This possibility was ruled out by pulsing EDTA-treated cells with $C^{14}$-uracil in the presence of either $K^+$ or chloramphenicol (and $K^+$), and then adding actinomycin to each culture. The decay of RNA was the same as shown in Fig. 4 for the respective conditions employed at the time of actinomycin D addition.

## DISCUSSION

T-even mRNA synthesized in the presence of chloramphenicol is more stable than that synthesized in the absence of this antibiotic (1, 13). The present investigation has shown that phage T4 mRNA is also more stable in the absence of $K^+$ than in the presence of the cation. Moreover, RNA is more stable in the absence of $K^+$ than in the presence of chloramphenicol. The evidence for these conclusions can be summarized as follows.

(i) During normal infection in $K^+$-containing medium, $C^{14}$-uracil is incorporated into T4 mRNA linearly for about 10 to 20 min and then virtually stops. In the presence of chloramphenicol (and $K^+$) or in the absence of $K^+$ (no chloramphenicol), the radioactive precursor is incorporated linearly into RNA for the duration of the experiments (at least 40 min), and the final total amount of radioactivity incorporated is two to three times that observed during normal infection.

(ii) $C^{14}$-uracil incorporated into T4 mRNA can be chased out of RNA in a normal infection in $K^+$-containing medium by the addition of excess nonradioactive uracil to the culture (1, 13, 17). Of the initial trichloroacetic acid-precipitable counts in RNA, 50% is lost in 30 min during incubation of phage-infected cells in $K^+$-containing medium. In contrast, only 10% of the radioactivity is lost in the chloramphenicol-containing culture, and none is lost in the culture chased in medium lacking $K^+$.

(iii) In experiments in which actinomycin is used to stop RNA synthesis, the results observed in (ii) are magnified. Of the T4 mRNA, 75% decays in 6 min after addition of actinomycin D to infected cells incubated in growth medium containing $K^+$, whereas only 55% of the RNA decays in the chloramphenicol-inhibited culture. In contrast, the mRNA is far more stable in the

absence of $K^+$, and decays at a rate less than 10% of that observed under either of the other two conditions.

The reason for the stability of T4 mRNA in the absence of $K^+$ is open to speculation. T4 messenger instability may depend on a $K^+$-activated nuclease such as the $K^+$-activated phosphodiesterase, which has been reported (14). Alternatively, the mechanism by which protein synthesis is inhibited could govern the relative stability of T4 messenger. Although the mechanism of action of chloramphenicol is still not completely understood, it is different from inhibition of protein synthesis by $K^+$ depletion (7, 15, 16). Finally, it might be argued that T4 mRNA decay requires energy, and that this is not available during $K^+$ depletion. $K^+$-depleted infected cells respire at 60 to 75% the rate of normally infected cells, whereas chloramphenicol-inhibited infected cells respire at the normal rate (*unpublished data*). The possible requirement for energy for mRNA decay can therefore not be ruled out by the present investigation, but seems unlikely since other energy-requiring reactions can proceed in the $K^+$-depleted cells.

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