Filamentous Bacterial Viruses

IV. Fate of the Infecting Parental Single-Stranded Deoxyribonucleic Acid

B. Y. TSENG, B. HOHN,¹ AND D. A. MARVIN

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520

Received for publication 18 April 1972

Some of the infecting fd single-stranded viral deoxyribonucleic acid (DNA) molecules were found to be degraded after having initiated infection. The degradation products were reused for synthesis of DNA, primarily bacterial. Degradation was most extensive when viral DNA was replicating.

F-specific filamentous bacterial viruses (Ff) contain single-stranded circular deoxyribonucleic acid (DNA) in the virion. After bacteria had been infected with a virion containing radioactive parental DNA, the radioactivity was found at the buoyant density expected for a duplex DNA molecule. If the infecting single-stranded DNA was labeled with heavy atoms as well as with radioactive ones, the DNA was found at the buoyant density expected for a hybrid duplex, half heavy and half light. These experiments indicated that Ff single-stranded viral DNA, like that of ϕ X174, is converted upon infection to a duplex molecule by new synthesis of a complementary DNA strand (16).

As infection proceeded, some of the parental label was found to be attached to fast-sedimenting components, perhaps the bacterial cell envelope. In part, the label was found in DNA molecules which sedimented faster than the infecting viral DNA; these molecules have been interpreted as replicative intermediates (5, 13, 24). After the initial involvement in replication, a portion of the parental label was released in progeny virions (1, 13, 17, 27). However, a significant fraction of the parental DNA label was neither released in progeny virions nor retained as intracellular virus-specific DNA; rather, it was incorporated into bacterial DNA (16).

Initially, we speculated that this DNA might be inserted into the host chromosome as an intact piece, like λ prophage or simian virus 40 DNA (21). This hypothesis was especially attractive because bacteria which are infected with Ff continue to grow and divide while retaining the ability to produce virus (9-11, 15). Incorporation of viral DNA into the chromosome could be directly related to the formation of such a carrier state. However, further experiments indicated that in-

corporation of parental viral DNA label into bacterial DNA is a consequence of degradation of parental DNA and reuse of the degradation products for new DNA synthesis. This degradation of parental DNA has already been briefly described (16).

MATERIALS AND METHODS

Solutions and media. N1 solution contained 0.5 g of NaCl, 8.0 g of KCl, 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 12.1 g of tris(hydroxymethyl)aminomethane (Tris), 1 liter of water, 0.087 g of KH_2PO_4 , and 0.1 ml of 1 M CaCl_2 ; the pH was adjusted to 7.4 with HCl.

N7 medium contained 10 g of tryptone (Difco), 1 g of yeast extract, 8 g of NaCl, 1 g of glucose, 0.5 g of Tris, and 1 liter of water.

N12 medium contained 300 ml of N1 solution plus 9.5 ml of the following mixture: 1 ml of 1 M CaCl_2 , 5 ml of 0.2 g/ml NH_4Cl , 1 ml of 1 $\mu\text{g/ml}$ FeCl_3 , 10 ml of 20% glucose, 10 ml of 5% Casamino Acids, and 5 ml of 2 mg/ml thiamine.

N13 medium was N12 medium without Casamino Acids.

Tris-EDTA solution was 0.05 M Tris-0.003 M ethylenediaminetetraacetate, adjusted to pH 8 with HCl.

Chemicals. CsCl was Sequanal Grade, from Pierce Chemical Co. Sarkosyl NL 97 was from Geigy Chemical Co.; chloramphenicol and ethidium bromide, from Calbiochem; mitomycin C, from Nutritional Biochemicals Corp.; tryptone, yeast extract, and Casamino Acids (vitamin-free), from Difco; and ^3H -methyl-thymine, ^3H -methyl-thymidine, and ^{32}P -orthophosphoric acid (carrier-free), from New England Nuclear Corp.

Bacterial and virus strains. Bacterial strain DM6 is strain 165/1, HfrH, Thy⁻, T1-resistant, obtained from F. Bonhoeffer. Bacterial strain DM48 is strain S26-6, HfrC, Sup⁻, pho⁻, λ^- , obtained from R. Wilhelm. Bacterial strain DM70 (12) is a male derivative of strain HF4704 (14). Bacterial strain DM117 is strain KMBL 42 F⁺ hcr⁻ (13, 27), obtained from P. H. Hofschneider. Wild-type fd was used throughout.

Preparation of radioactive virus. Preparation of fd

¹ Present address: Biozentrum der Universität Basel, Basel, Switzerland.

labeled with ^{32}P (^{32}P -fd) followed the scheme used for labeling ϕX174 (22). A 10-ml culture of bacteria was grown to 2×10^8 bacteria/ml in N12 medium. The bacterial strain used was homologous to the strain to be infected in subsequent experiments, to avoid modification and restriction problems (2). The bacteria were infected with fd at a multiplicity between 1 and 10 plaque-forming units (PFU)/bacterium, and 2 mCi of ^{32}P was added to the culture. After 4 to 6 hr of incubation with shaking, the bacteria were removed by low-speed centrifugation.

The supernatant fluid was passed over a Sephadex G-100 column (2 cm by 15 cm). The column was prepared by autoclaving (10 min, 15 psi) a suspension of Sephadex in 0.05 M Tris-hydrochloride, pH 7.5, which was also the buffer used for washing and eluting the column. The virus in the peak of radioactivity (detected with a hand radiation monitor) was further purified and concentrated by CsCl equilibrium centrifugation. To each 4.5 g of liquid, 2.0 g of CsCl was added, and the mixture was centrifuged for 18 hr at 40,000 rev/min and 4°C in a Spinco SW 50.1 rotor. About 20 fractions were collected, and the peak fractions were combined and dialyzed against 0.05 M Tris-hydrochloride, pH 7.5. To avoid loss of virus titer during dialysis, Visking dialysis tubing was boiled three times in 5% NaHCO_3 -0.5% sodium citrate, with rinsing between boilings; it was then boiled again three times in distilled water and stored in 50% ethanol. Before use, the tubing was rinsed and boiled again in distilled water.

The yield of purified virus was 10^{12} to 10^{13} PFU from 10 ml of culture, with a specific activity of about 10^{-5} counts per min per PFU. N12 medium contained about 22 μg of P/ml (20 μg /ml in KH_2PO_4 and about 1.7 μg /ml in the Casamino Acids). Therefore, addition of 0.2 mCi/ml gave a specific activity of 1 ^{32}P atom per 32,000 ^{31}P atoms, or 0.2 ^{32}P atom per virion. This is 7×10^{-6} disintegrations per min per virion. A preparation with only 0.2 ^{32}P atom per virion contains very few virions with more than 1 ^{32}P atom, so virtually every virion that has been inactivated by a ^{32}P decay between the time of preparation and the time of use will be invisible to detection. This is not true for virions labeled with ^3H -thymine. Because of the long half-life of ^3H , a preparation with a specific activity of 10^{-6} counts per min per virion has several hundred ^3H atoms per virion. Since each ^3H decay has a 50% chance of being lethal (6), such a preparation contains an appreciable fraction of dead virions only a few days after preparation, and virtually all of the dead virions are radioactive. Therefore, ^3H should be used with caution for labeling stocks of small virus.

Virus labeled with 5-bromouracil (BU) as well as ^{32}P were grown in strain DM6 in the presence of BU (26).

Mitomycin C treatment. Bacteria were treated with mitomycin C as described by Lindqvist and Sinsheimer (14), except that the bacteria were concentrated five-fold before being treated with 50 μg of mitomycin C/ml for 10 min. The treated bacteria were washed and resuspended in fresh media before infection.

Lysis of bacteria. For analysis of DNA by ultracentrifugation, bacteria were washed three times with

Tris-EDTA in a Sorvall SS-34 centrifuge at 5,000 rev/min for 5 min at 5°C, and were resuspended with 100 μg of lysozyme/ml in Tris-EDTA with 0.01 M EDTA. Sarkosyl was added to give 0.5%. The extract was analyzed directly by ultracentrifugation, or it was first digested with 500 μg of Pronase/ml for 2 hr at 37°C, or it was extracted with phenol (18) before centrifugation.

Centrifugation. Neutral CsCl equilibrium gradients contained 2.65 g of CsCl and 2.0 g of sample in Tris-EDTA. Ethidium bromide-CsCl equilibrium gradients contained 2.25 g of CsCl, 2.3 g of sample in Tris-EDTA, and 230 μg of ethidium bromide. Alkaline CsCl equilibrium gradients contained 4.0 g of CsCl and 2.9 g of sample in 0.05 M Na_2PO_4 -0.003 M EDTA, adjusted to pH 12.5 with 1 N NaOH.

Sucrose gradients were to 5% sucrose in 0.5 M NaCl, 0.05 M Tris, and 0.003 M EDTA, pH 8.

Samples for shearing were collected from a CsCl equilibrium gradient and mixed with sufficient bacterial DNA to be detected with the ultraviolet optics of a Spinco model E ultracentrifuge. The mixture was sheared (8) in a VirTis 45 homogenizer, at about 35,500 rev/min at 0°C for 1 hr. A portion was analyzed by ultracentrifugation to determine the value of $S_{20,w}$ in 1 M NaCl. The mean weight was calculated from the sedimentation coefficient (25). The remainder of the sample was analyzed by equilibrium centrifugation to determine the buoyant density of the radioactive DNA.

Samples for determination of the amount of radioactivity were either collected directly on Whatman GF/A filters or they were collected in wells of a plastic tray (Linbro Chemical Co.) and a portion was transferred to a filter. The filters were washed with 10% trichloroacetic acid and acetone, and were dried for radioactive counting in 3 ml of toluene scintillation fluid (8). Overlap of ^3H radioactivity into the ^{32}P channel was less than 10^{-4} .

Acid-soluble pool. Samples (2 ml) of a culture infected with ^{32}P -fd were transferred into 0.1 ml of 20% sodium dodecyl sulfate (SDS) at 70°C. The mixture was incubated for 2 min and then chilled in an ice bath. Next 0.2 ml of a 70% trichloroacetic acid solution was added, and the mixture was stored for at least 30 min in ice. The samples were poured over B-6 membrane filters (Schleicher & Schuell Co.) 2.4 cm in diameter. The solution which came through the filter was collected and mixed with Triton X-100 scintillation fluid, to measure acid-soluble radioactivity. This measurement of acid-soluble radioactivity was reproducible to $\pm 20\%$. The filters were washed further, dried, and counted in toluene scintillator fluid, to measure acid-precipitable radioactivity. The counting efficiencies of ^{32}P in the two methods were similar. Results are presented as the percentage of total recovered radioactivity which was found in acid-soluble form.

RESULTS

Replication of parental viral DNA. The buoyant density of the viral duplex which contains the infecting parental DNA is 1.701 g/cm³, slightly

less than the density of bacterial DNA, 1.710 g/cm³ (20). Therefore, these two types of DNA can be distinguished in CsCl equilibrium gradients by labeling them with different radioactive isotopes (16; Fig. 1a). In the experiment shown in Fig. 1, the effective multiplicity of infection was

FIG. 1. Fate of parental viral DNA. A 20-ml culture of DM6 was grown at 37°C in medium N13 supplemented with thymine (20 µg/ml), deoxyadenosine (100 µg/ml) and ³H-thymidine (1 µCi/ml) for 3 hr, to a density of 3×10^8 bacteria/ml. Then 60 µg of chloramphenicol/ml was added, and 10 min later

about 0.3 PFU/bacterium, so few of the bacteria could have received more than one parental DNA molecule. After replication had proceeded for 10 min in the presence of BU, a large fraction of the parental label was found at hybrid density, as expected for semiconservative replication of duplex DNA (Fig. 1b). The buoyant density of the hybrid viral DNA was slightly less than that of hybrid bacterial DNA, as expected from the fact that the complementary strand of the viral duplex has a lower proportion of thymine than does bacterial DNA. When this replicated viral DNA was recentrifuged in the presence of ethidium bromide, most of it was found at a density greater than that of bacterial DNA, as expected for form I, the closed circular duplex (Fig. 1d). After replication had proceeded for 60 min in the presence of BU, the parental label was found largely at the density of hybrid bacterial DNA (Fig. 1c). When this DNA was recentrifuged in the presence of ethidium bromide, most of it was found at the same density as bacterial DNA (Fig. 1e). We interpret this experiment to indicate that parental duplex DNA first replicates semiconservatively, but then becomes incorporated into bacterial DNA. Some of the ³²P parental label was recovered at the density of light bacterial DNA at 10 min after the addition of BU (Fig. 1b). This indicates either that some parental viral nucleotides are incorporated into bacterial DNA at the growth point before BU displaces the residual thymine pool or that parental viral nucleotides are incorporated into bacterial DNA which has not yet replicated semiconservatively, perhaps as a result of "repair replication."

2×10^8 PFU of radioactive fd/ml (labeled with ³²P at a specific activity of 10^{-5} counts per min per PFU, 4 days old) was added. At 30 min after infection, the bacteria were centrifuged, washed, and resuspended in N13 containing 100 µg of deoxyadenosine/ml. A 2-ml portion was removed for sample a and for titer (9×10^7 PFU/ml), and 20 µg of 5-bromouracil (BU)/ml was added. At 10 min after the addition of BU, an 8-ml portion was removed for sample b and for titer (2×10^8 PFU/ml); the remainder was collected at 60 min after addition of BU for sample c and for titer (3×10^8 PFU/ml). Samples a, b, and c were washed, lysed, and analyzed by CsCl equilibrium gradient centrifugation. Half of each fraction from samples b and c was preserved, and the other half was measured in a liquid scintillation spectrometer. The remaining portions of fractions 14–19 from sample b and 15–18 from sample c were separately pooled to give samples d and e, and were re-run in CsCl equilibrium gradients containing ethidium bromide. The radioactivity recovered in the peak heavier than bacterial DNA is 65% of the total for d and 25% for e. Bacterial (³H) DNA, ●; in gradient b, the hybrid bacterial DNA is plotted on an expanded scale, ▲. Parental viral (³²P) DNA, ○.

Incorporation into bacterial DNA. The shift in buoyant density of parental DNA label, from the density of the duplex viral DNA to the density of bacterial DNA, is most easily explained by a physical incorporation of viral DNA into bacterial DNA. Linkage of viral DNA to bacterial DNA by a protein was ruled out by extracting the DNA with hot phenol or with SDS and phenol, or by digesting with Pronase. In all cases, most of the intracellular parental DNA label was found at a density of 1.710 g/cm³ by 60 min after infection.

Although the input multiplicity (number of PFU added per bacterium) was as high as 50 PFU/bacterium in some of these experiments, the effective multiplicity (number of radioactive virions per bacterium after washing, determined from the specific activity) was of the order of one or less, because the number of adsorption sites is limited. However, to be sure that the incorporation into bacterial DNA is not solely a function of superinfecting virions which do not initiate infection, we infected with an input multiplicity of 0.1 PFU/bacterium. Again, the label in parental DNA moved to the density of bacterial DNA.

The kinetics of this shift in density were studied by taking samples for equilibrium gradient centrifugation at various times after infecting bacteria with ³²P-fd. The change in mean density of the radioactivity, from 1.701 g/cm³ to 1.710 g/cm³, started by 10 to 15 min after infection, and was largely complete by 30 min after infection (Table 1). The time course of the shift was roughly the same whether the bacteria were grown in a rich medium (N7), where they have a doubling time of 20 min, or in a poor medium (N13), where they have a doubling time of 100 min. Therefore, the shift does not take place at only one specific time during a bacterial generation. Between 50 and 90% of the parental label which was present in viral duplex DNA at zero time was recovered at bacterial density when the shift was complete, in several experiments.

The shift in density was averted by some conditions which also prevent replication of viral DNA. The shift did not take place after infection with viral strains which carry an *amber* mutation in gene 2, a gene required for viral DNA replication throughout the virus life cycle (B. Y. Tseng, Ph.D. Thesis, Yale Univ., New Haven, Conn., 1972). The shift did not proceed in bacteria which were transferred to nonpermissive temperature after permissive infection with a viral strain carrying a temperature-sensitive mutation in gene 2. The shift did not take place after infection in the presence of 100 µg of chloramphenicol/ml, which prevents viral DNA replication (18).

The shift in density was also averted by condi-

TABLE 1. Kinetics of the shift of parental viral radioactivity from viral duplex density to bacterial duplex density^a

Medium	Start time (min)	Finish time (min)	Fraction remaining (%)	Eclipse period (min)	Doubling time (min)
N7.....	6	18	50	10	22
N12.....	10	30	60	12	60
N13.....	10	25	50	16	100

^a Bacterial strain DM48 was grown in the medium indicated to about 10⁸ bacteria/ml and was infected with ³²P-labeled fd at a multiplicity of 10 to 100 PFU/bacteria. At various times after infection, samples were analyzed by CsCl equilibrium centrifugation, as described in Fig. 2a. The start time is the earliest time at which parental label was detectable at bacterial density; the finish time is the time beyond which no increase in parental label at bacterial density was observed. Fraction remaining is the fraction of parental label present at zero time (after washing the bacteria) which was found in the bacteria at the finish time. Eclipse period was determined in a separate experiment, done with a low multiplicity of infection. Doubling time is the doubling time of the infected bacteria.

tions which prevent replication of bacterial DNA, and by some defects in bacterial DNA recombination. The shift did not take place in bacteria treated with mitomycin C, which do not synthesize bacterial DNA but do synthesize viral DNA (18). The shift did not take place in the presence of concentrations of nalidixic acid sufficient to prevent both bacterial and viral DNA replication. The shift was substantially reduced in bacteria which have defects (4) in the *recB* or *recC*, but not *recA*, loci (Tseng, Ph.D. Thesis).

In band centrifugation on sucrose gradients, parental viral DNA sedimented independently of bacterial DNA at early times after infection, but sedimented rapidly, with bacterial DNA, at later times. This was most clearly seen when viral DNA label was selected from an equilibrium gradient and recentrifuged in a velocity gradient, but it was also seen when the entire intracellular DNA was examined by velocity gradient centrifugation. In one such experiment (Fig. 2), we used strain DM117, a strain previously used to study transfer of parental label to progeny virions (13, 27). The bacteria were labeled with ³H-thymidine before infection with ³²P-fd, and the sedimentation behavior of the extracted DNA was examined at 5-min intervals after infection. Analysis of the DNA by equilibrium centrifugation (Fig. 2a) showed viral DNA label initially separate from bacterial DNA label, but becoming virtually coincident as the infection proceeded. Therefore,

FIG. 2. Association of parental viral DNA with bacterial DNA. A 50-ml culture of DM117 was grown at 37 C in medium N7 to a density of about 10^7 bacteria/ml; 0.2 μ Ci of ^3H -thymidine/ml was added, and growth was continued to 2×10^8 bacteria/ml. Then 200 μg of thymidine/ml was added, followed by 6×10^9 ^{32}P -labeled fd, 6 days old. After 5 min of incubation, the bacteria were centrifuged and washed three times in N1 at 4 C and were resuspended in 50 ml of prewarmed N7 at 37 C. Samples of 5 ml were removed into 0.25 ml of 0.4 M NaN_3 at 0, 5, 10, 15, 20, 30, 40, 50, and 60 min after resuspension. The samples were washed, lysed, and analyzed by CsCl equilibrium centrifugation (a) and sucrose gradient velocity centrifugation in 0.5 M NaCl (b). Samples were also taken for bacterial titer (colony formers) at various times after resuspension: 0 min, 10^8 bacteria/ml; 20 min, 10^8 bacteria/ml; 40 min, 2.5×10^8 bacteria/ml. (a) Centrifuged in an SW 50.1 rotor, 27,000 rev/min, 48 hr, 17 C. (b) Centrifuged in an SW 50.1 rotor, 35,000 rev/min, 3 hr, 17 C. Arrows indicate the positions of markers in parallel gradients: fd, infectious virions; V, viral single-stranded DNA; I, form I, the supercoiled circular duplex; II, form II, the relaxed circular duplex. Bacterial (^3H) DNA, $\bullet$; viral (^{32}P) DNA, $\circ$.

in this strain, over half of the infecting viral DNA was incorporated into bacterial DNA.

Samples from the same experiment were also analyzed by velocity centrifugation (Fig. 2b). Initially, much of the viral DNA was found at the position of form I and form II, the closed and open duplex forms. However, by 40 min much of the viral label sedimented rapidly, with the speed of

bacterial DNA. Up to 30 min after infection, bacterial DNA sedimented in a broad band centered at about 50S, faster than viral DNA; but, later than 30 min after infection, the bacterial DNA was found towards the bottom of the tube, implying a sedimentation coefficient of 80S or more. Since all samples were treated identically, the observed change in sedimentation behavior of

the bacterial DNA suggests that it may be especially sensitive to partial degradation during extraction in the early stages of fd infection, although the effect has not been studied in detail. In this experiment, and some others, a considerable amount of radioactivity was observed at the sedimentation position of whole virions, although the bacteria were washed after adsorption and again before lysis of each sample.

Strain DM117 is essentially the same as the strain used by others to study attachment of parental DNA label to host membrane, and to study longer than unit length viral DNA label (13, 24). Since we found that parental viral DNA label sediments rapidly because it is incorporated into bacterial DNA, it should be demonstrated directly (perhaps by DNA-DNA hybridization, or by use of density-labeled parental DNA) that the rapidly sedimenting parental label found in the earlier experiments (13, 24) is contained in virus-specific DNA.

Parental viral DNA might be incorporated into bacterial DNA by a recombination event which inserts the viral duplex into the bacterial genome. If this notion were correct, then shearing the bacterial DNA that contains parental label to sufficiently small pieces should release a section of the viral genome, as demonstrated for lysogenized bacteriophage (8). However, shearing the bacterial DNA to pieces weighing about 1.9×10^6 atomic mass units (about half the size of the viral duplex DNA) revealed no shift in the buoyant density of the viral DNA label (Fig. 3). Several

experiments of this type, done with natural viral DNA (which yields a duplex slightly lighter than bacterial DNA) or with viral DNA labeled with ^{15}N , were all negative. In every case, viral DNA label was found at the same buoyant density as bacterial DNA, even after sufficient shear (by ultrasonic treatment) to reduce the size of the DNA molecules to less than 10% the size of viral duplex DNA.

Degradation of parental DNA. These experiments eliminated recombination between bacterial DNA and viral duplex DNA as an explanation for the incorporation of parental viral label into bacterial DNA. We then considered the possibility that parental viral DNA is degraded, and that the degradation products are reused for synthesis of DNA. Some parental DNA label was found in the acid-soluble pool of infected bacteria (Fig. 4). The amount of acid-soluble parental label was variable from one experiment to the next; for instance, in an experiment similar to that

FIG. 3. Shearing of DNA from infected bacteria. A 15-ml culture of strain DM6 was grown in N12 supplemented with $10 \mu\text{g}$ of thymine/ml to about 10^8 bac-

teria/ml, and $5 \mu\text{Ci}$ of ^3H -thymidine was added. Growth was continued to a density of about 3×10^8 bacteria/ml, and the culture was centrifuged and washed with medium N1 supplemented with $10 \mu\text{g}$ of thymine/ml. The bacteria were resuspended in 3 ml of N12 containing $10 \mu\text{g}$ of thymine/ml and $50 \mu\text{g}$ of chloramphenicol/ml, and were incubated for 15 min at 37°C . Then 10^{10} PFU/ml of fd, labeled with 5-bromouracil and ^{32}P , were added and incubation was continued for 30 min. The bacteria were centrifuged, washed with N1, and resuspended in 15 ml of N12 containing $20 \mu\text{g}$ of thymine/ml. One portion of 10 ml was taken for CsCl equilibrium gradient analysis immediately after resuspension. Parental label in this sample (not shown) was recovered at hybrid density, as expected (16). The remainder was incubated for 60 min and then analyzed by CsCl equilibrium gradient centrifugation (a). After the initial washing, the sample contained 1.6×10^8 bacteria/ml and 1.4×10^8 PFU/ml. After 60 min of incubation (sample a), there were 3.1×10^8 bacteria/ml and 10^{11} PFU/ml. This gradient (a) was fractionated into 48 fractions. Samples were taken to measure refractive index and thereby the CsCl density. Half of fractions 31–35 were pooled, and bacterial DNA was added. Half of the pooled fractions was sheared and the other half was not. For the sheared fraction, $S_{20,w} = 13.1\text{S}$, as determined by band centrifugation of a portion of this fraction in a model E ultracentrifuge. The remainder of the fractions were run in parallel in CsCl equilibrium centrifugation. The gradients were fractionated into 35 fractions so fractions 23–24 correspond roughly to fractions 31–32 of gradient a. The position of hybrid density is not shown on the unsheared (b) and the sheared (c) gradients; in several experiments of this type, no peak of parental label was found at this position. The shoulder at fractions 18–19 in c is due to background fluctuation, as can be seen from a. The vertical arrow in a indicates the position expected for hybrid DNA. Bacterial (^3H) DNA, ●; viral (^{32}P) DNA, ○.

FIG. 4. Acid-soluble parental label. Bacterial strain DM70 was grown at 37°C in medium N12 supplemented with 10 µg of thymine/ml to about 10^8 bacteria/ml. A 40-ml sample was centrifuged and resuspended in 8 ml of N12 supplemented with 20 µg of thymine/ml. Of this culture, 4 ml was treated with mitomycin C and the other 4 ml was left untreated. The two samples were incubated at 37°C with 180 µg of chloramphenicol/ml for 10 min and then infected with ^{32}P -fd (14 days old) at a multiplicity of 10 PFU/bacterium; the samples were incubated for a further 30 min. Each culture was then centrifuged, washed, and resuspended in 20 ml of medium N12 supplemented with 10 µg of thymine/ml. Samples of 2 ml were taken at successive times after resuspension to measure the percentage of the total radioactivity which was recovered in acid-soluble form. In a control experiment, less than 0.5% of the ^{32}P -fd used for infection was in acid-soluble form. MC-treated bacteria, ○; untreated bacteria, ●.

shown in Fig. 4, the acid-soluble radioactivity in the presence of 100 µg of chloramphenicol/ml reached only 3% of the total radioactivity by 40 min after infection of bacteria not treated with mitomycin C. Presumably, the acid-soluble pool is continually depleted for new synthesis, and parental label is removed from the pool as new parental label enters it. Slight variations in the relative rates of these two processes would explain the observed fluctuations in results.

After infection of bacteria which have been treated with mitomycin C to prevent bacterial DNA synthesis, the acid-soluble parental label reached 40% of the total parental label by 30 min after infection (Fig. 4). In one such experiment, the viral duplex DNA was selected from a neutral equilibrium gradient and reexamined by alkaline

equilibrium centrifugation, which separates (19) viral-strand DNA (1.763 g/cm³) from complementary-strand DNA (1.748 g/cm³). Considerable parental DNA label was found at the position of complementary-strand DNA (Fig. 5). This experiment further supports the notion that

FIG. 5. Alkaline CsCl equilibrium centrifugation of duplex DNA from mitomycin C-treated bacteria. Bacterial strain DM70 was grown in medium N12 supplemented with 10 µg of thymine/ml to 3×10^8 bacteria/ml and was treated with mitomycin C. The treated bacteria were resuspended in medium N12 supplemented with 20 µg of thymine/ml and incubated for 5 min at 37°C. Then 200 µg of chloramphenicol/ml and 6×10^8 PFU of ^{32}P -fd/ml were added and the bacteria were incubated for 20 min. The bacteria were washed twice with cold N1 and resuspended at 37°C in N12 supplemented with 20 µg of thymine/ml. At 0 and 40 min after resuspension, samples were removed and treated with lysozyme, sarkosyl, and Pronase, and twice extracted with phenol. The samples were centrifuged in an SW 39 rotor at 30,000 rev/min for 46 hr at 17°C in a CsCl equilibrium gradient. The region of double-stranded DNA was collected and dialyzed against Tris-EDTA, digested with deoxyribonuclease (18), and run on an alkaline CsCl equilibrium gradient with 2.7 g of CsCl and 1.9 g of 1 M Na₃PO₄, pH 12.5. The samples were centrifuged in an SW 39 rotor at 30,000 rev/min for 48 hr at 17°C. Arrows indicate the positions of markers in parallel gradients: I, form I, the denatured supercoiled circular duplex; V, viral single-stranded DNA; C, complementary-strand DNA (from denatured duplex form II). Bacterial (^{14}C) DNA, prepared in a separate experiment and added as a marker, ●; viral (^{32}P) DNA, ○.

labeled parental DNA is degraded and reused for synthesis of new DNA. In normal infection, when most DNA being made is bacterial DNA, the degraded material would be reincorporated to a large extent into bacterial DNA as well as into viral DNA, but, when bacterial DNA synthesis is inhibited by mitomycin C, a large fraction would be incorporated back into virus-specific DNA, both into the viral strand and into the complementary strand of the duplex. Labeled viral strands would be released in new virions, leaving intracellular DNA enriched in labeled complementary strands.

None of the parental ^{32}P was found in a form which was rendered acid-soluble by 1 N NaOH. Therefore, although parental DNA may be degraded to mononucleotides, ^{32}P is not released and incorporated into RNA.

DISCUSSION

Infecting fd viral DNA is converted from single-strand to duplex molecules, which replicate semiconservatively during the first 10 min after infection. At about 5 to 10 min after infection, parental label starts to disappear from viral duplex DNA. Some of this parental DNA is transferred intact to progeny virions (17). However, an appreciable fraction of the viral DNA molecules which have initiated infection are degraded to nucleotides and reused for DNA synthesis. This degradation requires active replication of virus-specific DNA. We do not know whether viral strands of the duplex other than the infecting viral strand are degraded, or whether any of the complementary DNA strands are degraded. There are apparently several possible fates which await an infecting viral DNA molecule. After having served as a template for a complementary copy, it may be degraded or transferred to progeny virions. In the course of replication, it may be bound to fast-sedimenting material. The nature of the decision on the fate of infecting viral DNA molecules is unclear.

The cause of the degradation is also not clear. Preliminary experiments (Tseng, Ph.D. Thesis) on the shift in density of parental label in *recB*⁻, *recC*⁻, and *endo I*⁻ bacteria, and experiments on the appearance of parental label in the acid-soluble pool of these strains, suggest a tentative model: endonuclease I degrades a small fraction of the parental DNA to an acid-soluble form (perhaps oligonucleotides) not suitable for incorporation into bacterial DNA, and does so even in the absence of viral DNA replication; whereas the enzymes controlled by the *recB* and *recC* loci degrade parental DNA to a form which can be incorporated into bacterial DNA, but do so only

during active DNA replication. Replicating viral DNA may have some characteristics in common with the normal substrates for recombination enzymes, perhaps single-stranded regions (7, 23).

The loss of intact parental DNA strands shortly after infection is consistent with the idea that the complementary rather than the viral strand is important for further viral replication (3, 16).

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-06524 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

- Arber, W. 1966. Host specificity of DNA produced by *Escherichia coli* 9. Host-controlled modification of bacteriophage fd. *J. Mol. Biol.* 20:483-496.
- Arber, W., and S. Linn. 1969. DNA modification and restriction. *Annu. Rev. Biochem.* 38:467-500.
- Baas, P. D., and H. S. Jansz. 1972. Asymmetric information transfer during ϕX174 DNA replication. *J. Mol. Biol.* 63:557-568.
- Clark, A. J. 1971. Toward a metabolic interpretation of genetic recombination of *E. coli* and its phages. *Annu. Rev. Microbiol.* 25:437-464.
- Forsheit, A. B., and D. S. Ray. 1971. Replication of bacteriophage M13. VI. Attachment of M13 DNA to a fast-sedimenting host cell component. *Virology* 43:647-664.
- Funk, F., and S. Person. 1969. Cytosine to thymine transitions from decay of cytosine-5- ^3H in bacteriophage S13. *Science* 166:1629-1631.
- Goldmark, P. J., and S. Linn. 1970. An endonuclease activity from *Escherichia coli* absent from certain *rec*⁻ strains. *Proc. Nat. Acad. Sci. U.S.A.* 67:434-441.
- Hoffman, D. B., Jr., and I. Rubenstein. 1968. Physical studies of lysogeny. I. Properties of intracellular parental bacteriophage DNA from λ -infected sensitive bacteria. *J. Mol. Biol.* 35:375-399.
- Hoffmann-Berling, H., H. Dürwald, and I. Beulke. 1963. Ein fädiger DNS-Phage (fd) und ein sphärischer RNS-Phage (fr) wirtsspezifisch für männliche Stämme von *E. coli*. III. Biologisches Verhalten von fd und fr. *Z. Naturforsch.* 18b:893-898.
- Hoffmann-Berling, H., and R. Mazé. 1964. Release of male-specific bacteriophages from surviving host bacteria. *Virology* 22:305-313.
- Hofschneider, P. H., and A. Preuss. 1963. M13 bacteriophage liberation from intact bacteria as revealed by electron microscopy. *J. Mol. Biol.* 7:450-451.
- Hohn, B., H. Lechner, and D. A. Marvin. 1971. Filamentous bacterial viruses. I. DNA synthesis during the early stages of infection with fd. *J. Mol. Biol.* 56:143-154.
- Kluge, F., W. L. Staudenbauer, and P. H. Hofschneider. 1971. Replication of bacteriophage M13. Detachment of the parental DNA from the host membrane and transfer to progeny phages. *Eur. J. Biochem.* 22:350-354.
- Lindqvist, B. H., and R. L. Sinheimer. 1967. The process of infection with bacteriophage ϕX174 . XV. Bacteriophage DNA synthesis in abortive infections with a set of conditional lethal mutants. *J. Mol. Biol.* 30:69-80.
- Mach, F. 1970. Elektronenmikroskopische Untersuchung über die Vermehrung des filamentösen *Escherichia coli*-Phagen M13. *Z. Allg. Mikrobiol.* 10:321-328.
- Marvin, D. A., and B. Hohn. 1969. Filamentous bacterial viruses. *Bacteriol. Rev.* 33:172-209.
- Mechler, B., and W. Arber. 1969. Parental fd DNA is efficiently transferred into progeny bacteriophage particles

- even at low multiplicity of infection. *J. Mol. Biol.* 45:443-449.
18. Pratt, D., and W. S. Erdahl. 1968. Genetic control of bacteriophage M13 DNA synthesis. *J. Mol. Biol.* 37:181-200.
 19. Ray, D. S. 1969. Replication of bacteriophage M13. II. The role of replicative forms in single-strand synthesis. *J. Mol. Biol.* 43:631-643.
 20. Ray, D. S., A. Preuss, and P. H. Hofschneider. 1966. Replication of the single-stranded DNA of the male-specific bacteriophage M13. Circular forms of the replicative DNA. *J. Mol. Biol.* 21:485-491.
 21. Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40-transformed cells. *Proc. Nat. Acad. Sci. U.S.A.* 60:1288-1295.
 22. Sinsheimer, R. L., B. Starman, C. Nagler, and S. Guthrie. 1962. The process of infection with bacteriophage ϕ X174. I. Evidence for a "replicative form." *J. Mol. Biol.* 4:142-160.
 23. Sironi, G., H. Bialy, H. A. Lozeron, and R. Calendar. 1971. Bacteriophage P2: interaction with phage lambda and with recombination-deficient bacteria. *Virology* 46:387-396.
 24. Staudenbauer, W. L., and P. H. Hofschneider. 1971. Membrane attachment of replicating parental DNA molecules of bacteriophage M13. *Biochem. Biophys. Res. Commun.* 42:1035-1041.
 25. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* 11:373-390.
 26. Trenkner, E., F. Bonhoeffer, and A. Gierer. 1967. The fate of the protein component of bacteriophage fd during infection. *Biochem. Biophys. Res. Commun.* 28:932-939.
 27. Wirtz, A., and P. H. Hofschneider. 1970. Replication of the single-stranded DNA bacteriophage M13. Intracellular flow of parental DNA and transfer to progeny particles. *Eur. J. Biochem.* 17:141-150.