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Bacteriophage-induced Inhibition of Host Functions

II. Evidence for Multiple, Sequential Bacteriophage-induced Deoxyribonucleases Responsible for Degradation of Cellular Deoxyribonucleic Acid

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Degradation of bacterial deoxyribonucleic acid (DNA) after infection with T4 bacteriophage was studied in an endonuclease I-deficient host. The kinetics of degradation were similar to those seen in other hosts with a normal level of this enzyme. Irradiation of extracellular phage with ultraviolet (UV) destroyed the capacity of the infecting virus to induce extensive breakdown of host DNA, which was, however, converted to high-molecular-weight material. Addition of chloramphenicol to T4-infected cells provided data which can be interpreted to indicate the involvement of at least two endodeoxyribonucleases and one exodeoxyribonuclease having a high degree of specificity. A model is proposed showing the sequential action of two endodeoxyribonucleases followed by an exodeoxyribonuclease in the degradation of host DNA. The appearance of these hydrolytic enzymes requires protein synthesis. Infections leading to partial degradation only (UV-irradiated phages, gene 46 mutants) effectively inhibited the synthesis of bacterial messenger ribonucleic acid and of β-galactosidase.

Infection of *Escherichia coli* with T-even bacteriophages results in total and efficient utilization of the bacterial biosynthetic machinery in the multiplication of the virus. Synthesis of bacterial deoxyribonucleic acid (DNA), messenger ribonucleic acid (mRNA), ribosomal RNA, and proteins ceases rapidly and irreversibly (1, 4, 13, 19). Although the mechanism of phage-initiated inhibition of bacterial functions remains to be clarified, two processes appear to be involved, only one of which requires protein(s) synthesized under phage control (15). However, two recent studies (7, 11) have shown that, although bacterial protein synthesis stopped abruptly, significant amounts of host RNA continued to be made for several minutes after infection with T4.

In a recent publication (20), we presented evidence indicating degradation of bacterial DNA beginning 5 min after infection with T4, ultraviolet (UV)-irradiated T4 ($T4^{uv}$), amber mutants of gene 42 [N122, no DNA synthesis, hydroxymethylase-defective (21)], and gene 46 [N94, DNA arrest, defective in the complete degradation of host DNA (20, 22)]. We now present evidence that this hydrolysis of host DNA is genetically controlled by the infecting bacteriophage and requires de novo protein synthesis, apparently for the formation of at least three deoxyribonucleases. The pattern of degradation initiated by T4 in an endonuclease I-deficient host, ER22 (5), suggests further that this deoxyribonuclease has little involvement in this process. Our data show that infections resulting in only partial degradation of the bacterial genome are capable of effectively inhibiting host RNA and protein synthesis. Furthermore, extracellular irradiation of phage with UV light destroys the capacity to continue the degradation of host DNA without impairing its ability to inhibit host functions.

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MATERIALS AND METHODS

Bacteria. *E. coli* B3 thy⁻ at 37 C [suppressor⁻ (su⁻)], CR34 (thr⁻ leu⁻ thi⁻ ura⁻ thy⁻ su_{II}^+), and ER22, an endonuclease I-deficient su⁻ derivative of *E. coli* B (5), were used for DNA degradation studies. *E. coli* CR63 su_I^+ was used for the propagation of suppressible amber mutants of T4 bacteriophage.

Bacteriophages. T4D and amber mutants *am* N122 (gene 42), *am* N94 (gene 46), *am* B22 (gene 43, DNA polymerase), and *am* N82 (gene 44, DNA^-) were obtained from R. S. Edgar. Phages were irradiated with UV light provided by a germicidal lamp (G.E., G15T8, 15 w) at a distance of 50 cm. Irradiation for 1 to 2 min resulted in ca. a 10^4-fold drop in the viable

titer of phage. Multiplicity and photoreactivation were avoided by infecting at low multiplicity and keeping the infected cells in the dark.

Media. The glycerol-Casamino Acids-salts medium has been described (3).

β-Galactosidase induction and assay. Exponentially growing cells were induced with 5×10^{-4} M isopropyl-β-D-thiogalactoside (IPTG). At intervals, 100-μliter samples were pipetted into 0.9 ml of ice-cold medium containing 1 drop each of toluene and 2.5% sodium lauryl sarcosyl. After preincubation at 37 C for 10 to 20 min, 0.2 ml of 0.4% *o*-nitrophenylgalactoside (ONPG) dissolved in 0.5 M 2-mercaptoethanol-0.25 M sodium phosphate buffer, *p*H 7.0, was added and the incubation was continued for 15 min. An 0.5-ml amount of 1 M sodium carbonate was added, and the absorbance was read at 420 nm in a Gilford spectrophotometer.

Incorporation of uridine-5-^{3}H into acid-insoluble material. Samples (200 μliters) of bacteria were added to 0.1 μc of uridine-*5-^{3}H* (specific activity 0.5 μc/μg), and 50-μliter samples were spotted on Whatman 3 MM discs which were immediately dropped into ice-cold 5% trichloroacetic acid. Further processing was carried out as described previously (3).

Preparation of lysates, measurement of acid-soluble nucleotides, and zone sedimentation of DNA from infected and uninfected bacteria. These procedures have already been described (20).

Chemicals. Uridine-*5-^{3}H* was purchased from New England Nuclear Corp., Boston, Mass. Thymidine-methyl-^{3}H, thymine-*2-^{14}C*, and adenosine were supplied by Schwarz BioResearch, Orangeburg, N.Y. Puromycin and casein hydrolysate (vitamin and salt-free, acid-hydrolyzed) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. IPTG and ONPG were purchased from Sigma Chemical Co., St. Louis, Mo., and chloramphenicol (CM) was a gift from Parke, Davis & Co., Detroit, Mich.

RESULTS

Phage-induced degradation of DNA in an endonuclease I-deficient host. In a previous communication, we presented evidence showing a distinct decrease in the sedimentation rate of bacterial DNA within 5 min after infection with T4 bacteriophage and some mutants of the phage (20). In the present study, lack of participation of pre-existing endonuclease I in the conversion of the host genome into degraded but high-molecular-weight products was indicated by the characteristic degradation of cellular DNA seen after infection of *E. coli* ER22, an endonuclease I-deficient host (Fig. 1). A 90*S* product appeared within 5 min after infection with T4 and T4^{uv}. At a similar time (Fig. 2), infection with *am* N122 (no viral DNA synthesis) resulted in the appearance of 85*S* material. Although a decrease in the sedimentation rate of ER22 DNA 5 min after infection with *am* N94, a mutant incapable of producing acid-soluble material from DNA of

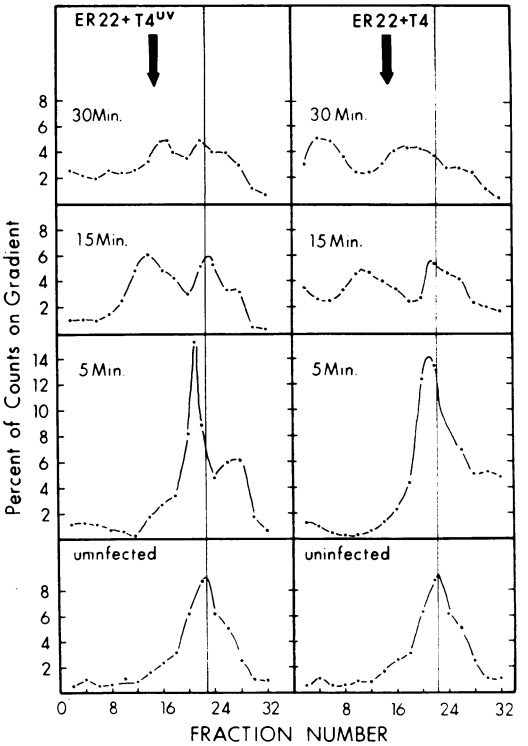

FIG. 1. *Sedimentation of E. coli ER22 DNA after infection with T4 and T4^{uv}. The cells were grown in the presence of 50 μc of thymidine-methyl-^{3}H per ml and 250 μg of adenosine per ml. Conditions of infection, preparation of lysates, centrifugation through linear 40 to 60% (v/v) glycerol gradients, and counting of fractions were done as described in reference 20. Multiplicity of infection = 2. Input per gradient = 8.5 to 9 × 10^3 counts/min. The position of differentially labeled (^{14}C or ^{32}P) T4 reference DNA in this and other sedimentation profiles is indicated by the arrow. The direction of sedimentation is to the right.*

nonpermissive hosts (20, 22), was not seen, the samples at 15 and 30 min after infection sedimented at 53*S* and 38*S*, respectively (Fig. 3). The similarity in the kinetics of degradation of ER22 DNA and of the DNA of *E. coli* B3 and CR34 after infection with T4 and these mutants, described in an earlier publication (20), indicated that the bacterial endonuclease I has a minor role in the breakdown of the host genome. This conclusion is further supported by the data presented in Fig. 4, showing the usual pattern (20) of production of acid-soluble nucleotides in ER22 infected with T4 and these mutants.

Absence of extensive degradation after infection with UV-irradiated bacteriophage. The capacity to induce complete degradation of host DNA into low-molecular-weight, acid-soluble

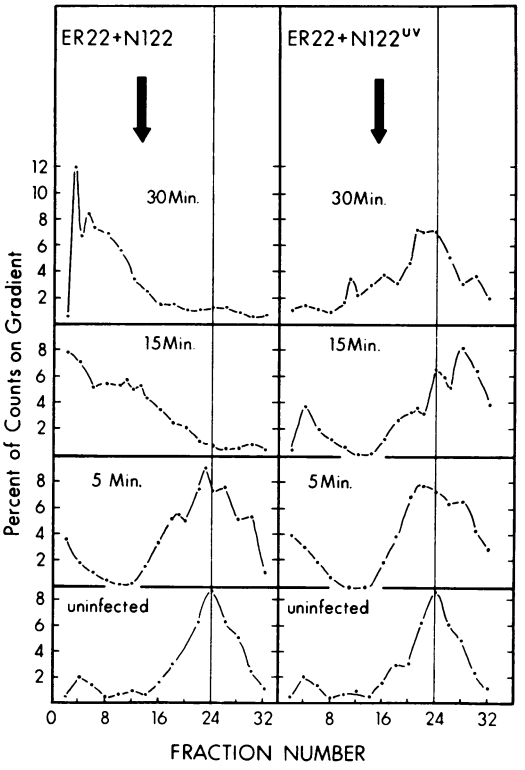

FIG. 2. *Sedimentation of E. coli ER22 DNA after infection with am N122 and am N122^{uv}. The cells were grown in the presence of 100 μc of thymidine-methyl-^{3}H per ml and 250 μg of adenosine per ml. Multiplicity of infection = 5. Input per gradient = 4.3 to 5 × 10^3 counts/min. Other conditions were as described in the legend to Fig. 1.*

material was highly sensitive to exposure of extracellular phage to UV irradiation (Fig. 1, 2). Infection of *E. coli* strains with T4^{uv} or *am* N122^{uv} resulted in the production, even after 30 min, of only high-molecular-weight materials, sedimenting at 50*S*. The data in Fig. 4 show that acid-soluble nucleotides were not produced from host DNA when UV-irradiated *am* N122 was used for infection. Efficient incorporation of breakdown products from host chromosome into newly synthesized viral DNA obscured the formation of acid-soluble nucleotides from bacterial DNA. This is clearly seen in Fig. 4 with T4 infection. With *am* N122 infection of the *su*$^-$ host, degradation is obvious in the absence of viral DNA synthesis (20, 21). Similar kinetics of production of acid-soluble nucleotides were obtained with other amber mutants defective in early functions, e.g., with *am* N82 (gene 44, DNA$^-$) and *am* B22 [gene 43, DNA polymerase defective but producing polymerase-associated nuclease (16)]. These results suggest that the formation of necessary deoxyribonucleases occurs in the absence of viral DNA and consequent late protein synthesis.

Requirement for de novo synthesis of protein. The foregoing results and those presented in an

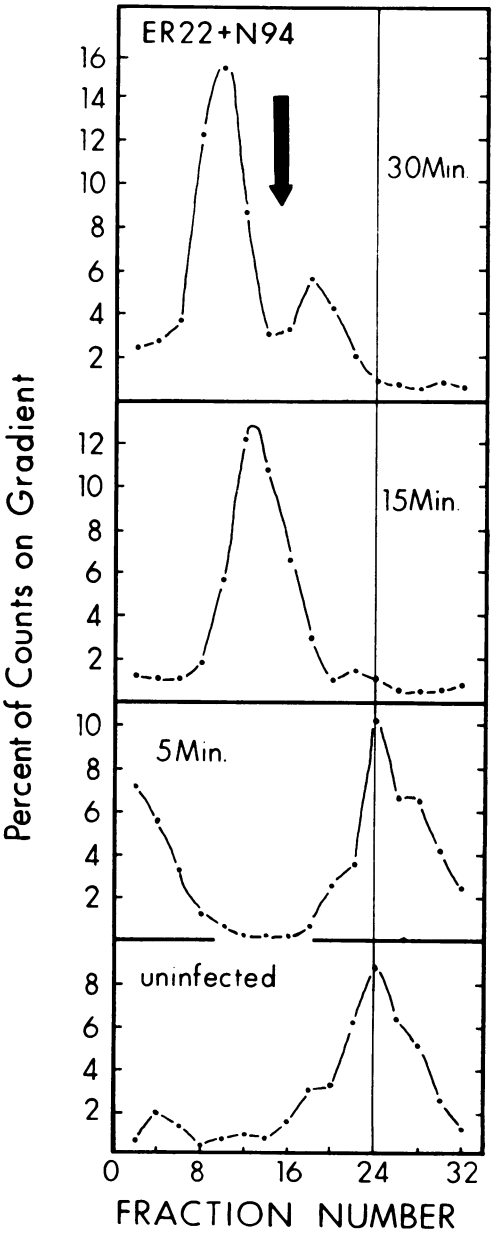

FIG. 3. *Sedimentation of E. coli ER22 after infection with am N94. Cells grown for the experiment represented in Fig. 2 were used. Other conditions were similar to those in Fig. 1.*

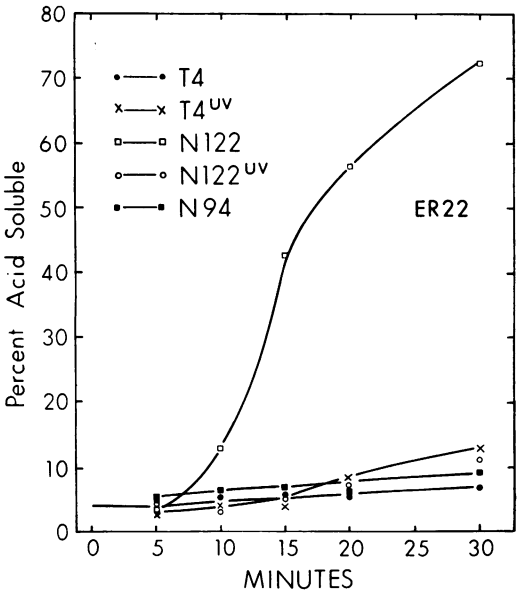

FIG. 4. *Production of acid-soluble nucleotides from the genome of E. coli ER22 after phage infection. Samples of bacteria, grown and infected as described in the legend to Fig. 2, were coprecipitated with unlabeled E. coli in 10% trichloroacetic acid at 0 C. The percentage acid-soluble determination is based on the number of counts in the supernatant fluid after cold trichloroacetic acid precipitation of the samples, relative to the number of counts after hydrolysis of duplicate samples in 10% trichloroacetic acid for 10 min.*

earlier publication (20) suggest that the enzymes catalyzing degradation of host DNA are synthesized after infection. The inability of a gene 46 mutant, *am* N94, to complete the degradation also indicates that several enzymes participate in the degradative process. The effect of CM added at various times after infection of *E. coli* CR34 and B3 with *am* N122 was studied. Samples of infected cells were treated with CM (100 μg/ml) and were incubated in the presence of the antibiotic. Sedimentation data in Fig. 5 show that degradation of host DNA was effectively blocked when CM was added at the time of infection, confirming the observations of Nomura et al. (14). Addition of CM at 5 or 7 min after infection, followed by incubation for an additional 25 or 23 min, resulted in "freezing" of the degradation of host DNA. Similar results (Fig. 6) were obtained by CM treatment of *am* N94-infected *E. coli* ER22. The kinetics of production of acid-soluble nucleotides from *am* N122-infected CR34 and B3 cells (Fig. 7) also indicated the arrest of hydrolysis of host DNA when CM was added at 0, 5, 7, and 10 min after infection. Degradation continued, however, when the antibiotic was added at 12 min or later, with incubation continued until 30 min after infection. Another inhibitor of protein synthesis, puromycin (200 μg/ml), gave identical results. In addition, mixing experiments with extracts from cells not treated with CM showed rapid hydrolysis of *E. coli* DNA in the presence of CM (S. K. Bose, *unpublished data*), indicating that deoxyribonuclease activity per se is not inhibited by CM or puromycin.

Inhibition of host functions in the absence of extensive degradation of host DNA. A sensitive test of phage-specific inhibition of host functions is the arrest of synthesis of β-galactosidase after infection of induced *E. coli* with T-even bacteriophages (2, 6). Does such an inhibition occur when the infecting phage is incapable of causing extensive degradation of the host genome? The data in Fig. 8 show that infections with $T4^{uv}$ or *am* N94, both of which cause only partial breakdown of the DNA of *E. coli* B3 (20), resulted in very efficient inhibition of synthesis of β-galac-

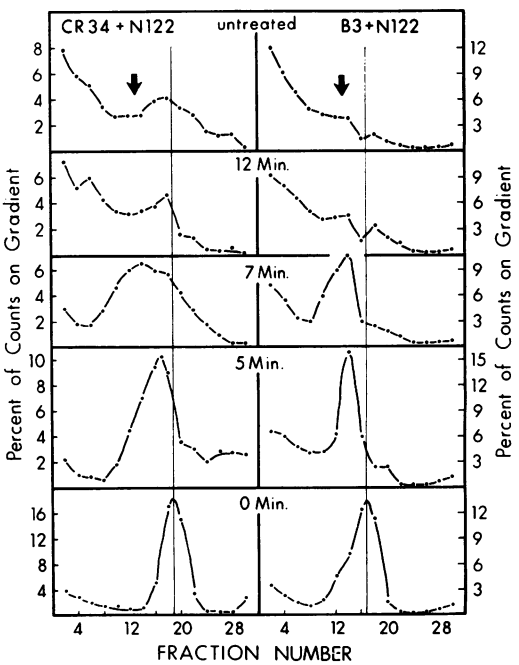

FIG. 5. *Effect of CM on the degradation of DNA of E. coli B3 and CR34 after infection with am N122. The cells were grown in the presence of 50 μc of thymidine-methyl-^3H per ml and other required supplements. Washed cells were infected at a multiplicity of 5. At the indicated times (0, 5, 7, and 12 min), samples of infected cells were removed to 100 μg of CM per ml, and incubation was continued at 37 C until 30 min after infection. Preparation of lysates, centrifugation, and analysis of samples were done as described in the legend to Fig. 1. Input per gradient = 1.2 to 1.8 × 10^4 counts/min.*

tosidase by the IPTG-induced host. Incorporation of uridine-5-^3H into acid-insoluble material (RNA) by the induced uninfected and infected bacteria is also represented in this figure. It is

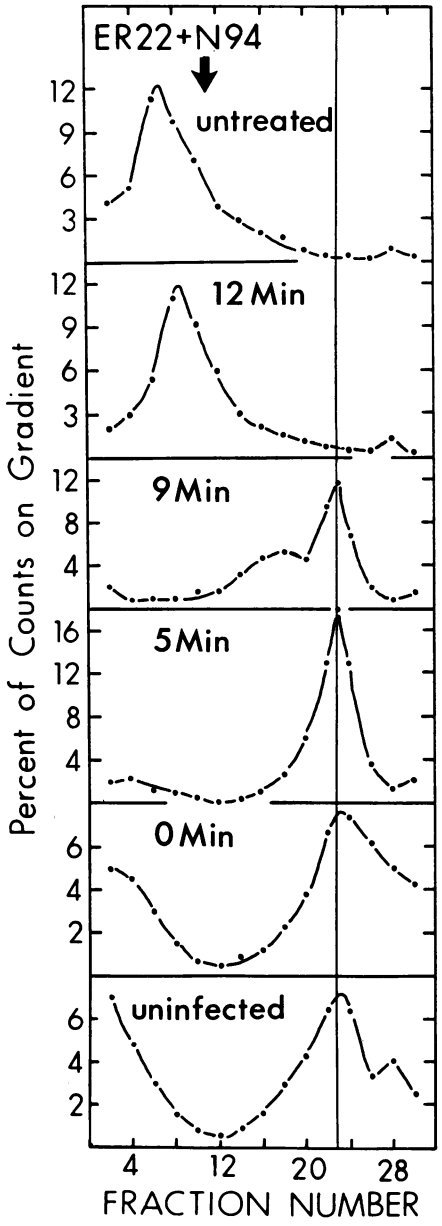

FIG. 6. *Effect of CM on the degradation of DNA of E. coli ER22 after infection with am N94. The cells were grown as described in the legend to Fig. 1 and were infected at a multiplicity of 5; samples of infected cells were removed to CM (100 μg/ml) at indicated times. After additional incubation at 37 C with aeration (total after infection = 30 min), samples were frozen in dry ice-ethyl alcohol, lysed, and centrifuged. Input per gradient = 10^4 counts/min.*

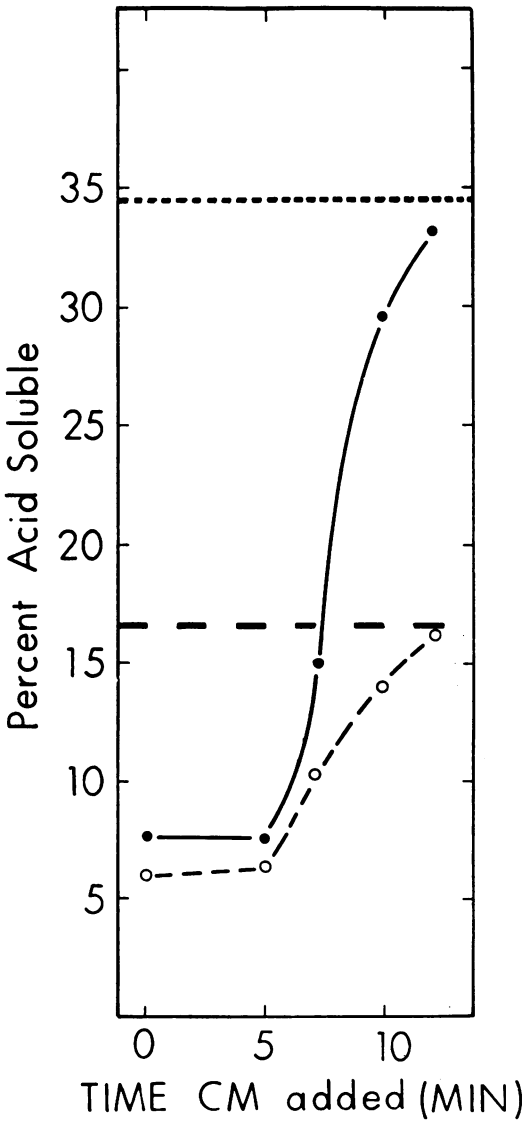

FIG. 7. *Effect of CM on the acid-solubilization of DNA in am N122-infected E. coli B3 and CR34. Samples of cells described in the legend to Fig. 5 were analyzed as detailed in the legend to Fig. 4. The time indicated on the abcissa is the time CM (100 μg/ml) was added. Symbols: CR34 + am N122, ○; B3 + am N122, ●. The dashed lines (small dashes, B3; large dashes, CR34) represent the maximum amount of acid-soluble nucleotides at 30 min in untreated infected cells.*

evident that net synthesis of RNA came to a halt rapidly after infection with T4, $T4^{uv}$, *am* N122, and *am* N94. These data suggest that the time of initiation of degradation of host DNA coincides fairly well with the establishment of inhibition of host-specific transcription and translation.

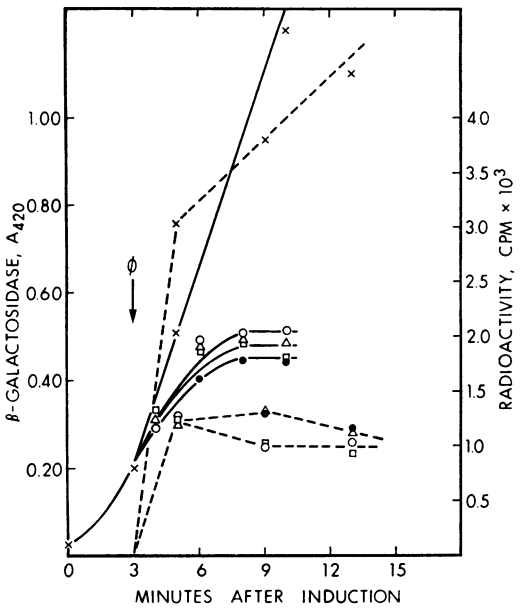

FIG. 8. *Inhibition of synthesis of β-galactosidase and uridine incorporation by E. coli B3 after infection with T4, T4^{uv}, am N122, and am N94. A 12-ml culture was grown to a density of 2 × 10^8/ml and induced with IPTG (5 × 10^{-4} M). Three minutes after induction, 2.0-ml samples were removed for infection with T4 (○), T4^{uv} (△), amN122 (●), and am N94 (□) at 10 phage per bacterium. From the uninfected culture, 0.20 ml was removed to uridine-5-^{3}H (0.1 μc, 0.2 μg), and 50-μliter samples were spotted on filter paper discs for measurement of uridine incorporation. The number of viable bacteria was also determined at this time (3 min). Samples of phage-infected cells were similarly treated for uridine incorporation and the number of survivors after infection. β-Galactosidase activity was determined in 100-μliter samples. Solid lines represent β-galactosidase activity, and dashed lines represent incorporation of uridine-5-^{3}H into trichloroacetic acid-precipitable material. The number of survivors decreased by 90 to 99% within 6 min after infection with all phages.*

DISCUSSION

Increase in the deoxyribonuclease activity after infection of *E. coli* with T-even bacteriophages has been noted in many laboratories (Bose and Nossal, Fed. Proc. **23**:272, 1964; 8, 9, 17, 18). It has, however, not been possible to establish that viral and not bacterial genes account for the increase in deoxyribonuclease activity. The demonstration (22) that the amber mutants in genes 46 and 47 were unable to induce degradation of DNA of a nonpermissive strain of *E. coli* to acid-soluble material was the first indication that viral genes were involved in this process. Our earlier data (20) showed, in addition, that more than one deoxyribonuclease may be involved in the postinfection breakdown of host genome. This conclusion was based on the sequence of appearance of characteristic products of degradation of *E. coli* DNA after infection with T4 and two amber mutants, N122 and N94.

The information provided in this report and in an earlier (20) report about the degradation of host DNA after T4 infection can be summarized as follows. (i) The first degradation product (detectable after 5 min) is a rapidly sedimenting (70 to 85*S*) material which has been found after infection with T4, T4^{uv}, and a number of amber mutants. (ii) This material appears after infection of an endonuclease I-deficient host, with characteristic degradation products arising with time, including the extensive amount of acid-soluble nucleotides after infection with *am* N122. (iii) The primary product is degraded to more slowly sedimenting material (40 to 60*S*) during the next 2 to 5 min (7 to 10 min after infection). During this interval, we have never detected any acid-soluble nucleotides arising from the host DNA. This second endonucleolytic cleavage is not seen after infection with UV-irradiated phage. (iv) Treatment with CM or puromycin immediately after infection blocks any degradation of host DNA. More importantly, the antibiotic added 5 or 7 min after infection with continued incubation of the infected bacteria does not result in any further alteration in sedimentation characteristics of cellular DNA. Measurement of acid-soluble nucleotides arising from host DNA in infected cells, treated with CM at various times and with a total infection period of 30 min, has provided confirmatory data. (v) Infections that do not lead to extensive degradation of cellular DNA, i.e., with UV-irradiated phage or with *am* N94, can effectively inhibit further synthesis of β-galactosidase by IPTG-induced *E. coli*.

Our interpretation of the foregoing data is that the first virus-induced endodeoxyribonuclease, which acts at a very limited number of sites, is made 5 to 7 min after infection with T4, T4^{uv}, *am* N122, *am* N122^{uv}, and *am* N94. The product is of high molecular weight. A second endodeoxyribonuclease is then synthesized, and this enzyme is probably not made after infection with UV-irradiated phages and *am* N94 (gene 46). The product of the second endodeoxyribonuclease becomes available to exonucleolytic attack by a virus-induced exodeoxyribonuclease(s) incapable of hydrolyzing high-molecular-weight DNA. Such an enzyme has been isolated from extracts of T2- and T4-infected *E. coli* (16, 17). Similar enzyme activity has been found after infection with *am* N122 and *am* N94 (16). Therefore, it

appears that the mere presence of an exodeoxyribonuclease in the infected cell does not result in the production of acid-soluble nucleotides, e.g., in T4^{uv}- or *am* N94-infected bacteria. The role of de novo synthesized proteins, as shown by the CM experiments reported here, provides further support for postulating sequential nuclease synthesis and activity in T4-infected bacteria. The possibility that the first endodeoxyribonuclease is unusually unstable has been considered as an alternative explanation of the kinetics of degradation in CM-treated infected cells; however, we have no other evidence to support this hypothesis.

Infection with UV-irradiated T2 leads to cell death and disorganization of the nuclear material (12), without breakdown of cellular DNA to acid-soluble material (4, 9). We have confirmed these observations and have shown, in addition, that endonucleolytic degradation of host DNA after infection with T4^{uv} is detectable. Since increased deoxyribonuclease activity can be found in extracts of *E. coli* infected with UV-irradiated T2 (Bose and Nossal, Fed. Proc. **23**:272, 1964; 9) and T4, it seems either that these deoxyribonucleases have very high specificity, at least in vivo, toward intracellular substrates or that they play a minor role in the degradation of host DNA. The latter may be ruled out by the results of our CM experiments.

Abolition of host-specific biosynthetic processes by infection with T4^{uv} and *am* N94, neither of which causes severe damage to the size of the host DNA, suggests that extensive breakdown of the host genome is not necessary. At present, little information is available about the localization of the bacterial genome and its relation to mRNA and protein-synthesizing machinery. It would be profitable to examine the effect of phage infection on such a complex.

Finally, although it seems attractive to propose that the rapid alterations in the sedimentation rate of host genome, reflecting degradation to smaller size or changes in the tertiary structure (or both), are responsible for the drastic inhibitions of host functions, we do not yet have any unequivocal evidence to support this hypothesis. Saturation hybridization experiments (7, 11) have indeed shown that synthesis of *E. coli* mRNA continues at a rate 15 to 30% of the normal rate between 3 to 4 min after infection with T4. On the other hand, this report shows that functional mRNA, at least for β-galactosidase, is shut off after infection with phages which do not cause extensive degradation of bacterial DNA. The mRNA measured by hybridization (7, 11) may be the result of a small fraction of host genomes which have not yet received the "inactivating" endonucleolytic cleavage.

The method, by which such cleavage may cause inactivation, is not defined by the available data. It is, however, not difficult to envision how disturbances by phage infection in the DNA-membrane-mRNA-ribosome complex constitute the mechanism mainly responsible for the death of the host.

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