The Action of Colicin E2 on Supercoiled λ DNA. I. Experiments in Vivo†

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ABSTRACT: A λ DNA supercoil system has been developed to study the effects of colicin E2 on DNA in vivo. Colicin E2, a protein antibiotic synthesized by strains of coliform bacteria that carry the Col E2 plasmid, has as its most conspicuous effect damage to the DNA of sensitive strains. Colicin E2 attacks the supercoiled molecules formed by labeled λ DNA in superinfected cells as well as it attacks the bacterial DNA. The rate and extent of acid solubilization of the λ supercoils and of host bacterial DNA induced by E2 treatment are nearly identical. Treatment of superinfected cells with colicin E2 results in the progressive conversion of λ DNA supercoils to open circles and/or linear full length molecules, and subsequently to fragments less than full λ in size. The first endonucleolytic reactions are single-strand and/or double-strand breaks. The rate of supercoil breakdown as well as the final percent supercoils remaining unconverted, the size of the final λ fragments, and the extent of solubilization are dependent on the multiplicity of colicin used. Addition of trypsin to E2-treated superinfected cells results in a cessation of further breakdown of the λ molecules, presumably as a result of digestion of accessible colicin molecules. Energy is essential for an early event in colicin E2 action. The host enzymes, endonuclease I and Rec BC, may be instrumental in the nucleolytic process caused by colicin E2: endonuclease I in a reaction preceding cell killing and Rec BC in a secondary degradation of the bacterial DNA.

Treatment of sensitive bacteria with colicin E2 has as its most conspicuous effect damage to the bacterial DNA leading to acid solubilization, as shown by Nomura (1963) and others. In 1970 Obinata and Mizuno, using neutral and alkaline sucrose density gradients, demonstrated the occurrence of double-strand breaks in the bacterial DNA 7 min after E2 addition at 37°. Ringrose (1970), in an analysis of bacterial DNA from E2-treated *Escherichia coli* cells, detected single-strand nicks as early as 2 min after E2 treatment and double-strand breaks at 5 or 7 min, depending on the E2 concentration. Acid-soluble counts were detected about 10 min after E2 addition. Ringrose proposed three stages in E2 action: stage I, corresponding to single-strand breakage; stage II, with double-strand breakage; and stage III, acid solubilization.

Almendinger and Hager (1972, 1973) have presented findings that appear to implicate endonuclease I in the E2-initiated reaction series. They found (1973) that colicin E2 plus endonuclease I, but not either by itself, causes degradation of DNA in *E. coli* spheroplasts. Yet, recent findings of RNase activity initiated by colicin E3 in vitro (Boon, 1971, 1972; Bowman et al., 1971) raise the possibility that E2 may possess a nuclease activity.

The present work employed a new system that appeared potentially promising both for studies in vivo and in vitro: the action of colicin E2 on λ DNA supercoils which are formed upon superinfection of an immune λ lysogen with a coimmune λ phage and persist undamaged for a reasonable length of time. This paper presents results on the effects of colicin E2 on λ supercoils in vivo. The accompanying report (Saxe, 1975) contains evidence suggestive of an in vitro endonucleolytic action of this colicin on the DNA supercoils.

Experimental Procedure

Bacterial Strains. The bacterial strains used are listed in Table I.

Bacteriophage Strains. The following bacteriophage strains were obtained from E. Signer's collection: λ, λ_{CI857}, $\lambda_{CI857S7}$, λ_{C60}, λ_{CI}, λ_{vir}, $\lambda_{ind-831}$, λ_{imm434}, and λ_{imm21}.

Media. Tryptone broth consists of 1% tryptone and 0.25% NaCl. It was supplemented with yeast extract (0.01%), maltose (0.2%), and B1 (1 μg/ml) (= medium λ YMB_1). LB broth contains 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH adjusted to 7.0. Add agar (1.5%) for LB agar. Soft top agar consists of 0.8% nutrient broth, 0.5% NaCl, and 0.65% agar. Medium 1 × 63, used to prepare radioactive phage, contains per 1 l.: 5.3 g of KH_2PO_4, 1.1 g of K_2HPO_4, 2 g of $(NH_4)_2SO_4$, and 3.0 ml of 1 *M* KOH. Glucose (0.3%), casamino acids (0.3%), $MgSO_4$ (10^{-3} *M*), and B_1 (1 μg/ml) were added.

Chemicals. Sodium dodecyl sulfate (SDS,[1] 95%) was recrystallized one time from 100% ethanol. CsCl (99.95% purity) was purchased from VarLacOid Chemical Co. 2,4-Dinitrophenol (2,4-Dnp) was recrystallized two times from ethanol. Lysozyme (salt free, two times crystallized) and trypsin (two times crystallized, washed free of salt) were obtained from Worthington Biochemical Corp. The source of bovine serum albumin (crystallized, BSA) was Miles Laboratories, Inc. Pronase (Calbiochem, Grade B) was pretreated as described by Young and Sinsheimer (1967).

Assay of Colicin E2. Colicin E2 activity was assayed either (1) by spot assay yielding arbitrary units (AU) or (2) from bacterial survival assay yielding killing units (KU) (Fields and Luria, 1969). The killing multiplicity *m* was calculated from the equation $m = -\ln(S/S_0)$ where S/S_0 is

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; 2,4-Dnp, 2,4-dinitrophenol; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; AU, arbitrary units; KU, killing units.

Table I: Bacterial Strains.

E. coli K12 Strain	Properties	Source
3000	HfrH	M. Wright
MRW25	Endo I$^-$ (end A) from 3000	M. Wright
AB1157	*pro*$^-$*leu*$^-$*thr*$^-$*arg*$^-$*his*$^-$*lac*$^-$*gal*$^-$-*xyl*$^-$*ara*$^-$*mtl*$^-$*thi*$^-$*str*^{r}T_6^r	S. E. Luria
JC5519	Rec B21 Rec C22 from AB1157	A. J. Clark
QR47a	$B_1^-su_{II}^+rec^+UV^r$	E. R. Signer
QR48	Rec A from QR47a	E. R. Signer
QW7	Endo I$^-$ (1100)$su_{II}^+gal^-rec^+$	E. R. Signer
QW8	Rec A_{56} from QW7	E. R. Signer
QW9	Rec B_{21} from QW7	E. R. Signer
WD5028	$gal_{1,2}^{2^c}str^rT_3^rT_7^r(\lambda C_{I857}S_7)$	E. R. Signer
M5177	$gal^-str^rT_1^r(\lambda C_{I857}S_7)\lambda^r thy^-$	E. R. Signer
Salmonella typhimurium cys 36 (Col E2)$_2$		S. E. Luria

the survival ratio of sensitive bacteria. All colicin dilutions were carried out in 0.85% saline containing 2 mg/ml of BSA.

Preparation of Colicin E2. The in vivo studies were carried out using crude E2 prepared from strain *Salmonella typhimurium* cys 36 (Col E2)$_2$ by the method of Nomura (1964).

Solubilization of Labeled Bacterial DNA. Overnight cultures in λ YMB$_1$ medium were diluted 1:100 into fresh medium and grown at 34° to a Klett value of 10. [2-^{14}C]Thymidine (0.5 μCi/ml) and deoxyadenosine (250 μg/ml) were added and the culture incubated to a Klett value of 40. The cells were then chilled, collected on filters, washed with medium, resuspended in medium containing 200 μg/ml of thymidine, shaken 20 min at 34°, and chilled on ice. Colicin dilutions and cells were prewarmed separately and mixed in equal proportions. Samples were removed at 10 min for viable counts and at various times for assay of acid-soluble counts. The values were normalized to the percent counts available for solubilization (the total number of acid-precipitable counts): (% soluble − % background)/(100% − % background). Acid-soluble radioactivity was determined according to Howard-Flanders and Theriot (1966). Samples (0.4 ml) of Cl_3CCOOH supernatants were added to 10 ml of Triton X100–toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene containing 0.8 ml of H_2O. Radioactivity was counted in a Beckman scintillation counter. Radioactive compounds were obtained from New England Nuclear Corp.

Preparation of λ Phage and λ DNA. ^{14}C- and ^{3}H-labeled phage were prepared from strain M5177 by heat induction of $\lambda_{CI857S7}$ phage in medium 63 supplemented either with [^{3}H]thymidine at 2 μCi/μg of thymidine or with [^{14}C]thymine at 1 μCi/15 μg of thymidine. The phage were partially purified by one cycle of differential centrifugation.

To prepare λ DNA, λ phage were further purified by CsCl equilibrium centrifugation in the IEC A321 fixed angle rotor for 24 hr at 25,000 rpm, 4°, and the DNA was extracted three times with redistilled phenol.

Superinfection of λ Lysogens. Superinfection of λ lysogenic strains was carried out as described by Boyce and Tepper (1968) with several modifications. An overnight culture of strain WD5028 carrying the ind$^-$ prophage $\lambda_{CI857S7}$ was diluted 1:50 into 100 ml of fresh λ YMB$_1$ and grown to a Klett value of 50 at 32°. The culture was chilled; the cells were washed and resuspended at 1 to 2 × 10^9 cells/ml in 10 m*M* potassium phosphate adsorption buffer (pH 7.0) containing 10 m*M* $MgSO_4$ and 100 μg/ml of thymidine. ^{3}H-Labeled λ_{CI} were added at a multiplicity of 5 to 10. The cells were incubated 20 min at 0° followed by 10 min at 32°, washed to remove nonadsorbed phage, resuspended in growth medium (120 ml) supplemented with 100 μg/ml of cold thymidine, and shaken 60 min at 34° to allow supercoils to form. Two 2-ml aliquots were removed. One served as untreated control; the other received colicin E2 at a low multiplicity (1×) for survival assay. E2 at the appropriate concentration (5 to 10×) was then added to the culture; 10- or 15-ml samples containing 2 to 4 × 10^9 cells/ml were taken and placed on ice at various time intervals. KCN was added to 10 m*M* and the samples were lysed as described below. Four-tenths milliliter samples were taken at intervals for determination of acid-soluble counts. Samples were also removed at 15 min for assay of viable cells.

Lysis of Superinfected Cells and Sucrose Gradient Analysis. The lysis procedure followed derives from the methods of Bode and Kaiser (1965) and Young and Sinsheimer (1967). Before sucrose gradient centrifugation the lysates were diluted to a sucrose concentration of 5% with 10 m*M* Tris (pH 8.0)–1 m*M* EDTA, and sheared four times with a 1-ml glass blowout pipet.

The gradient analysis procedure used is that of Botstein (1968). Gradient size was 12 ml. Neutral gradients for analysis of λ supercoils were centrifuged 2.5 hr and alkaline ones (pH 12.1) 2 hr at 38,000 rpm, 15°, in an IEC SB283 rotor. Gradients for study of linear λ DNA were centrifuged 4 hr. Fractions of about 0.3 ml were collected by pumping from the bottom through a micropipet inserted into the gradient. Eight-tenths milliliter of H_2O and 10 ml of Triton X100–toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene were added to each fraction and the fractions were assayed for radioactivity in a Beckman scintillation counter. The data were corrected for background and ^{14}C and ^{3}H spillover by IBM 360/65 computer analysis.

Results

(a) *Acid Solubilization of Bacterial DNA in Lysogenic and Nonlysogenic Strains and the Effects of Colicin E2 on Growth of Phage* λ. Several workers have observed acid solubilization of the bacterial DNA after treatment with colicin E2 (Nomura, 1963; Reynolds and Reeves, 1969; Holland and Holland, 1970). In the present experiments, cells prelabeled by growth in medium supplemented with [^{14}C]thymidine and deoxyadenosine were treated with E2 at various multiplicities; samples were taken for assay of viable cells and for determination of acid-soluble counts as described under Experimental Procedure. A consistent increase in acid-soluble counts was detected by 10 min after E2 addition, the rate and extent of solubilization being dependent on the E2 multiplicity. At high multiplicities 60 to 80% of the counts had been rendered soluble by 60 min (see Figure 5a).

It is known that acid solubilization of DNA by E2 is markedly reduced in cells carrying a λ prophage (Nose and Mizuno, 1971; Hull and Reeves, 1971). The effects of E2 on a pair of isogenic bacterial strains, one nonlysogen and the other lysogenic for λ ind$^-$, were tested. The inhibition of DNA breakdown is evident only at the high E2 multiplicity and at late times (Saxe, 1974). To determine which λ genes are involved, the effect of several mutant prophages was tested. The results indicate that the reduction in solubilization is due to the product of the rex gene of the λ prophage (Saxe, 1974).

Endo et al. (1963) reported that colicin E2 induces the development of λ from its prophage. In the present experiments, E2 at multiplicities lower than 2 induced the full development of λ from lysogenic cells. At higher multiplicities, the yield of phage was reduced. At a multiplicity of 6, a burst size of about 1 was obtained. Addition of E2 at ratios of 1 (multiplicity = 0.7), 2, 4, 8, and 64 after heat induction of a heat-inducible λ_{CI857} lysogen reduced the burst size to 55, 29, 12, <0.1, and <0.01%. The interference with λ growth may be due to attack on λ DNA, as demonstrated in later sections of this paper, to damage to the cellular synthetic machinery, or to a combination of both.

(b) *Action of E2 on λ DNA Supercoils in Vivo.* λ-Lysogenic bacteria in which a superinfecting λ phage generated supercoiled circles of labeled DNA were used to study the stages of E2 action. The procedure utilized for the λ superinfection experiments follows that described in the Experimental Procedure.

It was necessary to check whether λ DNA molecules were subject to E2-induced acid solubilization, and, if so, whether the rate and extent of solubilization were the same as for the bacterial DNA. To do this, cells were prelabeled by growth in medium containing [^{14}C]thymidine and deoxyadenosine, superinfected with ^{3}H-labeled λ, and treated with E2 at various concentrations. Samples were taken for assay of survivors and acid-soluble counts. As illustrated in Figure 1, the rate and extent of acid solubilization were nearly the same for the superinfecting λ and endogenous bacterial DNA, indicating that the two DNAs were very likely subject to the same sets of reactions. The final extent of solubilization was less than 50%, as already noted above for the bacterial DNA in λ lysogenic cells. The relative extents of total breakdown of λ and bacterial DNA are different. For example, approximately 30% breakdown of five supercoil λ DNA molecules per cell and 30% breakdown of the bacterial DNA is a significant difference in terms of mass. One possible explanation is that colicin E2 attacks specific sites in the DNA, e.g., regions of local denaturation, and that these occur with equal frequency in the two DNAs. Possible competition between these two substrates must be considered in some of these experiments.

The sucrose.gradient analysis to be described next was based on the known properties of λ DNA molecules. A single-strand scission converts a supercoil to an open circular molecule, which sediments less rapidly; double-strand scissions convert circles to linear molecules. These species may be resolved by sucrose density gradients.

Figure 2 presents the results of an experiment in which the conversion of λ DNA supercoils after treatment of superinfected bacteria with colicin E2 was examined. Colicin E2 was used at a killing multiplicity of about 20, so that the survival ratio was approximately 0.001%. At various times after E2 treatment, samples were extracted and analyzed on both neutral and alkaline sucrose gradients. In comparing the 0-min and 4-min diagrams one observes a slight increase in the slowly sedimenting peak in the neutral and alkaline gradients. By 6 min the supercoil peak is decreased and linear species are present in neutral gradients, indicating the occurrence of two-strand breaks. At 10 and 20 min, as incubation continues, the supercoil peak is reduced; linear species and fragments shorter than full λ length in size are seen in both neutral and alkaline gradients. The observations in the gradients of Figure 2 are compatible with a series of events including initial single- and/or double-strand breaks in the λ molecules, leading to progressive

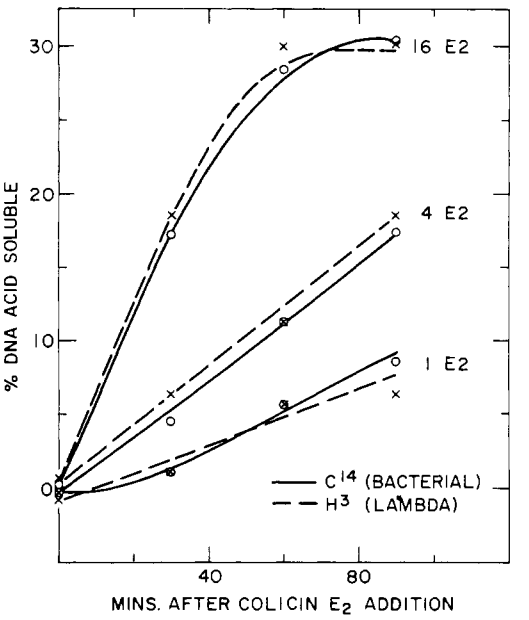

FIGURE 1: Acid solubilization of the bacterial and superinfecting λ DNA. A lysogenic strain (WD5028) was superinfected with λ labeled with [^{3}H]thymidine as described in Experimental Procedures except that the cells were prelabeled by growth in medium supplemented with 0.05 μCi/ml of [^{14}C]thymidine and 250 μg/ml of deoxyadenosine. E2 at 1-, 4-, and 16-fold concentrations was added to aliquots of cells. Samples were taken for assay of survivors at 16 min and at various time intervals for determination of acid-soluble counts. ^{3}H and ^{14}C counts were corrected for background and ^{3}H and ^{14}C spillover. The bacterial survival was 43, 7.6, and 0.01% at the three colicin concentrations (1:4:16).

changes from supercoils to circles and/or linear molecules and fragmented linear molecules. Measurements of acid-soluble label (which were included in every gradient experiment) confirmed that acid solubilization follows closely in time the appearance of the first molecular damages revealed by the gradients.

The time course of λ breakdown was followed in other experiments with altogether similar results. Figure 3 presents data from the experiment of Figure 2 and from another experiment in which colicin was used at a lower concentration. Samples were analyzed on neutral sucrose gradients and the percent supercoils remaining (counts under the supercoil peak relative to total counts recovered) were graphed against time. The course of supercoil loss (Figure 3a) is dependent on E2 multiplicity. At high E2 concentrations the supercoils are reduced to 10% or less by 20 min. At low E2 concentrations, 50 to 60% of the supercoils remain unaffected even when fewer than 1% of the cells remain viable. The percent acid-soluble counts (Figure 3b) and the size of the final λ DNA fragments are also related to the colicin multiplicity. At the lower colicin concentration the final fragments were linear and slightly shorter. In a separate experiment at a multiplicity of 40 the λ fragments were approximately 2×10^{6} daltons on neutral sucrose gradients, that is, about one-sixteenth the λ DNA size.

(c) *Trypsin Rescue of Cell Killing by E2 and Arrest of λ DNA Supercoil Breakdown.* Reynolds and Reeves (1963, 1969) first showed that treatment with trypsin within the first several minutes after addition of colicin E2 could reverse the lethal effect of the colicin. In the present work with colicin E2 trypsin rescue was tested by plating cells on LB agar containing trypsin at 100 μg/ml (Wendt, 1970). In experiments of this kind (see Figure 6a) one observes, after

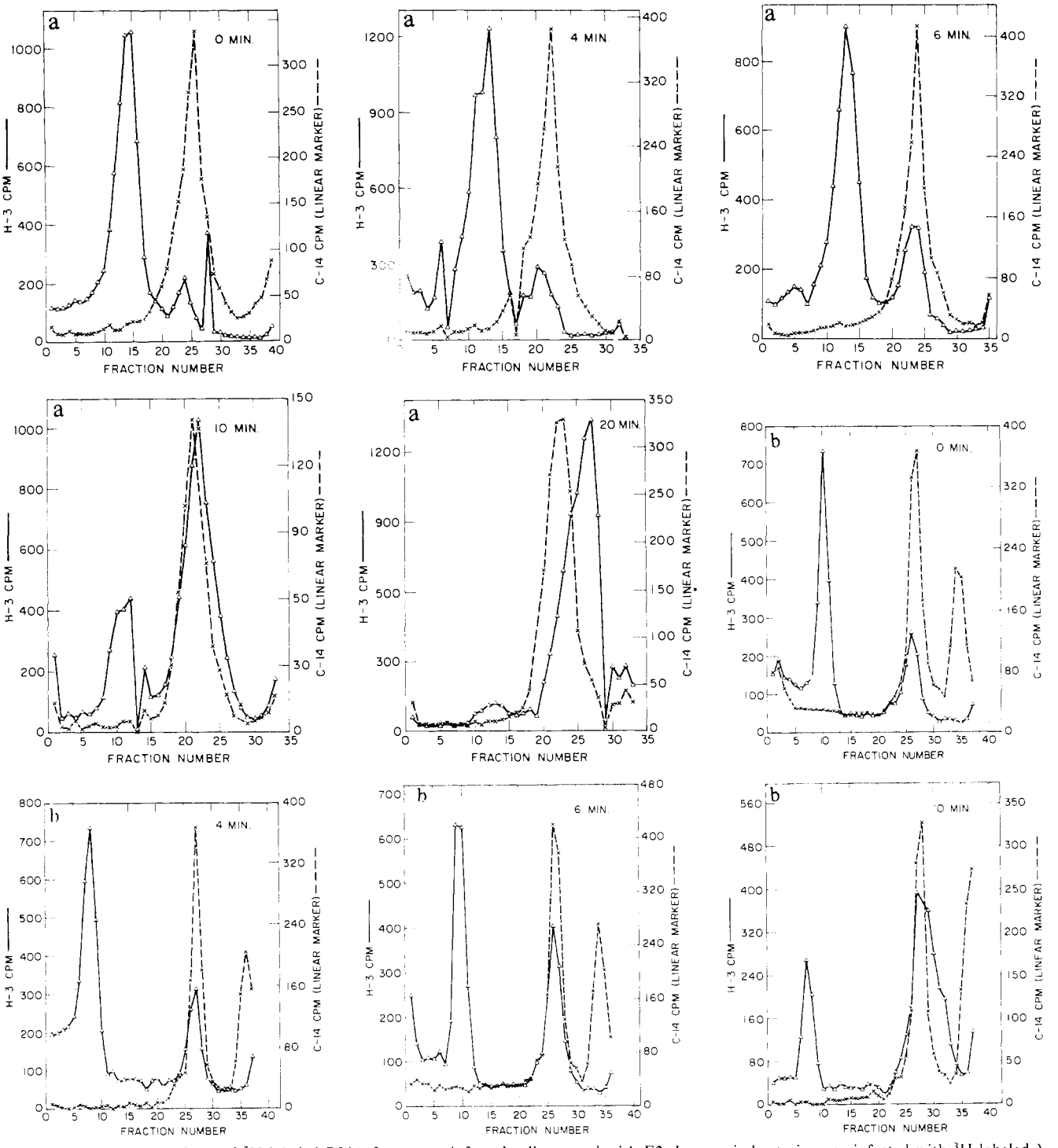

FIGURE 2: Sucrose gradients of ^{3}H-labeled DNA from superinfected cells treated with E2. Lysogenic bacteria superinfected with ^{3}H-labeled λ were treated with E2 (multiplicity ~20), lysed, and analyzed on neutral and alkaline sucrose gradients as described under Experimental Procedures: (a) DNA profiles in neutral sucrose; (b) DNA profiles in alkaline sucrose. The ^{14}C reference marker DNA in part b (broken line) was extracted from phage purified by one cycle of differential centrifugation; the counts at the top of the gradient (higher number fractions) represent acid-soluble counts.

a brief initial delay, an exponential loss of trypsin reversibility at a rate dependent on the multiplicity of colicin. The early delay could be due to repair of damage that had only reached some initial stage—for example, only single-strand breaks such as are found early (Ringrose, 1970).

Plate and Luria (1972) have defined for colicin K two stages in the action of colicins. In stage I a cell is still subject to trypsin rescue and physiological damage has not occurred. The transition from stage I to stage II, resulting in cell damage and absence of rescuability by trypsin, occurs with a constant probability per killing of colicin. The present results indicate that for E2 the transition starts with a few minutes delay and then proceeds exponentially as with other colicins.

Since trypsin can prevent the transition of colicin E2 to the damage-producing stage, it was important to examine the effects of trypsin on cells in which λ molecules were already being damaged by the action of E2. In Ringrose's (1970) experiments, if colicin E2 was followed by trypsin within 2 min after E2 addition, the molecular weight of the

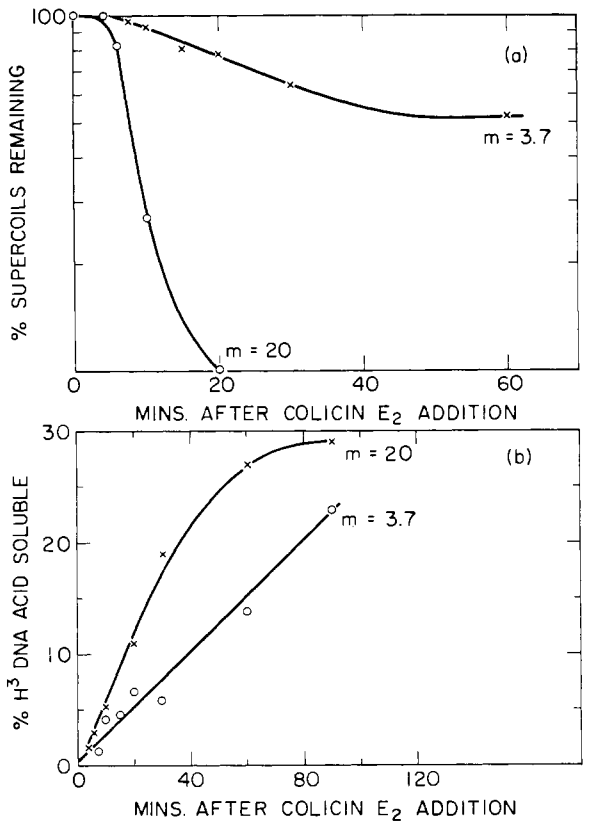

FIGURE 3: Loss of λ supercoils in E2-treated cells. Strain WD5028 superinfected with ^{3}H-labeled λ as described in Experimental Procedures was treated with E2 at low and high multiplicity in two separate experiments. Samples were taken at various intervals for gradient analysis in neutral sucrose gradients, for assay of acid-soluble counts, and at 20 min for viable counts. The percent supercoils remaining, obtained as the sum of the percent counts included under the supercoil peak normalized to the percent supercoils available for breakdown (percent supercoils in an untreated control), was calculated for each gradient sample. The multiplicity was 3.7 in experiment 1 and 20 in experiment 2: (a) percent supercoils remaining; (b) acid solubilization of λ DNA.

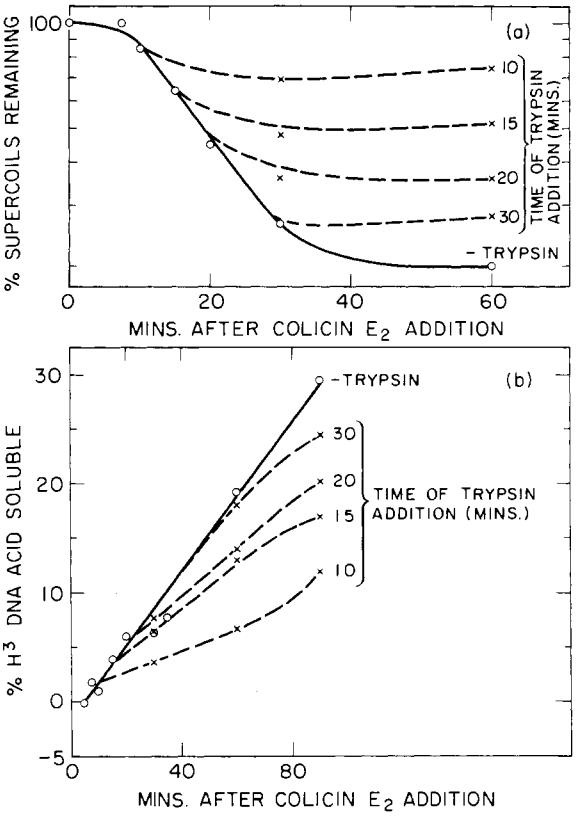

FIGURE 4: Effect of trypsin on λ DNA breakdown by E2. Lysogenic bacteria superinfected with λ as described under Experimental Procedures were treated with E2 (multiplicity 6.7). Sixteen-milliliter control samples were chilled on ice at 10, 15, 20, 30, and 60 min. Trypsin (2 mg/ml) was added to 30-ml cells at 10, 15, 20, and 30 min and samples were taken and chilled after 30 and 60 min. Lysates from all samples, prepared as described in Experimental Procedures, were resuspended in 0.2 ml of 1 *M* sucrose. The lysates were analyzed on neutral sucrose gradients and the percent supercoils remaining was calculated for each sample: (a) percent supercoils remaining; (b) acid solubilization of λ DNA.

single-stranded bacterial DNA after an initial decrease regained its original untreated value, presumably due to repair by polynucleotide ligase. Added at later times, trypsin was without noticeable effect.

In two experiments, with colicin E2 at different multiplicities, trypsin was added at various times to E2-treated, λ-superinfected cells and samples were taken at the time of trypsin addition and at various intervals afterwards for gradient analysis. Similar results were obtained for low and high E2 concentrations. As shown in Figure 4, at the low concentration of E2 the loss of λ supercoils continued for a few minutes after addition of trypsin and then stopped. Acid solubilization (Figure 4b) continued at a decreased rate, presumably from breaks or gaps formed prior to trypsin addition. Unexpectedly, the neutral gradients showed that not only the loss of supercoils but also the cleavage of the linear and the fragmentation of λ pieces was halted. Continued fragmentation by bacterial enzymes of the linear λ DNA and pieces may have been expected. Thus it appears that the continued presence of externally-accessible colicin is required, not only for a primary introduction of breaks into DNA molecules, but also for continued endonucleolytic cleavage of the DNA. The implication of this finding will be commented upon in the Discussion section.

No measurable increase of λ supercoils was observed after addition of trypsin (see Figure 4a), in contrast to Ringrose's (1970) finding of a restoration of bacterial DNA. This is reasonable since the initial λ molecules resulting from E2 attack may be linear molecules, or repair may be limited by the low amounts of available open circles not yet converted to linear forms.

(d) *Inhibition of Degradation of λ DNA Supercoils by Energy Inhibitors.* Energy inhibitors are known to block bacterial killing and DNA acid solubilization by colicin E2 (Reynolds and Reeves, 1963, 1969). To test whether they would block the damage to the λ DNA, λ-superinfected lysogenic cells were pretreated for 15 min with the inhibitors potassium cyanide (KCN), 2,4-dinitrophenol (2,4-Dnp), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or with colicin K (the gift of H. Unsöld), which is known to act at the level of energy metabolism (Fields and Luria, 1969). E2 was then added and samples were taken for gradient analysis at 7 min, when supercoils have started to decrease in the controls. In all cases no breakdown of supercoils occurred; killing and acid solubilization were also prevented. When KCN was added to an E2-treated culture 7 min after the colicin and samples were analyzed at 10 and 60 min after addition of E2, there was no further breakdown of supercoils, of linear molecules, or of fragments or any further killing or acid solubilization. These results indicate that energy is required for the first step in E2-initiated attack as well as for the later steps.

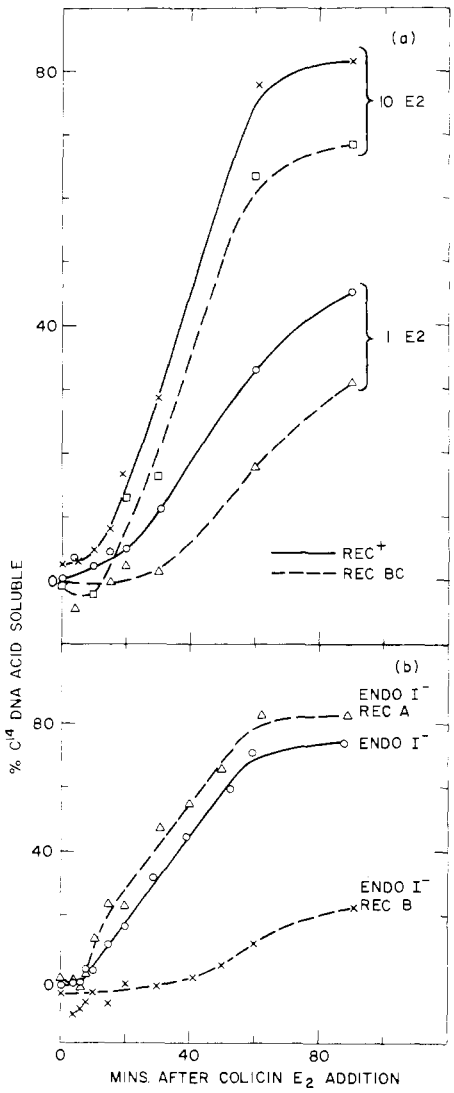

FIGURE 5: Acid solubilization of DNA in recombination-deficient mutant strains by colicin E2. (a) Rec$^+$ (AB1157) and Rec BC (AB1157 RecBC). Procedure as in Experimental Procedures. E2 concentrations differed by a factor of 10. For each concentration, the two strains were treated in parallel. The final bacterial survival was 4.9 and 0.0005% for Rec$^+$ and 0.8 and 0.001% for Rec BC at the two colicin concentrations. (b) Endo I$^-$ (QW7), Endo I$^-$ Rec A (QW8), and Endo I$^-$ Rec B (QW9). Procedure as in Experimental Procedures. The bacterial survival was 8.8% for Endo I$^-$, 0.8% for Endo I$^-$ Rec A, and 0.6% for Endo I$^-$ Rec B.

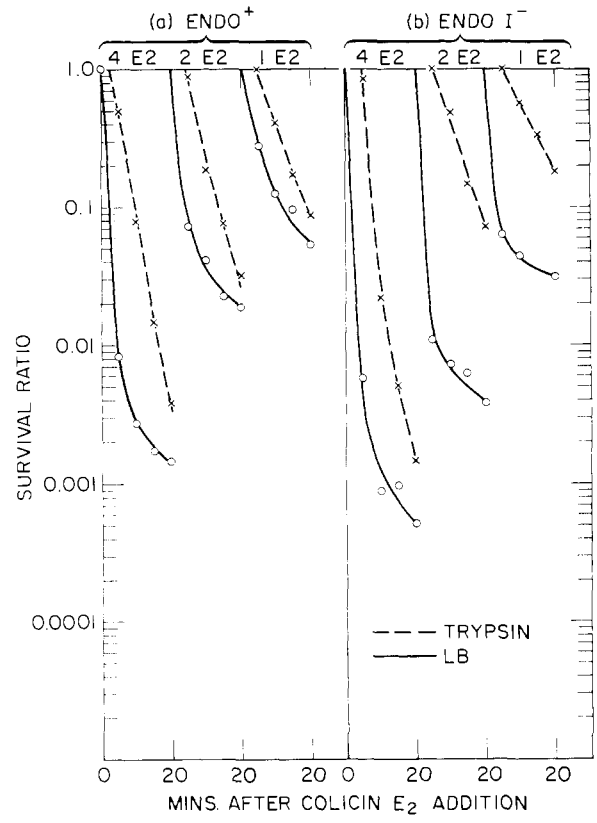

FIGURE 6: Trypsin rescue of E2-treated cells of HfrH (Endo$^+$) and MRW25 (Endo I$^-$). Overnight cultures of strains HfrH and MRW25 in λ YMB$_1$ medium were diluted 1:100 into fresh medium, incubated at 34° to a Klett value of 40, and then placed on ice. Colicin E2 was diluted gently in cold 0.85% saline containing BSA at 2 mg/ml, the final dilution being in λ YMB$_1$ medium. Various colicin dilutions and cell suspensions were prewarmed separately 3 min at 34° and 0.5 ml of colicin was added to 0.5 ml of cells. The mixtures were diluted 1:100 at 5 min to arrest further colicin adsorption. Aliquots were taken at 5-min intervals up to 20 min, diluted in cold λ broth, and plated by spreading on LB agar with or without trypsin (100 μg/ml). Parent and mutant strains were treated in parallel and plated on LB agar with or without trypsin. The calculated multiplicities were 2.9 at onefold E2 concentration, 3.9 at twofold, and 6.5 at fourfold for Endo$^+$ and 7.6, 5.5, and 3.5 for Endo I$^-$: (a) Endo$^+$; (b) Endo I$^-$.

(e) *The Effects of Certain Bacterial Mutations on E2-Initiated Cell Killing, Acid Solubilization of the Bacterial DNA, and Breakdown of λ DNA Supercoils.* The effects of colicin E2 on various mutants with defects in DNA metabolism were tested in an attempt to establish a role of bacterial enzymes in E2 action. Parent and mutant strains were tested in parallel for cell killing by E2, trypsin rescue, and acid solubilization of the DNA. The mutants included Rec A, Rec BC, Endo I$^-$, Endo I$^-$ Rec B, and Endo I$^-$ Rec A. The Endo I$^-$ strain, MRW25, has been shown to have less than 1% residual Endo I activity (Wright, 1971). Only the most marked effects will be mentioned.

The Rec A and Rec BC strains were somewhat more sensitive to E2 killing than the parental strains. Since, however, Rec BC and Rec A cells formed filaments and since approximately 50% of the cells yielded no colonies, the viable counts data may be inaccurate.

Acid solubilization was depressed in the Rec BC strain (which lacks endo- and exonucleolytic DNase activities and ATPase activity; Goldmark and Linn, 1972), starting from early times and at both low and high colicin multiplicities (Figure 5a). Solubilization was only slightly enhanced in the Rec A mutant at a low colicin multiplicity. Marked depression of the rate and extent of solubilization occurred in the Endo I$^-$ Rec B strain, while solubilization in the Endo I$^-$ Rec A strain at a low colicin multiplicity was slightly enhanced compared to the Endo I$^-$ strain (Figure 5b).

No significant difference in acid solubilization between the parent and the Endo I$^-$ mutant strains was detected, as also reported by Buxton and Holland (1974). The Endo I$^-$ strain exhibited a somewhat greater trypsin rescue, an indication that the trypsin-irreversible step may be delayed in this strain (Figure 6).

The effects of the Rec BC and Endo I$^-$ mutations on colicin E2 induced breakdown of λ supercoils in superinfected bacteria were then examined. A small amount of colicin E2 sufficient to produce a measurable effect on the parent strain was utilized. The pattern of disappearance of supercoils in the pair of strains Rec$^+$ and Rec BC was similar. With the Endo I$^-$ strain the loss of supercoils was delayed by a few minutes, suggesting that Endo I may be involved in

an early step of E2 action, although the mutation in the Endo I gene evidently does not cause failure to respond to the colicin. This finding, as well as the more persistent rescuability by trypsin mentioned above, supports the idea that the product of the Endo I gene plays some role in the early steps of colicin E2 action, as suggested by Almendinger and Hager (1972, 1973).

Discussion

The present report provides additional information about the in vivo action of colicin E2 on DNA.

The picture derived from the in vivo experiments with λ DNA circles does not strictly fit Ringrose's description of colicin E2 action on bacterial DNA. Some delay was observed before the λ supercoils were affected. A possible source of disparity is that Ringrose's experiments on bacterial DNA were carried out in minimal M9A medium at 37°, whereas the λ experiments were done in tryptone broth at 34°. It was not possible to ascertain in these experiments whether single-strand breaks precede double breaks, as reported by Ringrose.

The finding that λ DNA supercoils are subject to colicin E2 attack extends Ringrose's findings to include two conclusions: (1) DNA synthesis is not required for colicin attack since λ supercoils are nonreplicating; (2) a membrane association of the DNA substrate is not needed since λ DNA supercoils exist free in the cytoplasm (Sakakibara and Tomizawa, 1971). The data of Sakakibara and Tomizawa (1971), however, do not exclude the possibility that the association of λ supercoils to the DNA membrane may be labile and not easily detected. Ringrose (1970) suggested that the attack sites on bacterial DNA may be specific on the basis of the density heterogeneity of the final fragments. The sites could represent similar sequences in the λ and bacterial DNAs. Experiments on bacteria carrying smaller plasmids should be helpful in approaching this problem.

In contrast to the all-or-none killing action of a single colicin molecule, the process of supercoil loss is related to colicin multiplicity. This suggests that the active agent involved in the conversion is either unstable or is tied up in the course of the reaction. The fact that a certain percentage of supercoils remains undamaged could also be explained if these were less accessible to nuclease attack. Higher multiplicities of colicin could increase the likelihood that these species would be attacked.

An intriguing finding is the ability of trypsin to arrest the breakdown of DNA supercoils in colicin-treated cells. Apparently the endonucleolytic agent(s), either the colicin itself or one or more bacterial enzymes, must stop acting on DNA when trypsin-accessible colicin is removed. Trypsin may remove partially penetrated E2 molecules or E2 molecules that direct some bacterial endonuclease reversibly inward toward the cytoplasm.

How do these results fit Almendinger and Hager's (1972, 1973) interpretation of E2 action, namely, that it causes access of endonuclease I to intracellular DNA, either by causing its entry or by removing inhibitory RNA? In favor of it there is the fact that an Endo I^- strain, although fully sensitive to E2, shows a delayed start of supercoil degradation and a prolonged sensitivity to trypsin rescue. The strain used in the present work has supposedly only 1.0% the endonuclease activity of the wild type (Wright, 1971); yet even this small amount may be sufficient to mediate E2 action. E2 might also favor the access of other nucleases to DNA.

The alternative hypothesis, that E2 may itself be a nuclease and act directly on DNA, is also in agreement with the findings presented here and is supported by the in vitro experiments reported in the accompanying paper.

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