*EFFECT OF BACTERIOPHAGE INFECTION ON THE SULFUR-LABELING OF SRNA**

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Several examples of viral-induced modifications of cellular ribonucleic acids have been reported. After infection of *Escherichia coli* with T-even bacteriophage, Sueoka and Kano-Sueoka[1, 2] found quantitative as well as qualitative changes in leucyl-tRNA's when compared to uninfected cells by chromatography on methylated albumin-kieselguhr (MAK). More recently, Waters and Novelli,[3] using reverse phase chromatography, were able to confirm the early changes in leucyl-tRNA after T2 infection of *E. coli* and, in addition, observed two new leucyl-tRNA peaks which appeared very late after infection. Wainfan, Srinivasan, and Borek[4] have reported on the alteration in the relative activities of the base-specific methylases after T2 infection.

The presence of thiolated bases in sRNA and the demonstration that cell-free extracts catalyze the transfer of cysteine-sulfur to sRNA,[5, 6] prompted an inquiry as to whether any changes occurred in sulfur-containing RNA's after viral infection. The present communication describes changes in the MAK chromatographic profiles of S^{35}-labeled sRNA after *E. coli* infection with T-even bacteriophages. Evidence is offered which indicates that these changes are viral-induced, and that they require protein synthesis to be manifest.

Experimental.—Growth of cells and viral infection: *E. coli* B cells were grown in a medium that contained the following constituents per liter: 2 gm of NH_4Cl, 6 gm of NaCl, 0.01 gm of $MgCl_2$, 6 gm of Na_2HPO_4, 3 gm of KH_2PO_4, 0.026 gm of Na_2SO_4, 2 gm of glucose, and 10 mM Tris·HCl of pH 7.5. This medium was further supplemented with 0.04 vol of 3XD medium.[7] The cell suspension (2% inoculum of an overnight culture) was vigorously shaken in a gyratory apparatus at 37° (generation time approximately 50 min) until a density of 7×10^8 cells/ml was reached, at which time L-tryptophan was added to a concentration of 100 μg/ml, and then T4 phage at a ratio of 13 plaque-forming units per cell. Under these conditions, cell death was found to be greater than 99% after 2.5 min, and the number of infective centers was 95% or more of the bacterial cell count. The infected cell suspension was shaken at 37°, and after a given time infection was stopped by the rapid addition of 0.5 *M* NaN_3 (2 ml per 100 ml of culture) and crushed ice, and rapid cooling in an ice-salt water bath to 4°. The cells were collected by centrifugation and either handled immediately for RNA extraction or stored frozen at −20°. Infection with the coliphages T2, T7, MS2, and $\phi X174\rho^-$ was carried out in a similar manner except that the infecting ratio of phage/cell was 10, and *E. coli* K12W1485 and *E. coli* C were used as the respective hosts for MS2 and $\phi X174\rho^-$ phages.

S^{35}-labeling of cells: *E. coli* cells (either infected or uninfected) were labeled with S^{35} by the addition of radioactive Na_2SO_4 (New England Nuclear Corp.) to the culture medium. Cells were collected as described above except that nonradioactive 0.5 *M* Na_2SO_4 (2 ml per 100 ml of culture) was added to the suspension at the same time as the azide. "Prelabeled-chased" cells were grown in a 300-ml culture which contained 20 mc of $Na_2S^{35}O_4$. At a density of 5×10^8 cells/ml, the cells were centrifuged, washed with cold medium several times, and resuspended in 300 ml of fresh medium containing nonradioactive Na_2SO_4. The cells were grown to 1×10^9/ml and then divided into two portions; one served as the uninfected control and the other was infected with T4 phage.

Extraction of RNA: The pelleted cells were suspended in 2 ml of 10 mM $MgCl_2$–1 mM Tris·HCl, pH 7.5 (sodium dodecyl sulfate (SDS) was sometimes included), and extracted three times with an equal volume of phenol.[8] The nucleic acid was precipitated from the aqueous phase by the

addition of 0.10 vol of potassium acetate, pH 5.4, and 2 vol of ethanol. Subsequently, the RNA preparation was treated with DNase (RNase-free, Worthington Biochemical Corp.), then with phenol, and then deacylated by incubation for 45 min at 37° in 1 *M* Tris·HCl, pH 9. The RNA was precipitated and redissolved in the appropriate salt solution for MAK loading.

Chromatography: MAK chromatography was carried out essentially as described by Sueoka and Yamane.[9] In most experiments, 2–3 mg of nucleic acid was added to a 30-ml MAK column (approximately 3 cm in height) in 0.3 *M* NaCl–0.05 *M* Na_2HPO_4, pH 6.7, at a concentration of 20 μg per ml, washed with 300 ml of the same solution, and then eluted with a 240-ml linear salt gradient from 0.3 to 1.2 *M* NaCl containing the same phosphate buffer concentration as above. Prior to column loading and when necessary, the labeled sample was adjusted to contain approximately 2 mg of total nucleic acid with commercial *E. coli* B sRNA. The column was run at room temperature, and 2-ml fractions were collected. The fractions were examined for 260-mμ absorbing material and radioactivity by liquid scintillation counting. The samples were counted either directly, or by acid-precipitation and collection on Millipore disks (0.45-μ pore size); similar radioactivity profiles were obtained by both methods. In several experiments, the MAK fractions were treated with ribonuclease (10 μg/ml of pancreatic RNase and 1 μg/ml of T1 RNase) prior to radioactivity determinations. In these instances, acid-precipitable radioactivity was measured. Recovery of the S^{35}-RNA applied to MAK columns ranged between 80 and 95%.

Thiol-transferase activity measurements: Cells were grown as described above. Infected cells were harvested 20 min after T4 infection; 105,000 × *g* supernatants were prepared from infected and uninfected *E. coli* B extracts as previously described except that cell washing was omitted.[5] The soluble extracts were dialyzed for several hours against 0.01 *M* 2-mercaptoethanol–0.01 *M* Tris·HCl, pH 7.5, and the protein content was determined by the method of Lowry.[10] Thiol-transferase activity was measured by determining the amount of radioactivity incorporated into the RNA fraction from S^{35}-cysteine catalyzed by the above extract. Assay conditions were as previously reported.[5]

Results.—When *E. coli* is grown on a medium containing radioactive sulfate for several generations, the cellular RNA becomes readily labeled.[11–13] This radio-

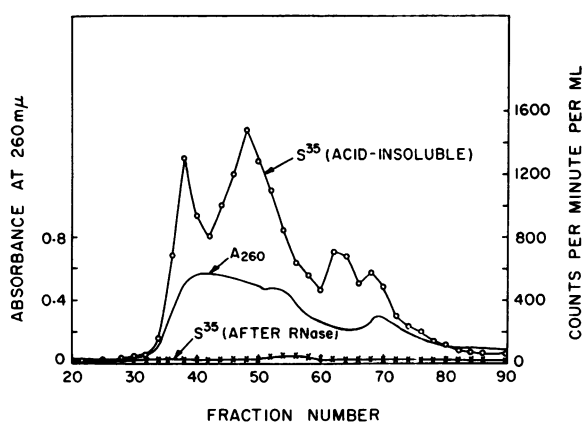

FIG. 1.—Chromatography of *E. coli* S^{35}-RNA on MAK. *E. coli* B was grown in 100 ml of medium containing 50 mc of $Na_2S^{35}O_4$, and labeled RNA was prepared from these cells as described under *Experimental*. Thirty-six μg of S^{35}-RNA (80,000 cpm) was mixed with 2 mg of carrier *E. coli* B sRNA and chromatographed on MAK.

activity is associated only with the low-molecular-weight RNA species. When RNA extracts are prepared from sulfur-labeled *E. coli* cells and subjected to chromatography on MAK, several radioactive peaks are usually observed in the region where sRNA is eluted (Fig. 1). The S^{35}-labeled material is acid-insoluble but is

readily converted to an acid-soluble form by treatment with RNase, but not with DNase.

RNA extracts prepared from T4-infected and uninfected cells, pulse-labeled for 17.5 minutes with S^{35}-Na_2SO_4, give similar radioactive profiles after centrifugation in a sucrose gradient (Fig. 2). In both cases, all of the label is found with the slowly sedimenting RNA species.[§] When these same RNA extracts are subjected to chromatography on MAK, a striking difference is observed in the radioactive elution pattern (Fig. 3). The RNA extract from uninfected cells shows two predominant radioactive peaks in the early part of the sRNA elution profile and a minor third radioactive region following, whereas the T4-infected profile appears to be reversed, the first and second S^{35}-peaks are sequestered, and the third peak is now predominant. As is the case for the labeled material from uninfected cells, the T4 S^{35}-labeled fractions are nondialyzable, acid- and alcohol-precipitable, and rendered soluble to these reagents by treatment with RNase.

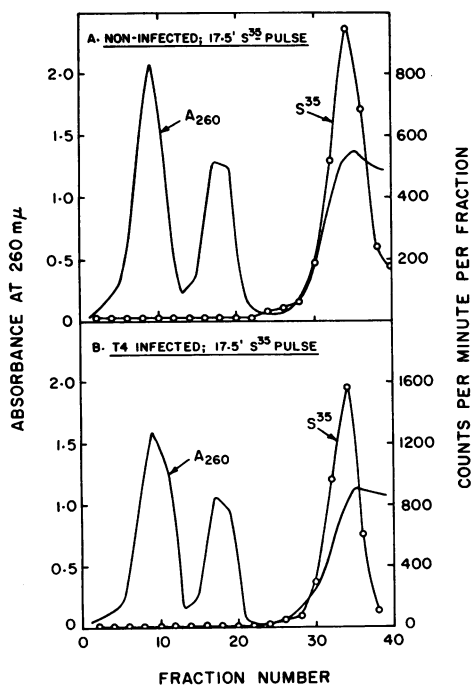

FIG. 2

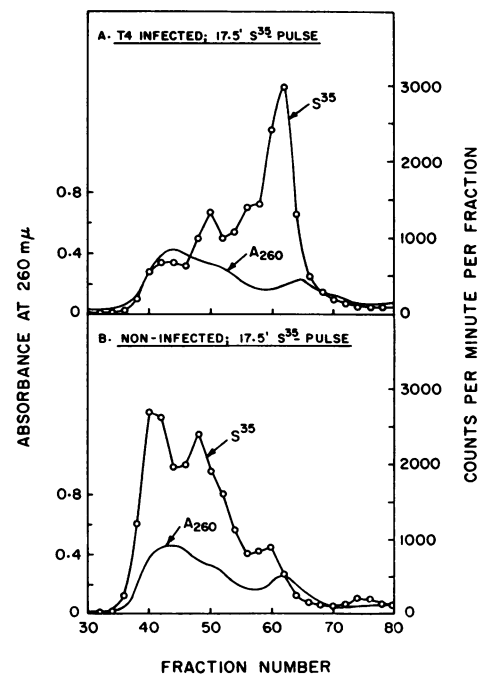

FIG. 3

FIG. 2.—Sucrose-gradient centrifugation of S^{35}-RNA from noninfected and T4-infected *E. coli*. *E. coli* B (400-ml culture) was pulsed for 17.5 min with 15 mc of $Na_2S^{35}O_4$. A second 400-ml culture was infected with T4 phage; 15 mc of $Na_2S^{35}O_4$ was added 2.5 min later and incubation proceeded for 17.5 min more. RNA was extracted from the collected cells as described under *Experimental*, (1% SDS was included in the phenol extraction procedure). Uninfected (840 μg) and infected (670 μg) S^{35}-RNA were subjected to sucrose-gradient centrifugation at 24,000 rpm for 19 hr at 4° in a Spinco SW-25 rotor, and fractions were collected from the bottom of the tube. The sucrose gradient was from 3 to 20% in 0.1 *M* NaCl–0.01 *M* Tris·HCl, pH 7.5, and the radioactivity shown represents acid-precipitable counts.

FIG. 3.—Comparison of MAK S^{35}-RNA profiles from T4-infected and uninfected pulse-labeled cells. Pulse-labeled S^{35}-RNA from T4 infected (*A*) and uninfected (*B*) *E. coli* B was prepared as described for Fig. 2. The extracted RNA's were subjected to MAK chromatography as described under *Experimental*.

Experiments were carried out to test the dependence of the observed chromatographic changes of S^{35}-RNA on viral infection. Pulse-labeling of T4 infected *E. coli* revealed that the first observable changes in the labeled RNA profiles occurred between 4.5 and 7.5 minutes after infection (Fig. 4). As compared with nonin-

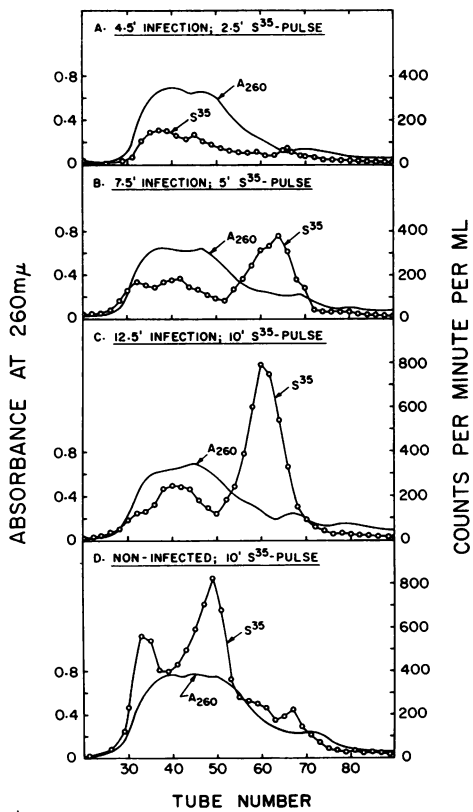

FIG. 4.—MAK profiles of S^{35}-RNA extracted from *E. coli* cells exposed to T4 infection for various periods. *E. coli* B was infected with T4 phage; $Na_2S^{35}O_4$ was then added several minutes later (5 mc/100 ml of culture) and infection stopped at various times after the isotope addition. The RNA was extracted from the infected cells and subjected to chromatography on MAK. (*A*) Isotope added 2.0 min after infection; infection stopped after 4.5 min. (*B*) Isotope added 2.5 min after infection; infection stopped after 7.5 min. (*C*) Isotope added 2.5 min after infection; infection stopped after 12.5 min. (*D*) *E. coli* B cells pulsed for 10 min with isotope (no infection). The amount of S^{35}-RNA used for chromatography was 1.8, 0.8, 0.4, and 0.48 mg for *A*, *B*, *C*, and *D*, respectively. Carrier *E. coli* sRNA was added to each of these prior to loading onto MAK, so that a total of 2 mg of sRNA was chromatographed.

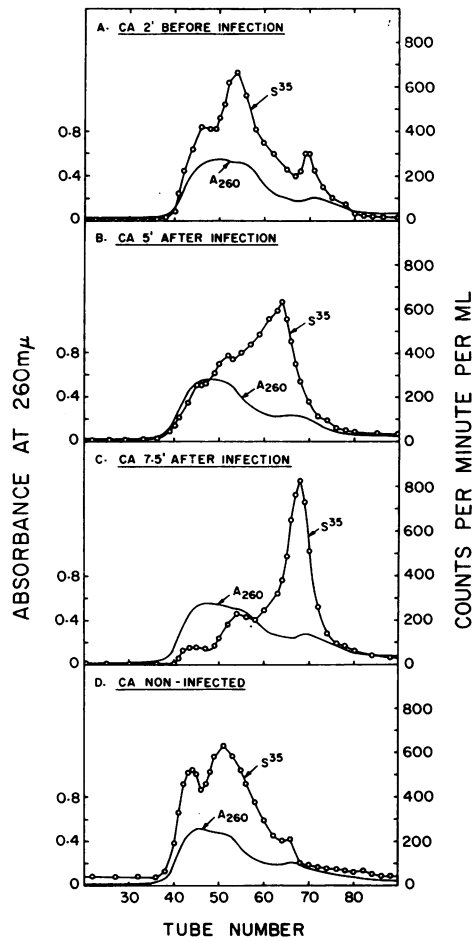

FIG. 5.—Effect of chloramphenicol on S^{35}-labeling of RNA in T4-infected cells. One hundred-ml cultures were employed for *A*, *B*, and *C*, and a 200-ml culture for *D*. In each case, 10 mc of $Na_2S^{35}O_4$ was added to the cultures and chloramphenicol (CA) was present at a concentration of 50 μg/ml. At the end of the incubation, cells were collected, RNA was extracted, and subjected to chromatography on MAK. (*A*) CA added 2 min before T4, and $Na_2S^{35}O_4$ added 2.5 min after infection. (*B*) CA added 5 min after T4, and $Na_2S^{35}O_4$ added 30 sec later. (*C*) CA added 7.5 min after T4, and $Na_2S^{35}O_4$ added 30 sec later. Infection was stopped 20 min after T4 addition in each of the above runs. (*D*) $Na_2S^{35}O_4$ added 2 min after CA, and cells collected after 17.5 min.

fected cells, infected *E. coli* showed a diminished rate of S^{35}-incorporation into the first two peaks and a marked increase in the rate of labeling of the peak 3 region.

The transition of the S^{35}-RNA labeling pattern from the "uninfected" to the "infected" profile is prevented by the addition of chloramphenicol to the medium just prior to, or at the same time as, initiation of infection (Fig. 5). Chloramphenicol by itself does not alter the S^{35}-RNA profile in normal cells. If chloramphenicol is added five minutes after infection, or later, the viral-induced transition is observed once more. Under the conditions employed here, chloramphenicol inhibited C^{14}-leucine incorporation into acid-insoluble material by 98 per cent, indicating an almost complete shut-off of cellular protein synthesis.

An anlysis of the sulfur-containing nucleotides in the early and late RNA fractions (alkaline hydrolysis and chromatography on DEAE cellulose[5]) revealed no great differences between uninfected peaks 1 and 2 and infected peak 3. In each RNA peak approximately 70–80 per cent of the S^{35}-label was found in 4-thioUMP and the remainder distributed in unidentified nucleotides. It seems unlikely, therefore, that the T4-induced transition in S^{35}-RNA is due to any large alteration in the ratio of the different thionucleotides in the various RNA peaks obtained by MAK chromatography. For this reason, it was thought possible that the sulfur-containing RNA of peak 3 might be derived from peaks 1 and 2 by specific chemical or structural modifications induced by viral infection. If this were so, the increase of peak 3 S^{35}-radioactivity after infection could be accounted for by the observed reduction in the labeling of peaks 1 and 2.

To test this hypothesis, *E. coli* cells were grown on S^{35}-Na_2SO_4 for several generations, collected, washed, and resuspended in a medium containing cold Na_2SO_4. After "chasing" for approximately one generation, the cells were divided into two portions; one was infected with T4 phage and the other served as an uninfected control. As shown in Figure 6, the sRNA extracted from the uninfected and infected "prelabeled-chased" cells had a similar S^{35}-radioactive profile, suggesting that the enhancement of peak 3 labeling and sequestration of peaks 1 and 2 labeling, normally seen after infection, are not due to an interconversion of the radioactivity from these fractions. So far, the changes described above in sulfur-labeling of RNA have only been observed with T4 and T2 bacteriophages; pulse-labeled RNA extracts prepared from *E. coli* infected with T7, $\phi X174\rho^-$, and MS2 viruses show S^{35}-RNA profiles similar to uninfected extracts when subjected to chromatography on MAK (Fig. 7).

An examination of the thiol-transferase activity in *E. coli* 105,000 $\times$ *g* soluble extracts indicates that whereas the initial rates of thiolation are similar for both the uninfected and infected enzyme preparations, the T4 reaction quickly deviates from linear kinetics and levels off early (Fig. 8). This phenomenon is found either with or without added acceptor sRNA (yeast); the over-all result is that the extent of thiolation catalyzed by the T4 extract is significantly less than for extracts prepared from uninfected cells.

Discussion.—Previous reports from several laboratories indicate that the replication mechanism for certain coliphages involves some modification of the host's translating machinery; phage-induced changes for certain tRNA's[1-3] as well as the appearance of a new tRNA synthetase[14] have been observed. As described in this report, chromatography on MAK reveals a striking alteration in the sulfur-labeled

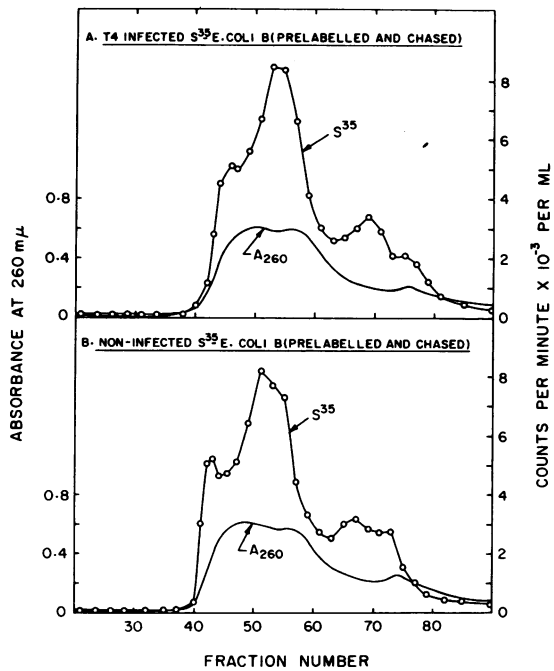

FIG. 6

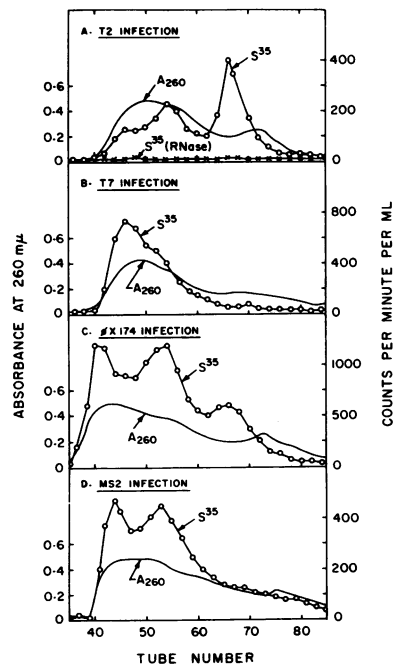

FIG. 7

FIG. 6.—MAK chromatography of S^{35}-RNA from T4-infected and uninfected "prelabeled and chased" *E. coli* cells. *E. coli* B cells prelabeled with S^{35} and chased for one generation were prepared as described under *Experimental*. (*A*) One portion of cells was infected with T4 phage and infection was stopped after 20 min. (*B*) Another portion was treated the same way, but infection was omitted. S^{35}-RNA was extracted from the collected cells and equivalent samples were subjected to MAK chromatography.

FIG. 7.—MAK chromatography of pulse-labeled S^{35}-RNA extracted from T2, T7, MS2, and $\phi X174\rho^-$ infected *E. coli*. Fifty-ml cultures of *E. coli* were infected with coliphage, and 2.5 min later 5 mc of $Na_2S^{35}O_4$ was added to the medium. Incubation was continued for 12.5 min before the infection was stopped and the cells were collected. The labeled RNA was prepared as described under *Experimental* and then subjected to MAK chromatography. The S^{35}-RNA chromatographic profiles from the various phage-infected cells are indicated above. The different infected cultures were identical in all respects except that the strain of *E. coli* host used was as indicated in *Experimental*. RNA extracted from control *E. coli* B pulse-labeled cells was also chromatographed on MAK; no difference from the usual uninfected S^{35}-RNA profile was observed (not shown here).

profiles of soluble RNA after T4 phage infection of *E. coli*. This phenomenon occurs within 7.5 minutes after infection and may therefore be considered as one of the early events in the viral replicative process. The changes described here appear to be viral-induced since (1) infection with only certain coliphages, T2 and T4, shows this response, and (2) the transition in sulfur-labeling can be prevented by the presence of chloramphenicol just prior to, but not 5 minutes after, infection, suggesting that some phage-induced protein synthesis is necessary.

The possibility that the changes observed in the S^{35}-RNA profiles by T4 infection might arise from S^{35}-exchange between different sRNA molecules seems unlikely, since RNA extracts prepared from infected and uninfected cells, prelabeled with S^{35} and "chased," show the same type of "uninfected" S^{35}-RNA distribution pattern on chromatography. Moreover, when the individual peaks are rechromatographed on MAK with the addition of cold carrier sRNA, the radioactivity elutes in the

same relative position. The experiment using prelabeled S^{35}-*E. coli* cells also indicates that the altered T4 S^{35}-RNA profile is not due to viral-induced alterations of preexisting host sRNA (e.g., methylation) and that the thiolation of sRNA molecules after infection must represent the *de novo* addition of sulfur.

The questions that remain unanswered are whether *de novo* sRNA synthesis occurs after T-even coliphage infection and, if so, whether it is host- or viral-directed. Earlier studies[15-17] have clearly shown that infection of *E. coli* with T-even phage results in a shut-down of host nucleic acid synthesis, although the extent of this shut-down has not been exactly established. Nomura, Hall, and Spiegelman[16]

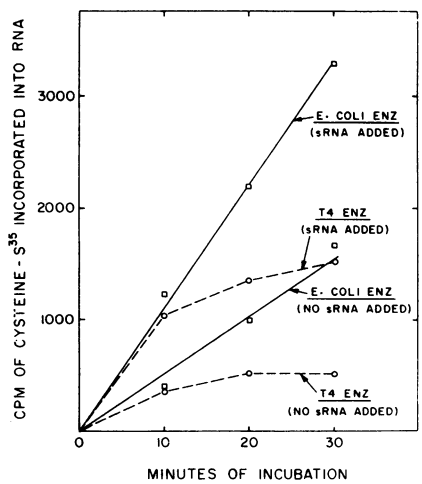

FIG. 8.—Thiol-transferase activity in uninfected and T4-infected *E. coli* extracts. The complete system, 0.50 ml, contained 50 μmoles of Tris·HCl (pH 8.2), 1.5 μmoles of ATP, 5 μmoles of 2-mercaptoethanol, 5 μmoles of $MgCl_2$, 0.1 μmole of S^{35}-cysteine (22×10^6 cpm/μmole), and 1.6 mg of protein from either uninfected or infected 105,000 $\times$ *g* extract. Where indicated, 500 μg of acceptor yeast sRNA was included in the reaction mixture. The reaction was incubated at 37° for the times shown above, and the RNA fraction assayed for S^{35} as described under *Experimental*.

found newly synthesized RNA in both the soluble and ribosomal fractions after pulse-labeling T2 infected cells with P_i^{32}; both labeled RNA fractions were identified as being T2-specific since base analysis indicated a composition similar to T2 DNA. It therefore appeared that very little, if any, host sRNA was being made. On the other hand, the method of analysis might not have permitted the detection of host sRNA synthesis at the level of a few per cent. At the same time, it has never been clearly established whether or not the T-even phage genome codes for any viral-specific RNA other than phage messenger RNA. In this regard it is interesting to note that preliminary hybridization experiments with highly labeled "peak 3" T4 S^{35}-RNA indicates that a significant degree of ribonuclease resistance is imparted to the S^{35}-RNA after annealing to T4 DNA; *E. coli* DNA is far less effective.

The *in vitro* assays for thiol-transferase activity indicate that viral-infected extracts thiolate sRNA at a slower over-all rate and to a lesser extent than extracts from normal cells. Furthermore, the level of endogenous acceptor sRNA for the transfer of cysteine-sulfur also appears to be lower in the infected extracts. These findings could explain the reduced sulfur incorporation in the early MAK-RNA fractions from infected cells, but not the increased sulfur incorporation into the peak 3 region. Several explanations are possible: (1) T4 infection induces the formation of an inhibitor that specifically prevents the thiolation of certain sRNA molecules but not others. (2) Viral infection initiates the synthesis of a specific

thiol-transferase and/or viral-specific sRNA acceptors. (3) Infection induces enzymes that specifically degrade or dethiolate certain sulfur-containing sRNA's. This last possibility seems unlikely since infected extracts show no greater rate of S^{35}-RNA degradation than uninfected extracts. No firm information has been obtained to support or deny the other two possibilities.

Finally, the use of radioactive sulfur as a tool for studying special aspects of RNA metabolism might offer certain advantages over other isotopes. Its primary uniqueness is that so far only the low-molecular-weight cellular RNA species appear to contain thiolated nucleotides;[9-11] therefore, it may serve as a specific labeling device for following the metabolism of sulfur-containing sRNA molecules. If sulfur-containing nucleotides are indeed absent in the higher-molecular-weight RNA species, as shown here, then the present results cannot be attributed to the degradation of other cellular RNA's, as might be the case if other isotopes were employed.

Summary.—Pulse-labeled studies of S^{35}-incorporation into *E. coli* RNA have been examined in normal and phage-infected cells. In both cases, S^{35} is incorporated into the sRNA fraction only. Chromatography on columns of MAK, however, reveals a significant change in the S^{35}-RNA profile after T4 infection. The alteration in S^{35}-labeling of sRNA occurs early after viral infection and can be blocked by chloramphenicol if this is present prior to host-cell infection. Only the T-even coliphages have been observed to effect these changes. The observed alterations after T4 infection do not appear to be attributable to changes in preexisting host sRNA molecules.

§ *Note added in proof:* The labeled RNA preparations have been examined by chromatography on columns of G-100 Sephadex which clearly resolves mixtures of 4*S* and 5*S* RNA into two separate and distinct peaks.[18] The S^{35}-RNA behaves like 4*S* RNA from both control and T4-infected cells; no radioactivity is found in the region where carrier 5*S* RNA is located.

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† Operated by the University of Chicago for the United States Atomic Energy Commission.

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