Breakdown and Exclusion of Superinfecting T-Even Bacteriophage in *Escherichia coli*

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In bacterial strains containing the deoxyribonuclease endonuclease I (endonuclease I⁺ strains), 70 to 80% of the injected superinfecting T-even phage deoxyribonucleic acid (DNA) is rapidly degraded to oligonucleotides having an average chain length of 8, the same value as that obtained by endonuclease I digestion of purified T-even phage DNA in vitro. In endonuclease I⁻ strains, less than 5% of the injected superinfecting T-even phage DNA is degraded to acid-soluble components. The superinfecting phage DNA is, however, fragmented into a large segment having a molecular weight of about 90×10^6 and 30 or more small acid-insoluble segments having molecular weights of less than 10^6 . In both endonuclease I⁺ and endonuclease I⁻ strains, over 80% of the DNA from adsorbed primary T2 or T4 phage, but only 50% of the DNA from adsorbed superinfecting T2 or T4 phage, is injected. Superinfecting T4 are genetically excluded as efficiently from endonuclease I⁻ strains as they are from endonuclease I⁺ strains. The excluded phage cannot complement defects in either early or late gene functions carried by the primary phage. The induction of both superinfection breakdown and superinfection exclusion requires a period of protein synthesis between primary infection and addition of the superinfecting phage. These observations seem best explained by failure of superinfecting DNA to enter the host cell cytoplasm, presumably as a result of changes in the cell envelope induced by the primary phage.

When *Escherichia coli* are infected simultaneously at high multiplicity with equal quantities of a T-even bacteriophage and a distinguishable mutant derived from the same phage (e.g., T2r⁺ and T2r), most cells produce mixed bursts, and the progeny consists of approximately equal quantities of both phage types (10, 12). If, however, a short interval separates the addition of the two phages, few of the infected cells produce mixed bursts, and the genetic markers of the secondary (or superinfecting) phage are largely excluded from the progeny. This phenomenon, known as "partial exclusion of the second kind" or more simply as superinfection exclusion, was first recognized by Dulbecco (12) although similar observations had previously been made in Delbrick's laboratory (9, 10). The induction of superinfection exclusion was shown to occur within 1 min of the primary infection and to require actively metabolizing cells (12). The extent to which the superinfecting phage is genetically excluded was shown to be dependent upon the multiplicity of infection (MOI) of both the primary and secondary phages (42).

A possible physical basis for superinfection exclusion had been established somewhat earlier by Lesley et al. (31) who had noticed that a large fraction of ³²P-labeled T2 deoxyribonucleic acid (DNA) became acid-soluble when labeled phage infected *E. coli* at high multiplicities. This breakdown was not found when the infections were carried out at low multiplicities, and they showed that the acid solubilization affected only late-adsorbing phage (which constituted a major fraction of phage in high-multiplicity experiments). When Lesley et al. (31) modified their protocol by performing a primary infection with unlabeled T2 and then allowed an interval as brief as 2 min before a secondary infection with ³²P-labeled T2, they observed that 50% of the adsorbed counts were rapidly solubilized. This phenomenon was called superinfection breakdown. Reviews of the early work on superinfection exclusion and superinfection breakdown have been presented by Graham (21) and by Adams (1).

Our interest in superinfection breakdown grew out of an earlier study (15) on the breakdown of nonglucosylated DNA from T*2 phage (T* restriction) in which it was shown that the

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periplasmic deoxyribonuclease of *E. coli*, endonuclease I, is not involved in restriction breakdown. To our surprise, a mutant of *E. coli* B deficient in endonuclease I (endonuclease I⁻ mutant) failed to carry out superinfection breakdown. This strain can, nevertheless, carry out superinfection exclusion. Fielding and Lunt (18) have recently reported a similar observation.

In this report we describe these observations as well as experiments concerning the nature of the changes caused by primary phage which lead to superinfection exclusion and breakdown. Breakdown appears to occur in at least two ways: superinfection fragmentation, the removal of about 25% of the superinfecting DNA as acid-insoluble pieces (occurs in endonuclease I⁻ strains); and superinfection solubilization, the reduction of nearly all of the injected superinfecting DNA to acid-soluble pieces (occurs only in endonuclease I⁺ strains). All of these phenomena may result from the failure (in both endonuclease I⁺ and endonuclease I⁻ strains) of injected superinfecting DNA to penetrate a cell envelope profoundly altered by the primary infection (35).

The following paper (2) describes autoradiographic experiments which indicate that superinfecting T4 DNA is in fact localized in the cellular envelope.

MATERIALS AND METHODS

Bacteria and phage. *Shigella sonnei* (ER58) and *E. coli* B (ER-1) were from the laboratory collection. Strains ER21 and ER22 are endonuclease I⁻ mutants isolated from *E. coli* B by Eigner and Block (15). The endonuclease I⁻ *E. coli* K-12 strain 1100 (ER130) and its endonuclease I⁺ parent 1000 (ER129) were originally obtained from H. Hoffman-Berling. Strain ER151 is an *E. coli* K12(λ) obtained from E. Goldberg, and strain ER218 (CR63) from S. Phillips is an *E. coli* K-12 (λ) Su⁺, permissive for amber mutants of T4.

Phage T2 was originally obtained from N. Melchen; T4r⁺ and T4rII were obtained from E. Goldberg; T4amN112 (defective in gene 42 product, deoxycytidylate hydroxymethylase) and T4amNG218 (defective in gene 7 product, tail endplate component) were obtained from S. Phillips; T4s (spackle mutant of J. Emrich) was provided by S. Silver. Purified T2-ghost particles were the gift of R. Fabricant.

Standard bacteriophage procedures were performed by the methods of Adams (1).

Media. L broth and L agar have been described (15). Top agar was Fisher Gram-Pac Phage Assay Overlay Agar. M9GC medium is the M9 medium of E. H. Anderson (3) supplemented with 2 g of technical Casamino Acids (Difco) per liter. TGCN medium, containing 11 μg of phosphorus per ml, was prepared by the method of Kozinski and Szybalski (27) except that 2 μg of niacin per ml was added, and Na₂SO₄ and CaCl₂ were omitted. Phage-diluting fluid (PDF)

contained 3 g of NaCl, 1 g of peptone (Difco) and 0.15 g of MgSO₄ per liter.

Enzymes. *E. coli* alkaline phosphatase, with a specific activity of 100 units per mg and more than 95% pure by electrophoresis, was the generous gift of M. J. Schlesinger. Endonuclease I was the partially purified enzyme obtained from the 35 to 67% ammonium sulfate precipitate of a Nossal-Heppel "stage I osmotic shockate" of a log-phase culture of *E. coli* B (34); the enzyme fraction had an activity of approximately 26,000 units per ml (a unit is defined as the acid solubilization of 1 nmole of DNA nucleotides in 30 min at 37°C).

Miscellaneous materials. Carrier-free ³²P-orthophosphate (sterile aqueous solution in 0.02 N HCl), thymidine-methyl-³H (³H-TdR; 1.9 Ci/mmmole), and ³H-leucine (1.9 Ci/mmmole) were obtained from Schwarz BioResearch, Inc. Rabbit anti-T4 serum (Gateway Immuno-Sera, Cahokia, Ill.) for determination of infective centers had a velocity constant of 246 per min at 37°C. Streptomycin and chloramphenicol were products of Eli Lilly & Co. and Parke, Davis & Co., respectively. Rifampin was obtained as a 5 mg/ml solution in 50% ethanol from D. Kennell.

Preparation of phage stocks and DNA. Stocks of ³²P-labeled T2 and T4 were prepared in log-phase cultures of *E. coli* B grown in TGCN. Cultures grown to 4 × 10⁸ cells per ml were centrifuged, resuspended in fresh TGCN, and infected at MOI of 5. After 5 min of incubation at 37°C, carrier-free ³²P-orthophosphate was added to the desired specific activity (2 to 20 μCi/μg of P). After 2 hr of further incubation, bovine serum albumin (BSA) was added to 300 μg/ml, and the culture was lysed by the addition of a small amount of chloroform. The lysate was clarified by low-speed centrifugation (3,000 × g, 10 min, 4°C), and the phage stock was purified by two cycles of high-speed pelleting (20,000 × g, 30 min, 4°C) followed by an additional low-speed centrifugation. The phage pellets were resuspended by gentle washing with small amounts of PDF containing 300 μg of BSA per ml. Labeled phage stocks generally had an optical density at 260 nm (OD₂₆₀) to plaque-forming units ratio of 0.7 × 10¹¹ to 1 10¹¹ and contained less than 2% acid-soluble radioactive material. Stocks of ³²P-labeled T*2 were prepared in an analogous manner on Sh/4₀, a T4-resistant, uridine diphosphoglucose-less mutant of ER58.

³H-TdR-labeled phage stocks were prepared on M9GC-grown *E. coli* B in a manner similar to that described above. Five minutes after infection, the culture was supplemented with ³H-TdR to 10 μCi/ml.

³H-leucine-labeled phage were prepared on *E. coli* B grown in M9. Seven minutes after infection, ³H-leucine was added to a concentration of 40 μCi/ml.

Unlabeled phage stocks were prepared on L broth or M9GC-grown cultures of the appropriate strain in a manner similar to that for the ³²P-labeled phage.

DNA was isolated from purified phage by a phenol procedure (41).

Measurement of superinfection breakdown. Most phage infection experiments were performed by the following protocol. The appropriate strain was grown to 2.5 × 10⁸ cells per ml in M9GC medium and

harvested at room temperature. The cell pellet was resuspended in the original volume of fresh M9GC medium, and a sample was transferred to a sterile flask for infection. Cultures to be superinfected with labeled phage were first infected with unlabeled phage at MOI 3 to 5 within 1 to 2 min after transfer of the resuspended culture to 37°C ("primary infection"). The labeled superinfecting phage was then added to the desired MOI (usually 0.5 to 5). When labeled phage were used in primary infections, they were generally added at low MOI (0.5 to 1.0) to reduce secondary infections resulting from late adsorbing phage.

Acid solubilization of the parental phage DNA was measured by removing 0.1-ml samples from the infected culture with an Oxford sampler. The samples were added to cold test tubes (10 by 75 mm) containing 0.1 ml of BSA-Na₃ mix (1.25 mg of BSA per ml, 0.026 M Na₃), and 0.1 ml of ice-cold 15% trichloroacetic acid was promptly added to each tube. After at least 10 min in an ice-water bath, the precipitate was pelleted by centrifugation ($6,700 \times g$, 10 min, 4°C), and 0.25 ml of the supernatant fluid was counted with 8 ml of TMC counting fluid in a liquid scintillation spectrometer (Packard model 3003). TMC counting fluid contained 9.3 g of 2,5-diphenyl-oxazole, 30 mg of dimethyl-1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, 375 ml of Methyl Cellosolve (ethylene glycol monomethyl ether), and 625 ml of toluene.

To measure the extent of phage DNA injection, a sample of the infected culture taken 15 min after primary infection was diluted into ice-cold M9GC medium containing 300 µg of BSA per ml (total volume 15 ml). The sample was transferred to an ice-cold Eberbach no. 8575 microblending container mounted on a Waring model 700B base and blended at top speed (ca. 10,000 rev/min) for 4 min. Samples (in duplicate) were assayed for total counts per minute and acid-soluble counts per minute before and after blending and for the complete culture and a supernatant fluid fraction (obtained by centrifuging 1.0 ml of sample for 1 min in an International model MB microcentrifuge equipped with a no. 923 rotor). The data obtained yielded per cent counts per minute cell-bound or free, and per cent counts per minute in each of these categories which were acid-soluble or acid-insoluble.

Control experiments with ³H-leucine-labeled T4 indicated that the blending regime sheared 70 to 80% of the phage-coat protein from the cell surface while lysing less than 10% of the culture (as measured by the release of induced β-galactosidase). The per cent of uninjected phage DNA was assumed to be equivalent to the per cent of acid-insoluble DNA counts released into the supernatant fluid fraction by blending.

Sedimentation analysis. Gentle lysates of infected cultures or blended samples were made by a slight modification of a procedure described by Frankel (19). Cells were separated from unadsorbed or "blended" phage particles by centrifugation at $3,000 \times g$ for 5 min in the cold and were gently resuspended in cold medium to about 3×10^8 cells/ml. To five volumes of

room-temperature "lysis juice base" [0.1 M NaCN, 0.05 M ethylenediaminetetraacetate (EDTA), pH 8.0] were added five volumes of cold cell suspension followed immediately by one volume of 30% (w/v) sodium lauroyl sarcosine. The lysates were mixed by gently rotating the tube and were then stored at room temperature.

The lysates were centrifuged through 10 to 35% (w/w) linear glycerol gradients which are equivalent in density and viscosity to the more commonly used 5 to 20% sucrose gradients. Neutral gradients contained 0.02 M tris(hydroxymethyl)aminomethane, pH 8.0; 0.01 M EDTA; and 0.1% sodium lauroyl sarcosine. Alkaline gradients contained 0.2 N NaOH, 0.01 M EDTA, and 0.1% sodium lauroyl sarcosine. The gradients (4.6 ml) were layered on 0.3 ml of Anglo-Conray (80% sodium iothalamate, Mallinckrodt Pharmaceuticals) in silicone-treated nitrocellulose tubes (6). Small volumes of ³H-TdR-labeled T4 or T4 DNA (or both) were added to the lysates to serve as markers. Neutral gradients were loaded with 0.2 ml of lysate; alkaline gradients were first overlaid with 0.15 ml of a denaturing solution (0.35 N NaOH, 0.1% sodium lauroyl sarcosine) and then with 0.15 ml of lysate. Loaded gradients were centrifuged in a Spinco SW-50 rotor.

After centrifugation, dibromoethylene or tetrabromoethylene (densities 2.170 and 2.964, respectively) was pumped into the bottom of the tube, forcing the gradient out through a special polypropylene cap at the top. The pumping speed was set to deliver a 0.2-ml fraction in 30 sec; each gradient yielded about 29 fractions. The bromoethylenes were used as pumping fluids because they are much denser than the gradient solutions or cushions, are completely immiscible with aqueous solutions, and do not affect counting efficiencies. Most gradients were collected directly into counting vials, each containing 8 ml of TMC counting fluid. The number of counts applied to each gradient was determined by mixing 0.1 ml of the lysate with an equal volume of 35% glycerol gradient solution and counting with 8 ml of TMC. The empty nitrocellulose centrifuge tubes were cut in half, and each half was dissolved in and counted with 15 ml of TMC. Recovery of label applied to the gradients was generally greater than 90%.

The procedure outlined above measures total counts from the gradient and was used for infections of ER22, where production of acid-soluble label is negligible. In this situation, total counts are equivalent to acid-insoluble counts. This conclusion was confirmed by trichloroacetic acid precipitation of fractions from selected control gradients.

Other procedures. Cell lysis was routinely assayed by the release of preinduced β-galactosidase into the medium. β-Galactosidase was assayed spectrophotometrically (25). The chain length of acid-soluble oligonucleotides was determined by measuring the fraction of monoesterase-sensitive phosphate by the method of Lehman et al. (30).

RESULTS

Superinfection solubilization: relationship to endonuclease I activity. Lesley et al. (32) demon-

strated that about 50% of the adsorbed DNA from ^{32}P -labeled T2 was degraded into acid-soluble material within a few minutes after the addition of the labeled phage to a culture of *E. coli* previously infected with T2, T4, or T6. We have confirmed this finding for the strains *E. coli* B (ER-1), *S. sonnei* (ER58), and *E. coli* K-12-1000 (ER129) with endonuclease levels (units per milligram with T2 DNA as substrate) of respectively 2,020, 934, and 165 (15). When these strains were superinfected with ^3H -TdR-labeled T2, 40 to 60% of the adsorbed phage label was rendered acid-soluble within a few minutes of superinfection. When the same phage stock was used to superinfect strain ER22, an endonuclease I⁻ mutant of ER-1 with 2 units per mg activity, or ER130, a mutant of ER129 with no detectable endonuclease I activity (15), only 5 to 10% of the adsorbed phage label was made acid-soluble.

Secondary phage injection. It was found that secondary phage adsorb as rapidly as primary phage, but "inject" DNA less frequently. The apparent correlation between solubilization and endonuclease I activity might have resulted from a specific failure of secondary phage adsorbed to the endonuclease I⁻ strains to transfer ("inject") their DNA. We therefore obtained an estimate of DNA transfer by measuring the fraction of adsorbed DNA which remained cell-associated after high-speed blending (23). Control experiments indicated that 70 to 80% of the adsorbed counts from ^3H -leucine-labeled phage could be removed from the cell surface with the blending procedure employed while less than 10% of the infected culture was lysed (*see above*). Five separate cultures of ER-1 and ER22 were infected at MOI 5 with unlabeled T4 and superinfected 5 min later with different fresh stocks of ^{32}P -labeled T4 (MOI 0.1 to 5); 15 min after primary infection, the superinfected culture was diluted into cold medium and blended. With ER22, $52 \pm 6\%$ of the adsorbed superinfecting DNA was blend-resistant; for ER-1, $62 \pm 8\%$ was blend-resistant. As a control, five cultures of ER22 were primary infected with the radioactive phage and blended 10 min afterwards. Here, $80 \pm 6\%$ of the adsorbed DNA was resistant to blending. Similar results were obtained with labeled T2. Thus, transfer of DNA from superinfecting phage particles adsorbed to the endonuclease I⁻ strain ER22 is nearly as efficient as transfer to the endonuclease I⁺ strain ER-1 ($t = 2.41$, $P < 0.05$). But superinfecting phage are significantly less successful than primary phage in transferring their DNA to *E. coli* ($t = 8.34$, $P < 0.001$ comparing secondary to primary in ER22; $t = 4.53$, $P < 0.005$, comparing secondary in ER-1 to primary in ER22).

No correlation between DNA transfer and MOI was noted for superinfecting phage, but for primary phage there was a reproducible inverse relationship with MOI. Thus, at MOI 7, the transfer was 70%; whereas, at MOI 1, the transfer was 80 to 85%. The likely explanation is that at high MOI, where many cells are multiply infected, the late adsorbing primary phage are in fact superinfecting phage with low transfer efficiency.

The decreased extent of transfer found for secondary phage was not due to slower adsorption since the initial adsorption rates of several unlabeled T4 stocks to ER22 grown in L broth were found to be about 6.0×10^{-9} ml per min (96% adsorption in 5 min) whether the culture had been previously infected or not.

Solubilization proportional to endonuclease I levels. In endonuclease I⁺ *E. coli* the process is rapid and extensive, and products appear in the medium. The kinetics of acid-solubilization after primary and secondary infection by ^{32}P -labeled T4 are given in Fig. 1. The extent of solubilization has been corrected for the noninjected fraction, and we see that 70 to 75% of the DNA from superinfecting T4 was solubilized within 15 minutes when ER-1 is the host. Superinfection of ER22, however, resulted in the solubilization of less than 6% of the injected T4 DNA; this same low level of acid solubilization was observed after primary infection of ER22 or ER-1 at low multiplicities.

Essentially identical results were obtained when T2 replaced T4. With T2, the solubilization was slightly slower, and it continued beyond 15 min. These differences can probably be accounted for by the slower adsorption of T2 to ER-1 and ER22.

An interesting feature of superinfection solubilization is that the acid-soluble products appear in the extracellular medium very rapidly. At 5 min after superinfection of ER-1, more than 70% (and at 10 min 100%) of the acid-soluble products were found in the supernatant fraction. There was no indication that these products were incorporated into progeny phage DNA at later times. The rapid excretion of the breakdown products suggests that the degradation process occurs very close to the cell surface, an observation consistent with endonuclease I involvement since this enzyme is found in the periplasm of the cell (7, 34).

The result of T4 superinfection of ER21, a derivative of ER-1 having 1% wild-type endonuclease I activity (15), is also shown in Fig. 1. In this mutant, both the rate and extent of superinfection solubilization are intermediate between values found for ER-1 and ER22.

Average chain length of products. The products of superinfection solubilization formed in vivo

FIG. 1. Superinfection solubilization of ^{32}P -labeled T4. Cultures of ER-1, ER21, and ER22 grown in M9GC were infected and treated as described in text. Superinfection was at 5 min after primary infection; samples of the culture were chilled and blended 10 min after addition of the labeled phage. The rate of adsorption of a nonradioactive T4 stock to a culture of ER22 grown in M9GC is given for comparison ($\bullet$). (a) Superinfection: ($\circ$) of ER-1: adsorption, 81.4% and injection (of adsorbed phage), 75.0%; ($\square$) of ER21: adsorption, 72.3% and injection, 68.8%; ($\triangle$) of ER22: adsorption, 81.1% and injection, 57.7%. (b) Primary infection ($\circ$) of ER22: adsorption, 89.8% and injection, 85.5%.

were found to resemble a limit digest of T4 DNA made in vitro by endonuclease I. At several times following superinfection of ER-1 by ^{32}P -labeled T4, samples of culture were chilled and precipitated with sodium formate (pH 3.5) or trichloroacetic acid. These acid-soluble fractions were assayed for monoesterase-sensitive phosphate, and the average number of nucleotides per chain was calculated (Table 1). A similar determination was also performed on acid-soluble samples taken 10 min after primary or secondary infection of ER22 and on the acid-soluble products from an in vitro digest of T4 DNA purified by phenol treatment. Approximately 3.5 nmoles of ^{32}P -T4 DNA was exhaustively digested with 10 units of partially purified endonuclease I. The final extent of digestion was 72%.

The acid-soluble products of superinfection breakdown had an average chain length of 8 nucleotides, and the chain length decreased only slightly during the course of the experiment

(Table 1). This result can be compared to the value of 8.2 obtained for the in vitro endonuclease I limit digest of purified T4 DNA [which is similar to the chain length of 7 obtained by Lehman et al. (30) for a limit digest of *E. coli* DNA by highly purified endonuclease I]. The small amount of acid-soluble material formed after either primary or secondary infection of ER22 appeared to have a chain length of about 2. Although this figure is relatively inaccurate due to the low activities of the samples (50 to 100 counts per min per sample), it rules out the possibility that the limited solubilization observed in infections of ER22 resulted from residual endonuclease I activity.

Superinfection solubilization requirement for primary phage-directed RNA and protein synthesis. In an early study, Graham (21) showed that the induction of superinfection breakdown is an energy-dependent process (it was blocked by the addition of 0.05 M NaCN added prior to primary phage infection), but a more specific definition of this requirement has not been reported.

When NaCN was added to a culture of ER-1 1.5 min after primary infection by unlabeled T2, it had little effect on the extent of breakdown of a ^{32}P -labeled T2 stock added 5 min after primary infection. Thus, it is unlikely that NaCN pre-

TABLE 1. Chain lengths of acid-soluble fractions from infections with ^{32}P -labeled T4 and from digestion (in vitro) of ^{32}P -labeled T4 DNA with endonuclease I

Expt	Time after primary infection (min)	Per cent inorganic ^{32}P ^b		Per cent ^{32}P released by monoesterase	Avg chain length ^c
		Monoesterase-treated	Untreated		
Secondary infection, ER-1	7	17.3	4.8	12.5	8.0
	10	17.6	4.8	12.8	7.8
	10 ^a	15.9	4.5	11.4	8.8
	15	16.1	5.0	11.1	9.0
	30	21.6	7.1	14.5	6.9
	30 ^a	22.4	4.1	18.3	5.5
Secondary infection, ER22	15	51.7	3.2	48.5	2.0
Primary infection, ER22	10	64	13	51	2
Endonuclease digest ^d		18.4	6.3	12.1	8.2

^a Cold sodium formate (pH 3.5) was added to 0.4 M final concentration as a precipitant; cold trichloroacetic acid was added to 5% final concentration in all others.

^b Inorganic ^{32}P was measured as counts/minute not adsorbed to Norit (30).

^c 100/per cent ^{32}P released.

^d See text for conditions of incubation.

vents superinfection solubilization by interfering with secondary phage DNA injection.

To characterize the induction process further, several specific inhibitors have been used. Rifampin (also known as rifampicin), which is known to prevent the initiation of messenger ribonucleic acid (RNA) synthesis in *E. coli*, was added to a concentration of 200 $\mu\text{g}/\text{ml}$ to a culture of ER-1 5 min prior to primary infection. As a control, rifampin was added to a second culture 3 min after primary infection, that is, 2 min before superinfection by ^{32}P -labeled T4. Rifampin added before primary infection suppressed the solubilization of superinfecting phage DNA to the level observed after superinfection of ER22 (about 5%), whereas, when added several minutes after primary infection, it had little or no effect (Fig. 2). The addition of rifampin before primary infection increased the efficiency of secondary phage injection to a level nearly as great as that usually observed for primary phage. The control experiment indicated that rifampin does not act directly to inhibit the ability of endonuclease I to degrade secondary phage DNA.

FIG. 2. Effect of rifampicin (rifampin) on the induction of superinfection solubilization in ER-1. Cultures of ER-1 grown in M9GC were infected and treated as described in the text except that to one culture ($\circ$) 200 μg of rifampin per ml was added 5 min prior to primary infection (MOI 6.7); 200 μg of rifampin per ml was also added to a second, control culture (Δ) 3 min after primary infection. Each culture was superinfected with ^{32}P -labeled T4 (MOI 0.8) 5 min after primary infection, and a sample of the culture was chilled and blended 15 min after primary infection. In each experiment, 88% of the labeled phage was adsorbed; the pretreated culture exhibited 74.8% injection of the adsorbed superinfecting phage and 63.8% was injected in the control culture.

Graham (21) reported that very high concentrations of streptomycin (5 mg/ml) added to a culture prior to primary infection prevented the solubilization of superinfecting T2 DNA. However, he also found that, when this experiment was repeated in a streptomycin-resistant derivative of *E. coli* B or when streptomycin was added between the primary and secondary infections, solubilization was again prevented. It can be concluded, therefore, that in his experiments the loss of superinfection solubilization did not result from a specific inhibition of protein synthesis.

To reexamine the role of protein synthesis, we added 100 μg of streptomycin per ml or 200 μg of chloramphenicol per ml to cultures of ER-1 several minutes before primary infection by unlabeled T4 $^{+}$ (Fig. 3). Streptomycin had little or no effect on the solubilization of the labeled secondary phage, whereas chloramphenicol prevented its solubilization. When 100 μg of chloramphenicol per ml was added 3 min after primary infection, however, more than 75% of

FIG. 3. Effect of streptomycin and chloramphenicol on the induction of superinfection solubilization in ER-1. Cultures of ER-1 grown in M9GC were infected and treated as described in the text. To one culture ($\bullet$), 100 μg of streptomycin (STR) per ml was added 3 min before primary infection; 200 μg of chloramphenicol (CAP) per ml was added 5 min before primary infection of a second culture ($\circ$). As a control, 200 μg of chloramphenicol per ml was added to a third culture 3 min after primary infection (Δ). Each culture was primary infected with unlabeled T4 (MOI 5) and 5 min later was superinfected with ^3H -TdR-labeled T4 (MOI 4.5). A sample from each culture was chilled and blended 15 min after primary infection.

the transferred DNA was solubilized within 10 min, and the rate of solubilization was indistinguishable from that found in untreated cultures of ER-1. This result indicated that chloramphenicol did not directly inhibit solubilization. Chloramphenicol added before primary infection also increased the injection efficiency of superinfecting phage to the level observed for primary infection. Although only 59.4% of the adsorbed superinfecting T4 transferred their DNA in the control experiment, 83.7% injected when chloramphenicol treatment preceded primary infection.

These experiments lead to the conclusion that RNA and protein synthesis are required for several minutes after primary phage infection to induce superinfection solubilization. The failure of streptomycin to inhibit this induction may have several explanations. (i) The synthesis of the critical protein or proteins necessary to induce superinfection solubilization may be relatively resistant to streptomycin (but not to chloramphenicol). (ii) Although the level of streptomycin used is sufficient to inhibit protein synthesis extensively in uninfected *E. coli*, the speed with which the drug acts may not be sufficient to block induction unless it is added well in advance of primary infection. According to Lesley et al. (32), *E. coli* B becomes maximally competent for the solubilization of superinfecting phage DNA 2 min after primary infection by T2. In a separate experiment, about 3% of the normal phage burst, or 10 phage per cell, was obtained from a culture of ER-1 to which 100 μ g of streptomycin per ml was added 3 min before infection. When streptomycin was added 15 min prior to infection, less than 0.03 phage per cell was produced. It would appear, therefore, that the addition of 100 μ g of streptomycin per ml 3 min prior to infection is insufficient to inhibit completely early phage-directed protein synthesis.

T2 ghost particles and induction of superinfection solubilization. If phage-directed protein synthesis is required to induce the superinfection breakdown mechanism, primary infection with ghost particles (empty phage capsids) should not be able to elicit superinfection solubilization. To test this prediction, a culture of ER-1 was grown as described (*see above*), except that isopropyl thiogalactoside (IPTG) was added to the culture about 1 hr before infection to induce β -galactosidase production. In place of the usual primary infection, the culture was "infected" with enough T2 ghost particles to kill 99.5% of the viable cells, and 5 min later the culture was superinfected with 32 P-labeled T4 r^+ at MOI of 0.82. The T2 ghost preparation used in this experiment had been purified by banding in a KBr

gradient and contained less than 0.001% surviving plaque-forming units.

No detectable solubilization of the superinfecting phage DNA was observed during the first 4 min after superinfection, and by 15 min only 5% of the added secondary phage DNA was solubilized. Ghost infection therefore failed to induce superinfection solubilization. We must interpret this result with caution since the fragility of the ghost-infected cells (25% of the preinduced β -galactosidase was spontaneously released by 15 min) precluded determination of the blender-sensitive fraction of superinfecting phage.

Ribonuclease I and "activation" of endonuclease I. Endonuclease I is strongly inhibited by RNA in the *in vitro* assay and, as isolated from the cell by sonic treatment or the osmotic shock procedure, the enzyme is 95% inhibited by bound RNA. Ribonuclease I, the major ribonuclease activity of *E. coli* B, is thought to be located in the periplasmic space along with endonuclease I (34). To see whether ribonuclease I is also involved in superinfection breakdown, perhaps by "activating" endonuclease I in response to a signal from the primary phage, the ribonuclease I $^-$ *E. coli* K-12 mutant ER93 (D10) lacking detectable ribonuclease I activity, but possessing the *E. coli* K-12 wild-type level of endonuclease I activity, was superinfected with 32 P-labeled T2 as described above. Within 15 min of superinfection, 40% of the adsorbed superinfecting T2 DNA had been degraded to acid-soluble fragments, and the kinetics of solubilization were indistinguishable from the result obtained with ER129, a ribonuclease I $^+$ *E. coli* K-12 strain.

T4s (spackle) mutant induction of superinfection solubilization. The spackle mutant of T4 was discovered as a mutation which phenotypically suppressed T4e (lysozyme-defective) mutants by allowing lysis to occur by some other means (16). It has been shown that cells infected with T4s and superinfected with T4s $^+$ do not display two of the characteristics normally associated with superinfected cells, namely lysis inhibition and resistance to lysis from without. It has been suggested that this mutant may be affected in a gene which helps to direct the synthesis of bacterial cell wall components (16). Thus, it was of interest to see whether T4s was capable of inducing superinfection solubilization.

In lysis-inhibition control experiments, T4s behaved as expected: a culture of ER-1 infected with T4s as primary phage (MOI 5) and T4 r^+ as secondary phage (MOI 8) began to lyse (measured by OD decrease) at 40 min after primary infection; in contrast, ER-1 infected first with T4R11

and then with $T4r^+$ did not begin to lyse until 2 hr after infection.

When a $T4s$ -infected culture of ER-1 was superinfected 5 min after primary infection with 3H -TdR-labeled $T4r^+$, 46% of the injected label became acid-soluble within 10 min.

Nonglucosylated T^* phage induction of superinfection solubilization. Nonglucosylated (T^*) phage fail to replicate in ER-1 (a phenomenon known as "restriction"), and about half of the glucose-free DNA from such T^* phage is degraded to acid-soluble size. The same results occur when T^* phage are added to ER22 (15). Superficially, restriction solubilization resembles superinfection solubilization: both are initiated rapidly after the addition of the appropriate phage, they are completed within a few minutes, and roughly 50% of the phage DNA is solubilized in each case. They can be distinguished, however, in several ways: the products of T^* phage restriction are mononucleotides which remain largely associated with the bacterial cell (*unpublished data*); the products of superinfection breakdown are oligonucleotides which are rapidly released into the surrounding medium; and, when the poor injection of secondary phage is taken into account, much more than 50% of superinfecting DNA can be solubilized. It was of interest to determine: (i) whether T^*2 were capable of inducing superinfection breakdown in the restricting host ER-1, and (ii) whether superinfecting T^*2 would behave more as superinfecting phage or as restricted T^* phage.

In an experiment analogous to one shown in Fig. 1, ER-1 was primary infected at MOI 10 with unlabeled T^*2 and 5 min later superinfected with ^{32}P -labeled T2 at MOI 0.6. In spite of the fact that, under these conditions less than 0.1% of the T^*2 -infected bacteria produce a progeny burst, these cells were fully competent to solubilize the superinfecting T2 DNA (Fig. 4).

Superinfecting T^* : superinfection solubilization rather than T^* restriction. To determine whether solubilization of DNA from superinfecting T^*2 occurred and by what mechanism, a culture of T2-infected ER-1 was superinfected with ^{32}P -labeled T^*2 at MOI 7.6. As expected, most of the injected superinfecting T^*2 DNA was degraded (Fig. 4). Several lines of evidence suggested that superinfection solubilization (i.e., endonuclease I action) was mainly responsible rather than restriction. The kinetics and extent of solubilization in this experiment closely resembled that found for superinfecting ^{32}P -labeled T2 (cf. Fig. 1), and all of the acid-soluble material appeared in the supernatant fluid fraction by 10 min after infection. Samples of the acid-soluble material were taken for chain-length

FIG. 4. Comparison of superinfection solubilization with restriction solubilization. Cultures of ER-1 and ER22 grown in *L* broth (except as noted) were infected and treated as described in the text. Symbols: (●) primary infection of ER-1 (in M9GC medium) by unlabeled T^*2 (MOI 10); superinfection by ^{32}P -labeled T2 (MOI 0.6): adsorption, 65.2%; injection of adsorbed label, 53.3%. (Δ) Primary infection of ER-1 by T2 (MOI 9.8); superinfection by ^{32}P -labeled T^*2 : adsorption, 54.4%; injection, 46.7%. (○) Primary infection of ER22 by T2 (MOI 8.0); superinfection by ^{32}P -labeled T^*2 (MOI 6.6): adsorption, 59.9%; injection, 39.2%. (◇) Primary infection (only) of ER22 by ^{32}P -labeled T^*2 (MOI 6.9): adsorption, 72.1%; injection, 63.6%; note: subtract 5 min from the abscissa in this case.

determination 10 and 25 min after superinfection. A sample of T^*2 DNA was also purified from the ^{32}P -labeled T^*2 stock and digested with endonuclease I as previously described for ^{32}P -labeled T4 DNA: 96% of the purified T^*2 DNA was solubilized in a 90-min incubation period with 60 units of partially purified endonuclease I. The acid-soluble fraction of the *in vitro* digest had an average chain length of 5.4 nucleotides, whereas the value for the *in vivo* sample was 4.6 at 10 min after superinfection and 1.6 at 25 min. This decrease in chain length with increasing time after superinfection contrasts with the relatively constant value found for the acid-soluble products of superinfecting T4 DNA (Table 1). Although the fragments of T^*2 DNA from both the *in vivo* sample at 10 min and the *in vitro* sample were shorter than comparable T4 samples, the fact that they were oligonucleotides indicates that they arose by superinfection solubilization

rather than restriction solubilization, which produces mononucleotides (*unpublished data*). The shorter chain length at 25 min is unexplained but suggests that the nonglucosylated oligonucleotides are sensitive to another nuclease present in *E. coli*.

Further evidence for the domination of superinfection solubilization over restriction comes from an experiment in which T2-infected ER22 was superinfected by a ^{32}P -labeled stock of T*2. Although nearly half of the injected DNA from primary T*2 is solubilized in ER22, the results in Fig. 4 show that only a small fraction (5% at the time of blending) of the injected T*2 DNA was solubilized when T*2 is the secondary phage.

Superinfection exclusion: relationship to endonuclease I activity. After the discovery of superinfection breakdown by Lesley et al., Dulbecco (12) demonstrated that the genetic markers of the secondary phage were selectively, although not completely, excluded from the progeny burst. Although Dulbecco's findings seemed at first to follow directly from the breakdown results of Lesley et al., at least one of Graham's (21)

subsequent results suggested that solubilization may be a result of the exclusion process rather than its cause. He reported that high levels of streptomycin (5 mg/ml) suppressed the solubilization of ^{32}P -labeled secondary phage (in a streptomycin-resistant derivative of *E. coli* B) but did not permit the transfer of ^{32}P -DNA from the superinfecting phage to progeny phage.

Using a T4 r^+ and T4rII system, we have examined the genetic exclusion of superinfecting phage in two endonuclease I $^-$ strains. As shown above, superinfection solubilization does not occur in these strains. The bacteria were either infected with a mixture of T4 r^+ and T4rII (mixed infection) or were primarily infected with one or the other phage (rII or r^+) and 5 min later superinfected with the remaining phage (r^+ or rII; superinfection). Samples from mixed infections and superinfections were assayed at 10 min for infective centers and at 90 min for progeny phage of both parental types (Table 2).

In the mixed infections of both endonuclease I $^+$ and endonuclease I $^-$ strains, the compositions of the progeny bursts reflected reasonably well expectations based on the MOI values of the

TABLE 2. Genetic exclusion of superinfecting T4 in endonuclease I $^+$ and endonuclease I $^-$ *E. coli*^a

Strain	Endo- nucle- ase I type	Expt	Phages used				Per cent IC found ^b		Per cent secondary (m) found in lysate ^b	Per cent secondary (m) expected ^c if no exclusion	Per cent secondary "escape-from- exclusion" ^a	
			Primary (M)		Secondary (m)		Primary (M)	Second- ary (m)			IC	Lysate
			r type	MOI	r type	MOI						
ER-1	+	Mixed Super Super	rII	2.7	r ⁺	0.4	80	42	33	35		
			rII	1.8	r ⁺	0.4	98	3.5	11	37	0.0	5.4
			r ⁺	7.5	rII	0.9	100	0.2	0	59	0.2	0.0
ER22	—	Mixed Super Super	rII	4.0	r ⁺	0.8	82	22	29	55		
			rII	4.1	r ⁺	0.9	96	3.4	5	59	2.4	4.0
			r ⁺	6.5	rII	0.8	100	2.0	ND	55	2.0	ND
ER129	+	Mixed Super	rII	3.6	r ⁺	0.7	72	91	31	51		
			rII	6.5	r ⁺	1.3	83	14	8	73	14	8
ER130	—	Super	rII	9.6	r ⁺	2.1	100	10	8	88	10	8

^a Abbreviations used: primary (M), primary phage in superinfections, or majority phage in mixed infections; secondary (m), secondary phage in superinfections, or minority phage in mixed infections; MOI, multiplicity of infection; IC, infective centers; ND, not determined.

^b Determination of r types: T4 r^+ from plaque count on *E. coli* K-12 (λ) strain ER151; T4rII from r -like plus mottled plaques on *E. coli* B strain ER-1; total T4 r^+ and T4rII from plaque count on ER-1. Plaque counts on ER151 were multiplied by factor 1.11 to account for slightly reduced plating efficiency of T4 on K-12 strains relative to *E. coli* B.

^c Calculated from the MOI data by using the Poisson distribution, assuming no interference and equal replication.

^d Per cent secondary IC or lysate titer in excess of titer resulting from bacteria uninfected in primary infection (from MOI and Poisson distribution). At high primary MOI (>5), essentially all bacteria are primary infected, and any secondary yield is considered "escape from exclusion." At low primary MOI (<5), a correction is made for those cells which received secondary phage only.

parental phages, and the number of infective centers of each type indicated that the r^+ and rII alleles were able to replicate in the same cell in a large fraction of the bacterial population.

In contrast, in the superinfection experiments the r^+ or rII marker from the secondary phage was substantially underrepresented in the progeny burst, and only about 10% or less of the infective centers or progeny "expected" to carry the allele of the secondary phage did so. This result was obtained with both endonuclease I^+ and endonuclease I^- *E. coli* even though solubilization of the superinfecting DNA does not occur in the latter strains. The normal degree of superinfection exclusion is therefore expressed in the absence of superinfection solubilization.

The incompleteness of superinfection exclusion noted by Dulbecco (12) can be seen in Table 2. The last two columns indicate the degree of this "escape-from-exclusion." It may correlate with the incomplete solubilization sometimes found in superinfections of endonuclease I^+ strains.

Complementation between superinfecting and primary phage. The finding that superinfection exclusion occurs without solubilization in endonuclease I^- strains prompted us to inquire whether the DNA from superinfecting phage could be transcribed and translated. An experiment was designed to measure the ability of superinfecting phage to rescue amber mutants of T4. ER22 is nonpermissive for amber mutants, but, if ER22 is simultaneously infected with a phage carrying the wild-type amber allele, the complemented amber mutant appears in the progeny in numbers comparable to the burst from a permissive host. Two T4 amber mutants

were tested: T4amN122 is defective in the gene 42 product, deoxycytidylate hydroxymethylase, which is an "early" protein; and T4amNG218 is defective in the gene 7 product, a tail end plate component, which is a "late" protein. A culture of ER22 infected with one of the amber mutants at MOI 5 to 10 was infected 5 min later with T4rII, also at high multiplicity. Samples of the cultures were taken before and after superinfection and assayed for infective centers of both parental types. Another sample was taken 90 min after infection for determination of progeny types. A parallel culture of ER22 was mixedly infected with the two phages, and comparable samples were taken.

These experiments (Table 3) demonstrated that superinfecting T4rII was unable to complement either the early or the late amber mutant. Whereas superinfecting T4rII produced a 100-fold increase in the number of T4amN122-producing infective centers, this enhanced level was still only 16% of that obtained in a similar mixed infection and was comparable to the 9.4% escape-from-exclusion observed in this experiment. Production of progeny phage was also low in this experiment although the T4amN122 burst was more than twice as large as expected. Similar results were obtained for T4amNG218, the tail end plate mutant. Very few progeny phage of either type were produced after superinfection in this case, even though for unknown reasons a relatively large fraction of the infected ER22 gave plaques on ER-1 before (and after) superinfection. Several other late amber mutants behaved similarly.

Superinfection exclusion protein synthesis requirement. In a previous section it was shown that the inhibition of protein synthesis before

TABLE 3. Complementation of T4am mutants by superinfecting T4am⁺ in endonuclease I^- *E. coli* ER22^a

T4am mutant	Chloramphenicol used ^b	Expt	IC ^c (4.5 min) ^d T4am	IC ^c (10 min)		Lysate ^c (90 min)	
				T4am	T4rII	T4am	T4rII
N122	No	Mixed	0.0007	0.38	0.43	100	116
		Super		0.056	0.040	32	4.9
NG218	No	Mixed	0.32	0.52	0.71	190	135
		Super		0.20	0.018	5.6	4.3
N122	Yes	Mixed	0.009	0.52	0.53	14	11
		Super		0.64	0.77	23	17

^a Abbreviations as in Table 2, footnote a. Titration data are expressed as plaques $\times 10^{-8}$ per 10^8 infected bacteria. Numbers of bacteria infected ranged from 1.26×10^8 to 2.18×10^8 . MOI values for primary and secondary infections were the same in each experiment and ranged from 5 to 10.

^b Where used, chloramphenicol was added to 200 $\mu\text{g}/\text{ml}$ 5 min before primary infection and removed 10 min after primary infection.

^c Determination of amber types: T4am, r^+ from plaque count on *E. coli* K-12(λ), Su⁺ (ER 218) multiplied by 1.11 (see footnote b, Table 2); T4am⁺, rII from plaque count on *E. coli* B, Su⁻ (ER-1).

^d Superinfections were at 5.0 min after primary infections.

primary infection prevented the acid-solubilization of DNA of T4 superinfecting ER-1. In experiments similar to those described in that section, chloramphenicol was added to cultures of ER22 5 min prior to primary infection and removed 10 min after primary infection by centrifuging and resuspending the cells in L broth without chloramphenicol. Both primary T4 α mN122 and superinfecting T4rII produced as many infective centers as in comparable mixed infections with or without chloramphenicol (Table 3). Progeny phage production was less than that seen in mixed infections without chloramphenicol, but substantial and equal numbers of primary and secondary phage were produced.

Superinfection fragmentation observed in endonuclease I⁻ strain. As we have seen, very little of the DNA from superinfecting T2 or T4 was acid-solubilized after infection of ER22; however, superinfecting phage were still genetically excluded in this strain and were unable to complement defective primary phage. Limited endonucleolytic attack on the superinfecting DNA, not detectable in the assay for acid-solubilization, might explain these observations. To test this possibility, gentle sarcosine lysates of superinfected cultures of ER22 were made, and the size of labeled superinfecting DNA was determined by centrifugation through glycerol gradients.

When control cultures of ER22 that had been primary infected with ^{32}P -labeled T4 (MOI < 1) were lysed 10 min after infection as described above and sedimented through neutral glycerol gradients at 49,000 rev/min for 75 min (*data not shown*), most of the injected DNA reproducibly sedimented to the Anglo-Conray cushion at the bottom of the gradient. Very little acid-insoluble material remained in the top six fractions, and only a few per cent of the labeled material sedimented at the position of 65S intact phage DNA (fraction 16). Under the above conditions, whole phage particles (800 to 1,000S) also sediment to the Anglo-Conray cushion at the bottom of the gradient. However, in gradients run at lower speeds, i.e., 16,000 rev/min for 75 min (*data not shown*), the labeled DNA of primary phage sedimented much faster than whole phage particles. "Fast-sedimenting complexes" between primary T2 (or T4) DNA and some cellular component (cytoplasmic membrane?) have been frequently observed (19, 26, 33).

Several independent lysates of blended ER22 cultures superinfected with labeled T4 were analyzed on neutral gradients. A typical experiment is shown in Fig. 5a; 42% of injected sec-

FIG. 5. Neutral gradients: superinfection of ER22 by T4, with and without blending. The culture was grown, infected, blended, centrifuged, and lysed as described in the text: primary T4 infection was at MOI 6.7; superinfection at 5 min by ^{32}P -labeled T4 was at MOI 6.1. At 15 min, 55.4% of the adsorbed superinfecting phage had injected and 3.4% of the injected material had become acid-soluble. The culture sample used to make the unblended lysate was otherwise treated as was the blended lysate sample. The figures given in parentheses (part a) have been corrected to indicate per cent total adsorbed phage (rather than per cent injected phage) and are, therefore, directly comparable to the percentages given in part b for the unblended lysate. The thin vertical line on the right of this and subsequent sedimentation profiles indicates the position of the Anglo-Conray cushion. Centrifugation was at 49,000 rev/min for 75 min at 18 C. (a) Lysate of blended culture. (b) Lysate of unblended culture. Symbols: (O) experimental ^{32}P -labeled T4 DNA from lysates, (●) added ^3H -TdR-labeled T4 marker DNA.

ondary DNA sedimented as a relatively homogeneous peak to a position 10% closer to the meniscus than intact T4 DNA, and this peak was somewhat skewed toward the meniscus; 33% was found near the top of the gradient; and 25% reached the cushion.

These results indicated that DNA molecules from superinfecting T4 did not enter a fast-sedimenting complex (the amount of label at the cushion is small and variable compared to that seen in primary infections: part of this material may be due to superinfecting phage which escape exclusion, the rest may be a sedimentation arti-

fact) (6). Rather, secondary DNA appeared to be fragmented into two size classes: large fragments (fraction 14) that sedimented 10% slower than intact T4 DNA and were therefore about 25% lower in molecular weight (90×10^6); and small acid-insoluble fragments (fractions 1 to 6) which, from their sedimentation behavior, probably have molecular weights of less than 10^6 (6).

To see whether the reduction in sedimentation rate of the large fragment could be an artifact of protein or membrane binding, samples of a sarcosine lysate of a T4-superinfected culture were either heated (65 C for 20 min) or extracted with phenol prior to sedimentation (Fig. 6).

FIG. 6. Neutral gradients: superinfection of ER22 by T4, with and without denaturing treatments. The culture was grown, infected, blended, centrifuged, and lysed as described in the text: primary T4 infection was at MOI 5; superinfection at 5 min by ^{32}P -labeled T4 was at MOI 4.2. At 15 min, 47.8% of the adsorbed secondary phage had injected. Centrifugation was at 49,000 rev/min for 70 min at 18 C. (a) Untreated sarcosine lysate with added ^3H -TdR-labeled T4 marker DNA. (b) Lysate heated to 65 C for 20 min. (c) Lysate mixed with ^3H -TdR-labeled T4 and DNA gently extracted with phenol. There was no significant loss of ^{32}P -label during the extraction. The per cent of the ^{32}P -label which was recovered adsorbed to the nitrocellulose centrifuge tube is indicated. Symbols: (○) ^{32}P -labeled experimental T4, (●) ^3H -TdR-labeled T4 marker DNA.

Both treatments caused about half of the material at the cushion to shift to the central peak. The position of the central peak, however, was unaffected. The amount of material remaining in the upper portion of the gradient was also unaffected. Thus, half of the rapidly sedimenting DNA was released as molecules indistinguishable from large fragments, and the sedimentation rate of large fragments was not altered by destruction of proteins and membranes.

Strands of the large fragment are intact. To determine whether the DNA strands of the large fragments were continuous or interrupted, the lysate described in Fig. 5a was denatured and sedimented through alkaline glycerol gradients as shown in Fig. 7b. Little or no increase was seen in the material appearing in the top six fractions of the alkaline gradients, and most of the remaining material (51% of the injected superinfecting T4 DNA) sedimented at approximately the same position as the denatured phage DNA marker (Fig. 7a). A detailed analysis of this (central) region showed that the label from the blended superinfected lysate was slightly skewed to the light side of the marker peak as had been found in the neutral gradient. The large fragments of superinfecting DNA contained few, if any, single-strand breaks.

Also shown in Fig. 7a is the alkaline gradient of a lysate of primary infected ER22. The bulk of the parental DNA in this lysate sedimented considerably faster than single-stranded T4 DNA of unit length. It would appear that the DNA from primary infecting phage is elongated through replication or becomes attached to some fast sedimenting, alkali-stable complex. Kozinski (26) has described a similar sedimentation profile for replicating parental T4 DNA from lysates made 7 to 25 min after infection. The contrast between the alkaline sedimentation profiles of lysates from primary and secondary infected cultures suggests that the DNA from superinfecting T4 does not enter the pool of replicating primary phage DNA even when solubilization of the superinfecting phage DNA is largely suppressed because of the absence of endonuclease I.

Release of DNA by superinfecting phage. A sample of the superinfected ER22 culture described in Fig. 5a and 7b was not blended but was lysed in the same way and sedimented in neutral and alkaline gradients to learn more about the superinfecting phage that failed to inject (Fig. 5b, 7b). Subtraction (data not shown) of pattern 5a from pattern 5b reveals the contribution of noninjecting particles to the neutral sedimentation profile. About half of the non-injecting counts appear in the central region, about half at the Anglo-Conray cushion, and

FIG. 7. Alkaline gradients: primary infection of ER22 by T4; superinfection of ER22 by T4, with and without blending; T4 phage alone. The lysate of the culture of ER22 primary infected (only) by ^{32}P -labeled T4 at MOI 6.7 was made 10 min after infection as described in the text; 76.5% of the adsorbed phage had injected by the time of blending. The lysates of the superinfected culture of ER22 are described in the legend to Fig. 5. Each profile came from a separate gradient. The ^{32}P -labeled phage stock was centrifuged in parallel with the two superinfected lysates, the lysate of the primary infection was centrifuged under identical conditions approximately 12 hr later and 36 hr after the phage stock was prepared. The phage stock had a specific activity of 4.3×10^{-4} counts per min per plaque-forming unit; thus, the average phage particle would have undergone approximately one ^{32}P decay by 36 hr. Centrifugation was at 49,000 rev/min for 90 min at 18 C. Symbols: (a) (●, lower percentages) ^{32}P -labeled phage stock, (○, upper percentages) lysate of primary infected culture; (b) (Δ, upper percentages) lysate of unblended superinfected culture, (○, lower percentages) lysate of blended superinfected culture.

very few in fractions 1 to 7. The central peak aligns more closely with intact T4 DNA marker than the large fragment peak of Fig. 5a. This result suggests that at least half of the non-injecting particles had released intact T4 DNA molecules during sarcosine treatment. T4 virions are not dissociated by this treatment. Some adsorbed but noninjecting particles are clearly

more labile than virions. Perhaps they had initiated release of DNA from their capsids but were unable to transfer it to the cell.

The additional material appearing at the bottom of the gradient could represent non-injecting particles which have the same resistance to sarcosine dissociation as unadsorbed virions, DNA incompletely separated from capsid protein, or dissociated DNA trapped in or bound to some cell envelope component.

Alkaline sedimentation profiles of the blended and unblended lysates are compared in Fig. 7b. Most of the increment from the noninjecting particles appears in the central region; this result was expected since alkali treatment dissociates intact virions.

Superinfection fragmentation RNA synthesis requirement. It has been shown that if the synthesis of phage-induced functions were prevented by drug treatment, the induction of both superinfection solubilization and superinfection exclusion would not occur. One might expect that drug treatment prior to infection would also prevent the fragmentation reaction observed after superinfection of ER22. When 200 μg of rifampin per ml was added to a culture of ER-1 prior to primary infection, the bulk of the injected DNA from superinfecting phage had a sedimentation coefficient indistinguishable from that of whole phage DNA, and virtually no small acid-insoluble fragments were found (Fig. 8).

FIG. 8. Neutral gradients: rifampin-treated ER-1 superinfected by T4. The culture was grown, infected, and processed as described in the text and in the legend to Fig. 2. Rifampin was added to a concentration of 200 $\mu\text{g}/\text{ml}$ 5 min before primary infection. Centrifugation was at 49,000 rev/min for 75 min at 10 C. After collection, the gradient fractions were trichloroacetic acid-precipitated and collected on membrane filters (Millipore Corp.). Symbols: (○) ^{32}P -labeled experimental T4 DNA from lysate; (●) added ^3H -TdR-labeled marker T4 DNA.

DISCUSSION

A single cell of *E. coli* is thought to have approximately 300 receptor sites for each of the T-even phages (43). Edgar and Steinberg (14) found that at least 30 phage particles could participate (through marker rescue) in the infection of a single cell, and other evidence indicates that at least four phage genomes per cell can express and replicate independently (17, 39). Although a single T-even phage particle will, upon adsorption, produce a successful infection with a probability approaching unity, the chance is small that two or more T-even phage will participate equally in a nonsynchronous multiple infection. Thus, the T-even bacteriophage induce a mechanism which acts as a defense against superinfecting phage particles. Within 1 min after adsorption of the initial phage, wild-type *E. coli* is so changed that the DNA from subsequently adsorbing phage is rapidly degraded into acid-soluble fragments (superinfection solubilization), and its genetic markers are excluded from the progeny burst (superinfection exclusion). Although these facts have been known for some time, the mechanisms involved have not been thoroughly investigated. This study has concentrated on the fate of the parental DNA from the superinfecting phage in wild-type *E. coli* and in a mutant unable to carry out superinfection solubilization.

Requirement of endonuclease I for superinfection solubilization. In wild-type *E. coli* B, 70 to 80% of the nonblendable superinfecting phage DNA is degraded to acid-soluble fragments having an average chain length of eight nucleotides. These fragments are rapidly released from the cell (in contrast, the products of DNA solubilization occurring in T^{*} restriction are mononucleotides which are largely retained by the cell). No significant change occurs in the average chain length of the soluble fraction between 2 and 25 min after superinfection. Purified endonuclease I in vitro rapidly degrades 70 to 80% of *E. coli* DNA or T-even phage DNA to acid-soluble fragments having a chain length of about eight nucleotides. Thus, the in vitro products of endonuclease I action resemble the in vivo products of superinfection solubilization, and the rapid release of the oligonucleotides in vivo is consistent with the presumed surface location of endonuclease I (7, 34).

In mutants of *E. coli* lacking endonuclease I activity, less than 5% of the injected superinfecting DNA is degraded to an acid-soluble form. Similar results for other endonuclease I⁻ strains have recently been reported by Fielding and Lunt (18). The 3 to 5% solubilization that is

observed in the endonuclease I⁻ strain ER22 seems to be real, but since the acid-soluble products appear to be mono- or dinucleotides they do not arise from residual endonuclease I activity. A similar amount of parental DNA solubilization was observed after primary infection, and this material also consisted of mono- or dinucleotides. Strain ER21 has about 1% of the *E. coli* B level of endonuclease I; when superinfected by T4, only 15% of the injected DNA was solubilized. These results indicate that endonuclease I is the major, and perhaps the only, nuclease involved in the terminal solubilization of the DNA of superinfecting T-even phage. This is the only known biological function of this enzyme.

E. coli B contains 4×10^{-7} units (nanomoles of T2 DNA nucleotides per 30 min) of in vitro endonuclease I activity per cell, assuming 100 μ g of soluble protein per 5×10^8 cells (15). One T2 chromosome contains 6×10^{-10} nmoles of nucleotides. Therefore, assuming that the specific activity of endonuclease I is identical in vivo and in vitro and that the enzyme is relieved of RNA inhibition, each cell contains sufficient endonuclease I activity to account for the complete solubilization of approximately 20 T2 chromosomes per minute. Other strains have been reported to have less endonuclease I activity than *E. coli* B. According to the data of Eigner and Block (15), ER58 (*S. sonnei*) contains 2×10^{-7} units per cell (10 chromosomes/min), ER129 (*E. coli* K-12-1000) contains 3.3×10^{-8} units per cell (1.6 chromosomes/min), ER21 contains 2.9×10^{-9} units per cell (0.14 chromosomes/min). Thus, if endonuclease I activity in strains ER-1, ER58, and ER129 were relieved of RNA inhibition after phage infection, the resulting activity would be sufficient to account for the rapid solubilization of superinfecting phage. The limited solubilization of superinfecting T4 DNA observed in ER21 indicates that a minimum of approximately 10^{-8} endonuclease I units per cell is required to observe the normal rate and extent of solubilization of superinfecting phage DNA.

Fragmentation of superinfecting T-even DNA in endonuclease I⁻ strains. Sedimentation analysis of lysates of *E. coli* infected with labeled T4 or T2 indicates that parental primary phage DNA rapidly enters a fast sedimenting complex (33). The formation of this complex is frequently interpreted as evidence for the attachment of such DNA to the cell membrane. In the endonuclease I⁻ strain ER22, the DNA from superinfecting T2 or T4 fails to enter a fast sedimenting complex even though little of this DNA is solubilized in the absence of endonuclease I.

An accompanying paper (36) reports that T4 DNA superinfecting ER22 fails to form the sarkosyl M-band complex characteristic of primary phage infections and DNA-membrane association (13).

The neutral sedimentation profiles do indicate, however, that superinfecting DNA is fragmented into several pieces. The fact that the large fragment sediments at about 58S (90×10^6 daltons) suggests that it contains all but 25% of the intact T4 DNA molecule. Alkaline sedimentation profiles show that it does not contain single-stranded scissions. The small fragments are acid-insoluble, sediment at 0.5 to 10S (less than 10^6 daltons), and usually account for about 25% of the recovered label, as would be expected if the large fragment represents 75% of the superinfecting DNA. (The large fragment sedimentation peak always contained substantially less than 75% of the label, but when label was transferred from the bottom fraction, as by phenol treatment in Fig. 6c, the amount in this peak approached 60% of the total.)

A simple explanation of these observations would be that an unknown endonuclease introduced a minimum of 30 double-stranded scissions in a single segment, representing about 25% of the phage chromosome. Such scissions might have been introduced into a unique genetic segment of each phage chromosome or into segments at one or both ends of the circularly permuted T-even chromosome. Either mechanism would generate similar sedimentation profiles (in the former case, the fast sedimenting peak would be skewed further towards the light side).

The DNA of superinfecting T4 (in ER-1) was not fragmented when the culture was treated with rifampin before primary phage infection, nor was there evidence in the sedimentation profiles that the DNA from primary phage was fragmented in a manner similar to that of the superinfecting phage DNA. These results indicate that the enzyme which is responsible for the fragmentation of a portion of the superinfecting phage chromosome is specific to the superinfection "defense" mechanism, but we do not know whether the fragmentation results from a host enzyme or a phage-induced enzyme normally involved in phage replication (e.g., a recombination nuclease). Bose and Warren (4) reported that after T4 infection the host cell chromosome is initially fragmented into pieces similar in size to the 90×10^6 dalton fragment. Superinfection experiments have not been performed with phage mutants unable to degrade the host cell DNA.

Superinfection exclusion. The discovery of strains in which superinfection solubilization did not occur prompted a reevaluation of super-

infection exclusion. The results of experiments similar to those of Dulbecco demonstrated that superinfecting T4 was excluded genetically as effectively from endonuclease I⁻ strains as it was from their endonuclease I⁺ parents. Further experiments showed that super-infecting T4rII could not complement either an early or a late T4 amber mutant used as the primary phage to an extent significantly greater than might be expected from exclusion escape (about 10%). It may be concluded, therefore, that much of the superinfecting phage genome is not transcribed or, if transcribed, not translated, even though the superinfecting phage chromosome remains largely macromolecular. Gene 42 and gene 7 are separated by about 25% of the physical map of T4; thus, it is possible, but unlikely, that both genes would be inactivated by the fragmentation of a single segment comprising 25% of the T4 chromosome.

A superinfecting phage genome might be non-functional for several reasons. Snustad (38) has shown that only four to six phage genomes can be expressed simultaneously. Primary infections in the complementation experiments were at MOI 7 to 10; thus, it is possible that most of the "transcriptional slots" had been filled before the superinfecting phage was added. Alternatively, the superinfecting phage genome may be physically isolated from the replicating primary phage chromosome and the transcriptional and translational machinery, as we shall discuss below.

Induction of superinfection breakdown and exclusion. Previously it was known that the induction of superinfection breakdown was an energy-dependent process; superinfection breakdown was not observed when cyanide was added to a culture prior to primary infection (21). Results of this study demonstrate that phage-induced synthesis of RNA and protein is required between the primary and secondary infections to observe solubilization of the superinfecting phage DNA. Superinfection breakdown was not observed when chloramphenicol or rifampin was added to a culture of *E. coli* B prior to primary infection nor was it observed after primary infection by T2 ghost particles. Since *E. coli* B becomes competent to induce the breakdown and exclusion of superinfecting phage DNA within 1 min of primary T-even phage adsorption, we can assume that one or more of the "pre-early" proteins coded by the primary phage is responsible, directly or indirectly, for these phenomena. It seemed at first that a likely candidate for this phage-directed protein would be a ribonuclease capable of activating endonuclease I. The latter enzyme is 90 to 95% inhibited by RNA as isolated from bacteria, but its activity in vivo is not known. Ribo-

nuclease I, another periplasmic enzyme, does not activate endonuclease I since superinfection solubilization was induced normally in the ribonuclease I-defective strain ER93. Kozloff (28) and Kunkee and Pardee (29) reported that, after T-even phage infection, an RNA-inhibited deoxyribonuclease activity was activated by the destruction of the RNA inhibitor. Stone and Burton (40) were not able to reproduce these results, and a preliminary investigation in this laboratory (*unpublished data*) did not reveal any change in the degree of RNA inhibition of endonuclease I at 5 min after primary infection in *E. coli* B in either sonic extracts or cell-free osmotic shockates (under conditions of minimum cell lysis but good recovery of endonuclease I). It remains uncertain whether the induction of superinfection solubilization involves activation of endonuclease I.

"Failure-to-penetrate" model for superinfection breakdown and exclusion. The following observations seem significant to us in developing a model for superinfection. (i) Whereas adsorption of superinfecting phage is as rapid and as complete as that of primary phage, injection or transfer of the phage DNA from capsid to cell (measured by resistance of adsorbed counts to blending) is much less complete for superinfecting phage. This effect has been noted by others (24, 36). (ii) As stressed above, the acid-soluble products of superinfection solubilization result from the action of an enzyme located outside of the cytoplasmic membrane; these products appear almost at once in the extracellular medium (in contrast to T* restriction products, believed to be generated within the cell). (iii) Also, in contrast to the T* situation in which some or all T* genes are expressed (22), superinfecting DNA does not seem to contribute genetic markers to progeny or complement missing functions of primary phage (except to the extent of the 10% escape-from-exclusion usually observed) even when solubilization is eliminated by using an endonuclease I⁻ host. (iv) Superinfecting T* phage undergo superinfection solubilization in endonuclease I⁺ *E. coli*; they are subject to neither superinfection solubilization nor T* restriction solubilization in endonuclease I⁻ *E. coli*.

All of these observations can be simply explained if the primary infection leads to changes in the cell envelope which hinder the ejection of DNA from adsorbed virions and prevent those molecules which do eject from penetrating the cytoplasmic membrane. The superinfecting DNA would then remain trapped in the cell envelope where endonuclease I would quickly reduce it to small oligonucleotides which could readily diffuse into the medium. In endonuclease I⁻ *E. coli*, the important result would be the same: the injected

DNA fails to enter the cytoplasm and is therefore unable to replicate or be transcribed; hence, it suffers superinfection exclusion. Finally, since T* restriction appears to occur within the cytoplasm, we would expect that superinfecting T* DNA never reaches the restriction enzymes and so behaves like glucosylated superinfecting T-even phage.

To test the model for superinfection exclusion presented here, we must show that excluded but nonsolubilized DNA does not cross the plasma membrane and that it is trapped in the wall layers outside the membrane where endonuclease I is found. High-resolution autoradiographic experiments described in an accompanying paper (2) show that excluded, nonsolubilized superinfecting T4 DNA (in endonuclease I⁻ *E. coli*) tends to be localized in the envelope. In addition, this DNA is not sensitive to extracellular deoxyribonuclease, so it is not on the outer surface of the cell. These experiments do not prove that the DNA did not penetrate the membrane, but they are consistent with the notion.

What kind of cell envelope change would be required to prevent the entry of superinfecting DNA into the cytoplasm, and what kind of evidence is there that primary phage affect the envelope (rapidly!)?

The primary phage could make a protein which attached specifically to each of the phage DNA entry portals in the envelope. In 1 min, a substantial amount of protein can be synthesized in a phage-infected cell. For example, if messenger RNA synthesis is rate-limiting at 700 codons/min (5), the infected cell could synthesize more than 300 molecules of a protein of molecular weight 20,000 in 1 min, enough to affect directly each of the estimated 300 phage-receptor sites. However, changes in the cell membrane at points distant from phage receptors might also adversely affect superinfecting DNA penetration (11). It is clear that primary infection does profoundly affect the cytoplasmic membrane. For example, Frankel et al. (20) have shown that a number of new proteins appear in the membrane fraction shortly after T4 infection, and Cronan and Wulff (8) have shown that there are changes in the phospholipid content of the cell membrane after T4 infection.

The well known leakiness of T even-infected cells, their sensitivity to lysis, their altered transport properties, etc., also indicate that the envelope, and specifically the membrane, is rapidly altered by primary infection (11, 37). Several T-even phage genes have been associated with changes in the membrane of infected cells (16, 37). We expect that a phage mutant can be found which is unable to induce superinfection exclusion and breakdown because it fails to make the

membrane change postulated above. So far, we have tested only the spackle mutant of Emrich (16); it was competent in causing superinfection solubilization. In addition, the rII mutant, whose rapid lysis phenotype implies that it may be involved with envelope stability, has been shown to induce superinfection solubilization (32) and exclusion (12).

The very simple view we have developed here states that exclusion results from failure of superinfecting DNA to reach the cytoplasm and that solubilization results from the presence of endonuclease I at the place where the injected but blocked DNA finds itself. Much of the information about superinfection is readily explained by this hypothesis. Nothing that we know about is in conflict with it.

Puck and Lee (35) arrived at a very similar model for superinfection, based on their ability to wash nearly all of the ^{32}P label of secondary T2 from *E. coli* B under conditions which removed only one-half of the label of primary T2.

Relation of fragmentation to superinfection exclusion. In our initial attempts to explain the existence of superinfection exclusion in strains unable to solubilize superinfecting DNA, we looked for evidence of a nuclease activity that would cause limited fragmentation of DNA from the superinfecting phage. We found that the superinfecting phage genome is fragmented in a strain lacking endonuclease I activity. Presently, we feel that this phenomenon, like solubilization itself, may result from failure to penetrate, mainly because a piece of DNA of 90×10^6 daltons should be able to express a number of functions if it were inside the cell. Residual endonuclease I activity undetectable in the usual assay or another periplasmic nuclease activity could be involved.

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