Limited Production of R17 Ribonucleic Acid Phage in the Presence of Rifampin†

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ABSTRACT: When rifampin, an inhibitor of bacterial RNA synthesis, is added to an R17 RNA phage-infected *Escherichia coli* culture, the subsequent accumulation of phage RNA and protein proceeds for only 25 min irrespective of the time of its addition, indicating that rifampin causes premature cessation of phage synthesis. Phage assembly is increasingly impaired with increasing length of rifampin treatment and phage RNA which fails to be incorporated into phage particles is subsequently degraded. Chloramphenicol, if added within 15 min of rifampin addition, allows continuous accumulation of phage RNA. The rifampin-induced cessation of phage macromolecular synthesis appears to be related to the duration of phage synthesis rather than rifampin treatment itself. It is postulated that in the presence of rifampin a host factor is depleted as a result of phage-specific synthesis, which in turn limits the extent of phage synthesis.

The antibiotic rifampin (or rifampicin) directly inhibits bacterial DNA-dependent RNA polymerase both *in vivo* and *in vitro* (Hartmann *et al.*, 1967; Wehrli *et al.*, 1968). *In vivo*, the inhibition of mRNA synthesis results in the cessation of bacterial protein synthesis. Rifmapin, however, does not affect RNA-dependent RNA synthesis (*e.g.*, the synthesis of infectious Qβ phage RNA) in an *in vitro* system (Bandle and Weissmann, 1970).

Fromageot and Zinder (1968) reported that rifampin, when added within 4-min postinfection, severely limits the growth of f2 RNA phage, whereas if the antibiotic is added 5 min after infection, the phage growth proceeds nearly normally. On the other hand, Friesen (1969) noted that the addition of rifampin even at 15-min postinfection causes a threefold reduction of f2 phage yield. Passent and Kaesberg (1971) reported that rifampin, when added at the time of infection, greatly inhibits production of infectious Qβ phage, while phage RNA and protein components are synthesized in nearly normal amounts as in the absence of rifampin. Thus, they inferred that the rate of assembly into phage particles must be low in the presence of rifampin.

The present paper presents evidence indicating that rifampin causes premature cessation of phage macromolecular synthesis and impairment of phage assembly. Also described are the observations suggesting that these rifampin-induced phenomena are related to the duration of phage protein synthesis in the presence of rifampin.

Materials and Methods

Growth of Cells and Infection. A strain of *Escherichia coli* K12, F74 (F^+, rel^+, *met*, *his*) (Friesen, 1969) was used as a host of R17 RNA phage throughout. Purified wild-type R17 phage (Enger *et al.*, 1963), was used throughout. The host cells were grown at 37° in Tris–maleate medium (Paranchych, 1966) supplemented to 50 μg/ml with the required amino acids. In this medium, F74 has a doubling time of approximately 60 min. When the cell density reached approximately 4×10^8 cells/ml, the host cells were infected with R17 phage at a multiplicity of 5 plague-forming units (PUF)/cell.

Rifampin Treatment. Rifampin was added in the form of powder to bacterial cultures to a final concentration of 500 μg/ml. Rifampin at this concentration caused nearly complete cessation of bacterial RNA synthesis within a few minutes and protein synthesis in 10 min. The optical density measurement of the cultures (after removing rifampin by membrane filtration) showed that the growth of infected as well as uninfected cells immediately ceased on the addition of rifampin.

Assay of RNA and Protein Accumulation. In order to assay accumulation of RNA and protein, aliquots of the cultures were mixed with one-tenth the volume of the RNA-labeling or protein-labeling mixture. The RNA-labeling mixture contained per milliliter: 5 μCi of [^{14}C]uracil, 10 μg of uracil, and 200 μg of cytosine. The protein-labeling mixture contained per milliliter: 1 μCi of [^{14}C]alanine and 100 μg of alanine. At intervals, 0.1-ml portions of the cultures were withdrawn and assayed for cold trichloroacetic acid insoluble radioactivity (labeled RNA) or hot trichloroacetic acid insoluble radioactivity (labeled protein) by the filter paper disk method (Bollum, 1968).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The [^{14}C]alanine-labeled culture (1 ml) was filtered through a membrane filter (0.45 μ pore size), and the cells on the filter were washed three times with equal volumes of 0.01 M EDTA (pH 11.0). The cells were then resuspended in 1.0 ml of 2% sodium dodecyl sulfate in 0.01 M EDTA (pH 11.0) and heated at 50° for 5 min. The alkaline pH cleaves the ester bond of alanyl-tRNA. The resulting clear lysate was then dialyzed against three changes of 0.2% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol for a total of 9 hr at room temperature. The dialysate was then mixed with an equal volume of glycerol and one-fifth the volume of 0.025% Bromophenol Blue. This sample mixture (50 μl) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in which the gels contained 10% acrylamide and 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969). After electrophoresis the gels were sliced into segments of 1.5 mm thickness. The radioactivity in the slices was determined as described by Ward *et al.* (1970).

Equilibrium Density Gradient Centrifugation. The [^{14}C]-alanine-labeled culture (1 ml) was filtered through a membrane

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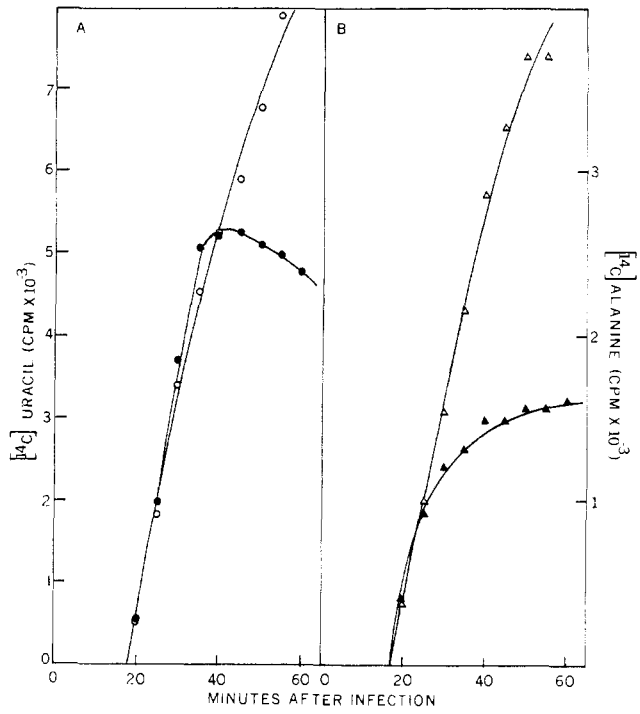

FIGURE 1: Effect of rifampin on cellular capacity to accumulate RNA and protein in R17-infected F74. An R17-infected F74 culture was divided into two portions. One portion was exposed to rifampin (500 μg/ml) at 15-min postinfection. The treated and untreated cultures were further divided into two portions. At 17-min postinfection, each portion was mixed with one-tenth the volume of either RNA-labeling or protein-labeling mixture. RNA accumulation (part A) and protein accumulation (part B) were assayed as described under Methods. The results in this figure have been expressed as the amount of accumulated radioactivity divided by the cell density (absorbance at 500 nm) of each sample to compare the cellular capacity at various times. RNA in the presence (●) or absence (○) of rifampin; protein in the presence (▲) or absence (△) of rifampin.

filter. The cells were washed three times with a buffer composed of 0.05 M Tris (pH 8.0), 0.005 M EDTA, and 0.05 M NaCl (TES buffer). The washed cells were resuspended in 1 ml of TES buffer containing 100 μg of lysozyme. After standing 15 min at 37°, 0.5 ml of 2% sodium *N*-lauroyl sarcosinate and 1 ml of TES buffer were added, and the mixtures were shaken gently until lysis was observed visually (usually within 10 min). The whole lysate was then mixed with 1.59 g of CsCl in polyethylene centrifuge tubes, and overlayered with light mineral oil to the top of the tube. Centrifugation was carried out for 24 hr at 300,000*g* at 5° in an SW 50.1 swinging-bucket rotor in a Spinco Model L3-50 ultracentrifuge. Five-drop fractions were collected from the bottom of the tube, directly onto filter paper disks, and hot trichloroacetic acid insoluble radioactivity was determined as previously described (Bollum, 1968).

Sucrose Density Gradient Centrifugation. The [^{14}C]uracil-labeled cultures (1 ml) was filtered through a membrane filter. The cells were washed three times with 0.0008 M EDTA in 0.03 M Tris buffer (pH 7.8) (Tris–EDTA buffer) and were resuspended in 0.5 ml of Tris–EDTA buffer containing lysozyme (100 μg/ml) and sucrose (20%, w/v). After 5-min incubation at 25°, 0.1 ml each of 0.1 M $MgCl_2$ and 5% Brij 58 (pH 7.8) were added. Lysis became evident within a few minutes. Portions (0.1 ml) of the lysates were layered on 4.8 ml of a linear gradient of 15–30% (w/v) sucrose in 0.01 M Tris buffer (pH 7.5) containing $MgCl_2$ (0.01 M) and NH_4Cl (0.06 M). Centrifugation was carried out for 80 min at 300,000*g* at 5° in an SW 50.1 swinging-bucket rotor. Ten-drop fractions were collected from the bottom of the tube directly onto filter paper disks. The cold trichloroacetic acid insoluble radioactivity was determined as previously described (Bollum, 1968).

In order to compare quantitatively the sedimentation patterns of the various samples, the degree of lysis of each sample was estimated by measuring the recovery of the trichloroacetic acid insoluble radioactivity into the supernatant of centrifugation at 30,000*g* and the data were corrected for these values.

Chemicals. Rifampin was obtained from Calbiochem; chloramphenicol, lysozyme, uracil, amino acids, sodium *N*-lauroyl sarcosinate, and Tris, from Signa Chemical Co.; uniformly labeled L-[^{14}C]alanine (156 Ci/mole) and [^{14}C]uracil (52.5 Ci/mole), from Amersham/Searle Corp.; Brij 58, from Atlas Chemical Industries; all reagents used in gel electrophoresis, from Eastman Kodak Co.

Results

Effect of Rifampin on Accumulation of Phage RNA and Protein. Figure 1 shows the effect of rifampin added at 15-min postinfection on the cellular capacity to accumulate RNA and protein. The data in this figure are presented as amounts of RNA and protein accumulation divided by the cell density of each sample since the infected cells in the absence of rifampin grow nearly normally until 60-min postinfection whereas the rifampin-treated cells do not. In the presence of rifampin, the infected cells accumulated RNA at the same rate as in the untreated control until 40-min postinfection, after which time net degradation of RNA began, whereas the rate of RNA accumulation in the untreated cells remained unchanged up to 60-min postinfection (Figure 1A). Similar comparison beyond 60-min postinfection is not possible due to the onset of lysis in the untreated control.

The fact that rifampin addition did not reduce the rate of RNA accumulation in the infected cells until 40-min postinfection suggests that in the infected cells, synthesis of bacterial-stable RNA had been greatly suppressed as previously reported (Hudson and Paranchych, 1967). This conclusion was further substantiated by examining the fate of radioactive uracil given as a 2-min pulse by means of sucrose density gradient centrifugation in a low magnesium (10^{-4} M) buffer. About 80% of the uracil that was incorporated into the infected cells, in the absence of rifampin, at 18- and 50-min postinfection could be chased into 80S phage particles, whereas 30S and 50S ribosomal subunits were not appreciably labeled. Therefore, the RNA being accumulated in the infected cells largely represents phage RNA, and the data of Figure 1A can then be interpreted to mean that rifampin causes the premature cessation of phage RNA, whereas in its absence accumulation of phage RNA continues.

Figure 1B shows that rifampin addition caused the decline in the rate of protein accumulation after 25-min postinfection and subsequent cessation, at about 40-min postinfection, at which time the rifampin-treated cells had accumulated only half as much protein as did the untreated cells. This is due to the fact that substantial synthesis of host proteins was occurring even at this time of infection as confirmed by comparison of sodium dodecyl sulfate–gel electrophoretic patterns of proteins labeled in the presence and absence of rifampin (data not shown). Although the electrophoretic patterns of proteins produced in the absence of rifampin cannot be

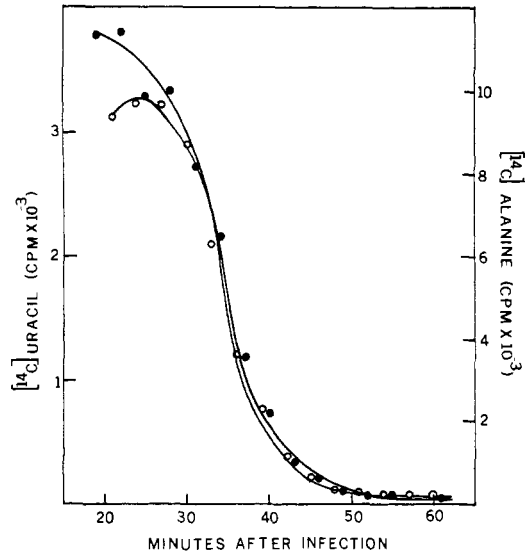

FIGURE 2: Rate of phage RNA and protein synthesis in the presence of rifampin. An R17-infected F74 culture was exposed to rifampin (500 μg/ml) at 15-min postinfection. Two 0.2-ml portions were periodically removed from the culture, and each portion was pulse labeled for 2 min either with [^{14}C]uracil (0.1 μCi/ml; 52.5 Ci/mole) or with [^{14}C]alanine (0.1 μCi/ml; 156 Ci/mole). After labeling, 0.1-ml portions were assayed for labeled RNA (●) or labeled protein (○).

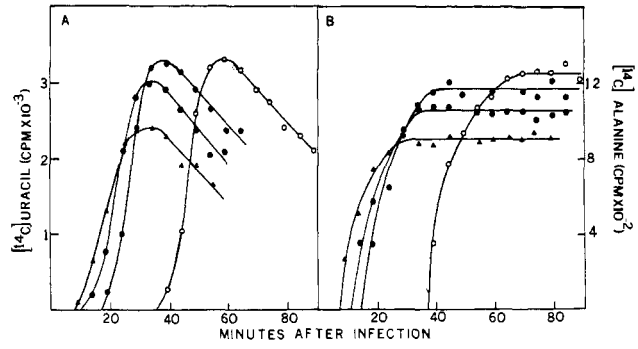

FIGURE 3: Effect of time of rifampin addition on accumulation of phage RNA and protein. Four portions of an F74 culture were exposed to rifampin (500 μg/ml) at different times after infection with R17 phage. An RNA- or protein-labeling mixture was added 2 min after rifampin addition. Part A represents RNA accumulation and part B, protein accumulation. The times of rifampin addition were 5 min (▲), 10 min (●), 15 min (●), and 35 min (○) after infection.

accurately analyzed due to the presence of bacterial proteins, it is clearly indicated that the phage coat protein continued to be synthesized beyond 40-min postinfection in the absence of rifampin but not in the presence of rifampin.

Rate of Phage RNA and Protein Synthesis. The RNA accumulation data (Figure 1A) may not permit us to determine the rate of phage RNA synthesis in the presence of apparent RNA degradation occurring in the rifampin-treated host. However, if we assume that the rate of degradation does not greatly exceed the rate of synthesis, the pulse labeling of RNA would give an approximate rate of RNA synthesis since the labeling of RNA must precede the degradation of the labeled RNA. Figure 2 shows that in the presence of rifampin added at 15-min postinfection the rate of phage RNA and protein synthesis sharply declined between 15 and 25 min after rifampin addition and their syntheses practically stopped by 30 min after rifampin addition. Therefore the net degradation of RNA observed after 25 min of rifampin treatment (Figure 1A) must represent the degradation of preformed RNA (in the absence of RNA synthesis).

Effect of Time of Rifampin Addition on Accumulation of Phage RNA and Protein. Figure 3 shows that these rifampin induced phenomena are not peculiar to rifampin addition at 15-min postinfection. Rifampin was added to F74 cultures at various times before and after infection. In all instances accumulation of phage RNA and protein proceeded for only 25 min and ceased. Thus, it is rifampin addition that is causing premature cessation of phage RNA and protein accumulation. As well, lower levels of RNA and protein accumulation occurred when rifampin was added earlier.

When rifampin was added 10 min prior to infection, accumulation of phage RNA and protein occurred but ceased approximately 30 min after infection. Rifampin added at the time of infection resulted in the cessation of phage RNA and protein accumulation at 30-min postinfection. Therefore it appears that the cessation of phage macromolecular accumulation is related to the duration of phage-specific synthesis in the presence of rifampin (which becomes evident approximately 5 min after infection) rather than the duration of rifampin treatment itself. Figure 3 also shows that the net degradation of phage RNA commenced immediately after its accumulation ceased, irrespective of the time of rifampin addition.

The lower levels of RNA and protein accumulated with the earlier rifampin addition (Figure 3) are partly ascribed to different cellular densities and different rates of phage-specific synthesis at the time of rifampin addition. These rifampin-induced phenomena are also observable at lower rifampin concentrations (*e.g.*, 50 μg/ml) and in other R17 phage hosts, F8 (*rel*), CP 78 F$^+$ (*rel*$^+$), and CP 79 F$^+$ (*rel*) (Watson and Yamazaki, 1972).

Effect of Rifampin on the Production of Infectious Phage. Since rifampin not only limits synthesis of phage RNA and protein but also causes a considerable degradation of phage RNA, a marked reduction of phage yield is expected. Table I shows that the addition of rifampin to an infected culture indeed reduced the number of infectious phage by a factor of about 4 when it was added at 15-min postinfection and by a factor of about 20 when it was added 5 min prior to infection. These results clearly indicate the marked inhibitory effect of rifampin on infectious phage production. Since artificial lysis markedly increased phage yield (10- to 20-fold), release of R17 phage from the rifampin-treated infected cells into the medium must be very low.

Phage Proteins Synthesized in the Presence of Rifampin. We have previously shown (Khan and Yamazaki, 1970) that approximately 70% of the phage RNA labeled in the presence of rifampin appeared as a single sharp peak in sucrose gradient centrifugation which is indistinguishable in size from authentic R17 phage RNA. Figure 4A clearly shows that all three known phage-specific proteins could be synthesized in the presence of rifampin. Figure 4B illustrates that the phage proteins synthesized within 10 min of rifampin treatment could be incorporated into the phage particles having the same density as authentic R17 phage. Thus, the phage macromolecules synthesized in the presence of rifampin appear to be functionally normal.

Effect of Chloramphenicol on Phage RNA Accumulation. Rifampin does not directly inhibit RNA phage production processes since it exhibits no effect on the *in vitro* replication

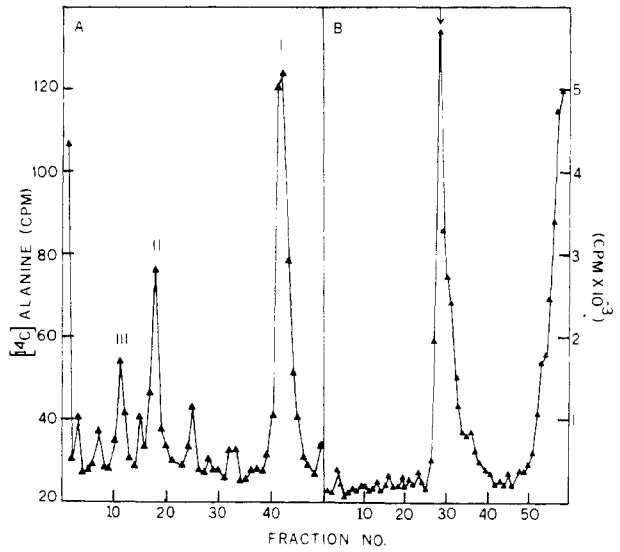

FIGURE 4: Phage proteins produced in the presence of rifampin. Part A: sodium dodecyl sulfate–gel electrophoresis of phage proteins. An F74 culture (4×10^8 cells/ml) was exposed to rifampin (500 μg/ml) 10 min prior to infection. The cells were labeled with [^{14}C]alanine (1 μCi/ml; 156 Ci/mole) between 0- and 20-min postinfection. One ml of the labeled culture was then analyzed on sodium dodecyl sulfate–gel electrophoresis as described under Methods. Similar analysis of proteins isolated from R17 phage permitted the identification of peak I as the coat protein and peak II as the maturation protein. Peak III is presumed to be the phage-specific components of R17 replicase. Electrophoretic migration is from left to right. Part B: equilibrium density gradient centrifugation. An infected F74 culture was exposed to rifampin (500 μg/ml) at 15-min postinfection. The cells were labeled with [^{14}C]alanine (1 μCi/ml; 156 Ci/mole) between 20- and 25-min postinfection; 1 ml of the labeled culture was lysed and analyzed by equilibrium density gradient centrifugation as described under Methods. The arrow indicates the peak position of authentic R17 phage particles synthesized in the absence of rifampin when they were similarly analyzed in the control experiment. The centrifugal force is from right to left.

TABLE I: Effect of Rifampin on the Production of Infectious R17 Phage.[a]

Rifampin	Phage Yield (PFU/ml)	
	Artificial Lysis	No Artificial Lysis
No addition	7.1×10^{11}	5.8×10^{11}
−5 min	3.4×10^{10}	5.4×10^{9}
+15 min	1.6×10^{11}	7.7×10^{9}

[a] A log-phase F74 culture (4×10^8 cells/ml) was infected with R17 phage at a multiplicity of 5. Two portions of the culture were exposed to rifampin (500 μg/ml) 5 min prior to infection (−5 min) or at 15-min postinfection (+15 min), respectively. At 60-min postinfection, one portion of the infected culture was assayed for infectivity without artificial lysis. The other portion of the culture was artificially lysed by exposing the culture to 0.01 M EDTA (pH 6.5) and lysozyme (100 μg/ml) for 5 min at room temperature and then to Brij 58 (0.5%) for 5 min at room temperature. More than 90% of the cells was lysed under this condition. Phage assay was performed in triplicate. Under our growth conditions, phage-specific lysis in the absence of rifampin became evident after 60-min postinfection.

of phage RNA (Bandle and Weissmann, 1970) and little effect on phage production in a mutant host possessing a rifampin-resistant RNA polymerase (Marino *et al.*, 1968; Passent and Kaesberg, 1971). The fact that rifampin causes the premature cessation of phage synthesis could then be explained by assuming that some bacterial factor(s) essential for phage production is depleted but not regenerated in the presence of rifampin. Since the rifampin-induced cessation appears to be correlated with the duration of phage synthesis in the presence of rifampin, it would be interesting to ask which phage synthesis (protein or RNA) is responsible for such presumed deletion.

Chloramphenicol addition to a bacterial culture permits accumulation of bacterial mRNA in the absence of protein synthesis (Gurgo *et al.*, 1969) though its mechanism is not well understood. Figure 5 shows the effects of chloramphenicol addition on phage RNA accumulation. The infected F74 cells were exposed to rifampin at 15 min postinfection and treated with chloramphenicol at various times thereafter. Chloramphenicol, when added within 15 min of rifampin treatment, allowed phage RNA to accumulate beyond 25 min after rifampin addition. However, if chloramphenicol was added later than 25 min after rifampin addition, it no longer allowed continued RNA accumulation nor prevented RNA degradation. Thus there seems to be a critical period between 15 and 25 min after rifampin addition. This period corresponds to the time of the sharp decline in the rate of phage macromolecular synthesis (Figure 2). The addition of chloramphenicol simultaneously with rifampin at 15-min postinfection resulted in a reduced rate of RNA accumulation. Presumably this reflects the effect of chloramphenicol on the synthesis of phage replicase which is still produced at this time. Since phage RNA accumulation continued until late infection in the presence of chloramphenicol added within 15 min of rifampin treatment, the host-specific factor in question does not appear to be deleted during phage RNA synthesis in the absence of protein synthesis.

Phage Assembly and Instability of Phage RNA. The observed degradation of phage RNA formed in the presence of rifampin (Figures 3 and 5) suggests that some of the phage RNA formed fails to be incorporated into stable phage particles. A question we would like to ask here is whether rifampin directly causes this impairment of phage assembly. Figure 6 illustrates the fate of phage RNA synthesized at two different times after rifampin treatment as analyzed by sucrose density gradient centrifugation. Phage RNA labeled between 5 and 10 min after rifampin addition (early label) was more efficiently incorporated into phage particles as compared to RNA labeled between 15 and 20 min after rifampin addition (late label). When both labels were subsequently chased with unlabeled uracil for 25 min, a larger proportion of the early label was chased into the phage particles (Figure 6A) whereas a substantial portion of the late label remained unincorporated, being subsequently degraded (Figure 6B). Figure 6B also indicates that some fraction of the late label which had been incorporated into the phage particles was also unstable. These results indicate that increasing length of rifampin treatment causes increasing impairment of phage assembly, suggesting that the impairment is a secondary effect of rifampin treatment.

In order to study whether the observed instability of phage RNA is specific to that synthesized in the presence of rifampin, the following experiments were performed. An R17-infected F74 culture was labeled with [^{14}C]uracil between 15- and

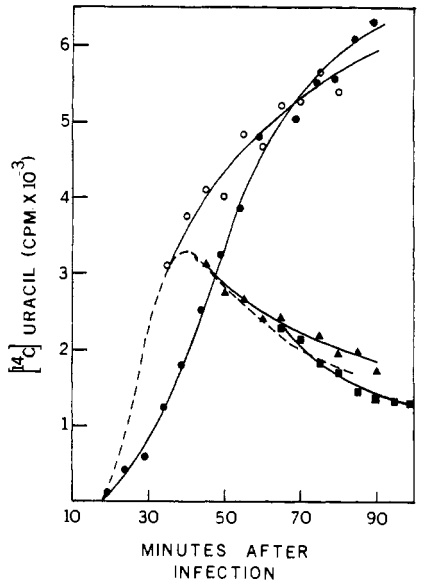

FIGURE 5: Effect of chloramphenicol on phage RNA accumulation. An R17-infected F74 culture was exposed to rifampin (500 μg/ml) at 15-min postinfection. Four portions of the culture were treated with chloramphenicol (100 μg/ml) at different times after rifampin addition: 0 min (●), 15 min (○), 25 min (▲), and 45 min (■), respectively. The broken line represents the control without chloramphenicol. The RNA-labeling mixture was added in all cases at 17-min postinfection. Labeled RNA was periodically assayed 5 min after chloramphenicol treatment.

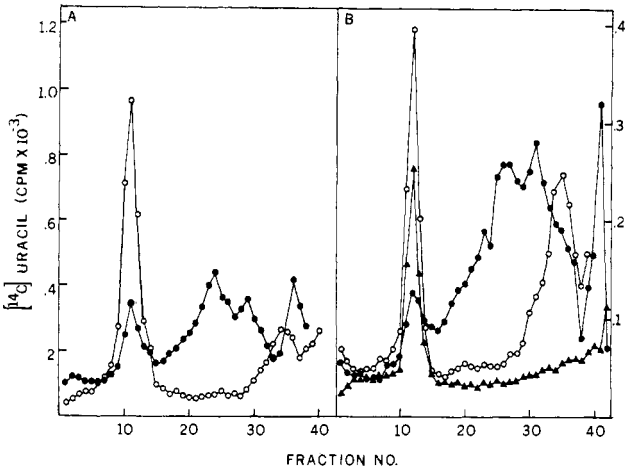

FIGURE 6: Fate of phage RNA synthesized in the presence of rifampin. An R17-infected F74 culture was exposed to rifampin (500 μg/ml) at 15-min postinfection, was then divided into two portions, and treated as below. Part A: the first portion of the culture was labeled with [^{14}C]uracil (1 μCi/ml; 52.5 Ci/mole) between 5 and 10 min after rifampin addition. At the end of the labeling 1-ml portion of the culture was prepared for sucrose density gradient centrifugation. 100-fold excess of unlabeled uracil was then added to the remaining culture to chase the label. After 25-min chasing, 1-ml portion of the culture was analyzed by sucrose density gradient centrifugation as described under Methods. (●) Sample labeled between 5 and 10 min after rifampin addition; (○) after 25-min chase. Part B: the second portion of the culture was similarly labeled with [^{14}C]uracil between 15 and 20 min after rifampin addition. Unlabeled uracil was similarly added at 20 min after rifampin addition to chase the label. (●) Sample labeled between 15 and 20 min after rifampin addition; (○) after 25-min chase; (▲) after 45-min chase. The peak between fractions 10 and 13 (parts A and B) corresponds to that of authentic R17 phage. Sedimentation is from right to left. Under the lysis condition used for preparation of samples, R17 phage particles were stable whereas 70S ribosomes were largely dissociated into the subunits. The data shown in these figures have been corrected for the differences in the degree of lysis which varied somewhat with the samples.

20-min postinfection in the absence of rifampin. After the labeling, the unincorporated label was removed by filtration through a membrane filter. The washed cells were resuspended in the growth medium and reincubated with or without rifampin. No significant change in the levels of trichloroacetic acid insoluble radioactivity was observed up to 60-min postinfection, whether subsequently treated with rifampin or not. A similar experiment was performed with an uninfected culture. The host-specific RNA (labeled in the absence of rifampin) was also stable whether subsequently treated with rifampin or not. Since the total RNA synthesized in the untreated infected cells is stable, the phage RNA (which is predominantly being synthesized at the time of labeling) must be stable during infection without rifampin. Therefore it is concluded that the observed instability of phage RNA is specific to the RNA synthesized in the presence of rifampin.

Passent and Kaesberg (1971) observed no degradation of Qβ phage RNA synthesized in Q13 host at least up to 50 min after rifampin addition. This may be ascribed to the fact that Q13 host lacks active ribonuclease I and polynucleotide phosphorylase, whereas we have used F74 which is the wild type with respect to these nucleolytic enzymes. We have confirmed that no degradation of R17 phage RNA synthesized in Q13 host occurs even after 100 min treatment with rifampin.

Discussion

In the absence of rifampin the R17-infected host supports active phage synthesis at least up to 60-min postinfection. In contrast, in the presence of rifampin the accumulation of R17 phage-specific RNA and protein proceeds for only 25 min, irrespective of the time of rifampin addition (Figure 3), clearly indicating that rifampin is causing the premature cessation of synthesis of phage RNA and protein.

In the presence of rifampin all known phage macromolecules are synthesized. The phage components made within 15 min of rifampin treatment appears to be functionally normal in that they are efficiently incorporated into stable phage particles, whereas a substantial proportion of the phage RNA formed after 15 min of rifampin treatment fails to be incorporated into stable phage particles and is subsequently degraded. This impairment of phage assembly can be understood if the synthesis of phage protein components required for the assembly becomes limited during rifampin treatment. Phage RNA accumulated in excess of its relative requirement for phage assembly is degraded by the action of bacterial nucleases. Since the instability of phage RNA is not observable in the absence of rifampin, the synthesis of phage RNA must be regulated in relation to the synthesis of other phage components in normal infection without rifampin.

Since rifampin does not directly affect phage production processes (Bandle and Weissmann, 1970; Marino *et al.*, 1968; Passent and Kaesberg, 1971) and the rifampin-induced cessation of phage synthesis appears to be related to the duration of phage macromolecular synthesis, the rifampin-induced phenomena could be explained by assuming that some bacterial factor(s) essential for phage production is depleted during phage synthesis but cannot be regenerated in the presence of rifampin. Since chloramphenicol added within 15 min of rifampin treatment allows continued accumulation of phage RNA, the factor in question must be stable during

phage RNA synthesis in the absence of protein synthesis, suggesting that the factor is depletable during phage protein synthesis. Such depletion may in turn limit further synthesis of phage proteins. Although the present results are consistent with this notion, other possible modes of rifampin action can not be eliminated at the present time.

Acknowledgments

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References

Bandle, E., and Weissmann, C. (1970), *Biochim. Biophys. Acta 199*, 551.
Bollum, F. J. (1968), *Methods Enzymol. 12B*, 169.
Enger, M. D., Stubbs, E. A., Mitra, S., and Kaesberg, P. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 857.
Friesen, J. D. (1969), *J. Mol. Biol. 46*, 349.
Fromageot, H. P., and Zinder, N. D. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 184.
Gurgo, C., Apirion, D., and Schlessinger, D. (1969), *J. Mol. Biol. 45*, 205.
Hartmann, G., Honikel, K. O., Knüsel, F., and Nüesch, J. (1967), *Biochim. Biophys. Acta 145*, 843.
Hudson, J. B., and Paranchych, W. (1967), *J. Virology 1*, 529.
Khan, S. R., and Yamazaki, H. (1970), *J. Bacteriol. 102*, 702.
Marino, P., Baldi, M. I., and Tocchini-Valentini, G. P. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 125.
Paranchych, W. (1966), *Virology 28*, 90.
Passent, J., and Kaesberg, P. (1971), *J. Virology 8*, 286.
Ward, S., Wilson, D. L., and Gillam, J. J. (1970), *Anal. Biochem. 38*, 90.
Watson, R., and Yamazaki, H. (1972), *Biochemistry 11*, 611.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wehrli, W., Nüesch, J., Knüsel, F., and Staehelin, M. (1968), *Biochim. Biophys. Acta 157*, 215.

Reactions of Tetranitromethane. Mechanism of the Reaction of Tetranitromethane with Pseudo Acids†

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ABSTRACT: The reactions of tetranitromethane with five pseudo acids (CH) have been investigated [CH_3CN, $CH_3SO_2CH_3$, CH_3COCH_3, $CH_2(CN)_2$, and $CH_3CH_2NO_2$]. Kinetic studies, quantitative product analyses, and limited electron spin resonance (esr) studies $C(NO_2)_4$ with CH_3CN, CH_3COCH_3, and $CH_3CH_2NO_2$ were carried out. The reactions of $C(NO_2)_4$ with the pseudo acids CH_3CN, $CH_3SO_2CH_3$, and CH_3COCH_3 were found to give pseudo-first-order kinetics with respect to trinitromethane anion formation and were found to obey the following rate expression: $k_{obsd} = k_{HO^-}[HO^-] + k_1[C^-] + k_2[C^-][CH]$, where $[CH]_T = [CH] + [C^-]$. For the reaction of $CH_2(CN)_2$ with $C(NO_2)_4$, no hydroxide rate was detected at the pH values employed and the rate expression was simply first order in C^- at the lower carbon acid concentrations used. Due to the fact that pK_a' of nitroethane is located in the accessible pH range, greater kinetic detail could be obtained with this pseudo acid. The pH dependence of the reaction of $CH_3CH_2NO_2$ with $C(NO_2)_4$ in deoxygenated solutions was found to be quite complex, the pseudo-first-order rate constant being provided by an expression describing a bell-shaped pH–log k_{obsd} profile with a discontinuity from slope +1 at low pH. The complicated bell-shaped pH–rate profile for the reaction of $CH_3CHNO_2^-$ with $C(NO_2)_4$ is suggested to arise not only from the reaction of the free carbanion with $C(NO_2)_4$ (giving rise to a shoulder in the pH–rate profile at the pH 6–8) but also from the reaction of the intermediate, $CH_3CH(OH)\ddot{N}(O^-)_2$, with $C(NO_2)_4$. This intermediate has been suggested to occur in the basic hydrolysis of nitroethane, and its conjugate acid [$(CH_3CH(OH)\ddot{N}(OH)_2$] is thought to be an intermedite in the hydrolysis of *aci*-nitroalkanes (Nef reaction). In order to account for the formation of nitrite ion as well as the esr results indicating considerable radical formation, the mechanism of the reactions of $C(NO_2)_4$ with pseudo acids is interpreted to be a nucleophilic attack of $C(NO_2)_4$ followed by subsequent formation of radical intermediates. The reaction of $CH_3CHNO_2^-$ at high pH values in oxygenated solutions was found to be zero order in the formation of $C(NO_2)_3^-$. The reaction of $CH_3CHNO_2^-$ with $C(NO_2)_4$ in the presence of oxygen is suggested to occur *via* superoxide anion $O_2^{\cdot -}$, with $C(NO_2)_4$ reacting with the $O_2^{\cdot -}$ formed from the oxidation of carbanion by O_2.

Since the introduction of tetranitromethane, $C(NO_2)_4$, as a nitrating agent for tyrosyl residues in proteins (Riordan *et al.*, 1966), studies on model systems have been undertaken to determine the specificity of the reagent (Sokolovsky *et al.*, 1966). It is now apparent that $C(NO_2)_4$ reacts with thiol (Riordan and Christen, 1968; Sokolovsky *et al.*, 1969), indolyl (Spande *et al.*, 1969), and imidazole (Ivanetich, 1971)[1] groups.

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[1] Private communication.