The Action of Colicin E2 on Supercoiled λ DNA. II. Experiments in Vitro†

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ABSTRACT: An in vitro system has been developed to test whether colicin E2 possesses DNase activity. Purified colicin E2 preparations introduced one single-strand scission in supercoiled λ phage DNA. Glycerol gradient fractionation of colicin E2 supports the association of in vitro action with in vivo cell-killing activity. Colicin E2 preparations also attacked superhelical SV40 DNA yielding open circles and fragments and single-stranded fd DNA molecules causing one or more endonucleolytic breaks. The possible role of contaminating nucleases in the activity of colicin E2 preparations is discussed.

Colicin E2, following its adsorption to susceptible cells of *Escherichia coli,* initiates a process that leads to progessive damage to cellular DNA (Ringrose, 1970). This is evidenced by the appearance, in DNA extracted from colicin-treated cells, of single- and double-strand breaks and of acid-soluble fragments. It has been reported (Ringrose, 1972) that purified colicin E2 has no nucleolytic activity in vitro (on bacterial DNA, 10^7 mol wt). Its action on DNA in vivo has generally been interpreted as a secondary effect in line with Nomura's (1963) hypothesis that the action of colicins is exerted indirectly from the cell surface. In particular Almendinger and Hager (1972, 1973) have proposed that E2 allows the enzyme endonuclease I to enter the endoplasm from the periplasmic space, therefore allowing attack on the DNA.

We present here evidence suggestive of a specific endonucleolytic activity on DNA for colicin E2. We have followed the effects of E2 on supercoiled DNA of phage λ produced by superinfection of λ lysogens with λ phage labeled with [^{3}H]thymidine. The conversion of these supercoiled DNA molecules to open circles, linear molecules, and smaller fragments can be followed in sucrose gradients with greater precision than the degradation of bacterial DNA. Having established (Saxe, 1975) that the in vivo degradation of the DNA supercoils in E2-treated cells followed the pattern observed by Ringrose (1970) for bacterial DNA, we proceeded to test the effect of purified E2 on isolated λ supercoils in vitro.

Experimental Procedure

(a) *Strains and Procedures.* Most strains and procedures used are described in the preceding paper (Saxe, 1975). Table I lists additional strains.

(b) *Media.* In addition to the media used previously (Saxe, 1975), medium M9A base was used for preparation of λ DNA supercoils. It contains per liter: 3 g of KH_2PO_4, 5.8 g of Na_2HPO_4, 1 g of NH_4Cl, and 0.5 g of NaCl. The following supplements were added: $MgSO_4$ (10^{-3} *M*), glucose (0.4%), casamino acids (0.3%), vitamin B_1 (1 μg/ml), and thymine (2 μg/ml).

(c) *Chemicals.* tRNA (sRNA, *E. coli*) was purchased from General Biochemicals. RNase (Code R), from Worthington Biochemical Corp., was preheated as described by Wickner et al. (1972).

(d) *Preparation of Colicin E2.* E2 was prepared from strain QR47 (Col E2) and was purified according to the procedure of Herschman and Helinski (1967). Twelve liters of cells (Klett value of 120) was induced with mitomycin C (0.2 μg/ml), covered with a dark cloth, and allowed to incubate with gentle bubbling at 37° for 2 hr. The culture was chilled on ice and all subsequent operations were performed at 0°. The cells were collected and washed three times with 1 *M* NaCl in 10 m*M* potassium phosphate buffer (pH 7.0) using a Waring Blendor. The washes were pooled and colicin E2 activity was precipitated by a 20–40% $(NH_4)_2SO_4$ fractionation. This precipitate was purified by chromatographic fractionation on DEAE-Sephadex followed by CM-Sephadex. The peak fractions after CM-Sephadex chromatographic fractionation were pooled (preparation I); fractions surrounding the peak were also retained (preparation Ia). Protein concentrations were determined by the method of Lowry et al. (1951) or spectrophotometrically according to Herschman and Helinski (1967).

Colicin E2 killing units (Saxe, 1975) were assayed using strain C600. The specific activity of preparation I was 2.0×10^{14} KU/mg.[1] Assuming a molecular weight of 60,000 this equals about 50 molecules per killing unit. The specific activity of preparation Ia was about 1.4×10^{14} KU/mg.

Colicin E2 was purified also from strain 1100 (Col E2) (preparation II). Col E2 factor was transferred to the endonuclease I defective strain 1100 (Dürwald and Hoffman-Berling, 1968) from strain M72 (Col E2, F) by the mixed culture technique (Kennedy, 1972). Colicin E2 production was about tenfold lower in this strain. The final specific activity was 0.6×10^{14} KU/mg of protein.

An aliquot of a peak fraction (fraction 101) after CM-Sephadex chromatographic fractionation of a third colicin

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[1] Abbreviations used are: BSA, bovine serum albumin; IP-E2, immunity protein E2; IP-E3, immunity protein E3; KU, killing units; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; RNase, ribonuclease; SDS, sodium dodecyl sulfate; SLS, sodium lauryl sarcosinate-30.

E2 preparation (preparation III) from strain QR47 (Col E2) was retained and not pooled. The specific activity of the fraction was 0.9×10^{14} KU/mg. Adjacent fractions were also retained.

Colicin E2 preparation IV (CM-Sephadex eluate, 1.0×10^{14} KU/mg) and preparation V (DEAE-Sephadex eluate, 0.7×10^{13} KU/mg) were the kind gift of K. Jakes.

The purity of various fractions was assayed by electrophoresis on polyacrylamide disc gels (see below). In addition to the main colicin band, a band that migrates with the dye marker was observed in all preparations. This band may represent the E2 immunity substance, analogous to the E3 immunity protein, whose molecular weight is 10,000 (Jakes et al., 1974; Sidikaro and Nomura, 1974). This band is the only other band in E2 preparation III, a highly purified fraction. The substance is probably bound to the purified colicin E2 molecule (Jakes et al., 1974; see Discussion). Excluding this band, colicin preparation I appeared to be more than 90% pure; preparation II was about 70% pure and preparation III, 99%.

(e) *SDS Disc Gel Electrophoresis.* The procedure of Laemmli (1970) was followed for SDS disc gel electrophoresis. The gels contained 7.5% polyacrylamide, 0.4% (twice the usual amount) *N,N*-methylenebisacrylamide, 0.1% SDS, and 5% glycerol. The samples were adjusted to 1% SDS, 1% β-mercaptoethanol, 10% glycerol, and 2–5 m*M* $PhMeSO_2F$ to inhibit proteolysis (except when otherwise stated). Five microliters of 0.1% Bromophenol Blue was added as a tracking dye. The samples were immersed for 1 min in a boiling water bath to dissociate the proteins. Electrophoresis was carried out at constant voltage (125 V) until the Bromophenol Blue marker reached the bottom of the gel. The gels were stained with a solution of 0.1% Coomassie Blue in 25% isopropyl alcohol–10% acetic acid and diffusion destained by repeated washings in 10% acetic acid–10% isopropyl alcohol. The stained gels were then scanned at 550 mμ in a Gilford recording spectrophotometer.

(f) *Sucrose Gradient Analysis.* The procedures were described by Saxe (1975). Preparative sucrose gradients contained 5–20% sucrose, 10 m*M* Tris (pH 8.0), and 1 m*M* EDTA. Alkaline sucrose gradients used to resolve linear and circular λ, SV40, and fd DNA molecules contained 10–30% sucrose, 0.3 *M* NaOH, 0.7 *M* NaCl, 0.0025 *M* EDTA, and 0.015% SLS. They were centrifuged for 12 hr for λ and for 20 hr for SV40 and fd at 38,000 rpm, 10°, in an IEC SB283 rotor.

(g) *λ DNA Supercoils.* ^{3}H-Labeled λ DNA supercoils used in the in vitro experiments were prepared by heat induction in minimal M9A medium containing [^{3}H]thymidine (0.5–1.0 μCi/μg of thymine) of strain AS2D *rec*A and carrying a heat-inducible, recombination-deficient λ prophage (M. Gefter, personal communication). They were purified by centrifugation in cesium chloride–ethidium bromide density gradients. Ethidium bromide was removed either by passing the pooled supercoil fractions over a Dowex-50 column or by extraction with isopropyl alcohol. For some experiments the supercoils were further purified by centrifugation in sucrose density gradients.

(h) *Endonuclease I Assay.* The reaction mixtures contained: 150 m*M* Tris-HCl (pH 7.5), 5 m*M* Mg^{2+}, 5 m*M* β-mercaptoethanol, 0.25 mg/ml of BSA, 0.25 mg/ml of RNase, 25% glycerol, 25 μg/ml of [^{3}H]poly[d(A-T)], and enzyme in a total volume of 20 μl. The assay mixtures plus tRNA were supplemented with 1.23 mg/ml of tRNA, and contained 0.5 mg/ml of BSA and no RNase. Dilutions of endonuclease I were performed in 50% propylene glycol–10 m*M* Tris-HCl (pH 8.0). Reaction mixtures were incubated at 37° for 30 min. To stop the reaction 20 μl of salmon sperm DNA (2.5 mg/ml) was added, the reaction mixtures were chilled, and 10 μl of 6% perchloric acid was added. The mixtures were allowed to sit 15 min on ice, then centrifuged at 15,000 rpm, 5°, for 10 min in the Sorvall SS-34 rotor. Forty microliters of the supernatants was counted in 10 ml of Bray's scintillant containing 5 μl of 2 *N* NH_4OH.

Table I: Bacterial Strains.

E. coli K12 Strain	Properties	Source
QR47 (Col E2)	$B_1^- S_{II}^+ rec^+ UV^s$	S. E. Luria
M72 (Col E2) (F)	$lac^-am\, trp^-am$	S. E. Luria
C600	$thr^- leu^- B_1^- T_1^r T_6^r lac^-$	S. E. Luria
AS2D	*rec* A	M. Gefter
1100	B_1^- *Endo* I^-	J. Eigner

Results

Action of Purified Colicin E2 on λ DNA Supercoils in Vitro. Pooled λ DNA supercoils labeled with [^{3}H]thymidine were exposed to colicin E2 preparation I in a reaction mixture containing Tris-HCl (pH 8.0) (10 m*M*), $MgCl_2$ (10 m*M*), and colicin E2. The mixtures were incubated at 37°. The reaction was stopped by the addition of EDTA to a final concentration of 20 m*M* and SDS to a final concentration of 0.2%. An aliquot of ^{14}C-labeled linear λ DNA molecules, preheated to 75° for 10 min and then chilled for neutral gradient analysis (to separate annealed sticky ends), was added as a marker and the samples were analyzed on either neutral or alkaline sucrose gradients. Typical results are shown in Figure 1. Approximately half the supercoils were converted to open circles or linear molecules during a 2-hr incubation at a 2:1 ratio of DNA to colicin E2 (preparation I) corresponding to a molar ratio of 1:1000 of supercoils to colicin molecules. No fragments smaller than full λ DNA size were detected on alkaline sucrose density gradients (Figure 2).

The extent of the reaction is defined as the amount of DNA removed from the supercoil peak normalized to the total supercoil DNA available for breakdown. The dependence of the reaction on the concentrations of colicin and of DNA supercoils is illustrated in Table II. The total extent of the reaction (DNA cut, micrograms per milliliter) increases with increasing concentrations of colicin and of DNA.

Comparable results were obtained with colicin E2 preparation II from strain 1100 (Col E2), which is deficient in endonuclease I activity (Table II). Preparation III, a 99% pure fraction from a CM-Sephadex chromatographic fractionation, exhibited in vitro activity very similar to that of the other preparations (Table II).

EDTA added to a final concentration of 20 m*M* blocked the reaction. No reaction occurred in the presence of 2.5 m*M* EDTA and no added Mg^{2+} ions. Mg^{2+} ions or other divalent cations, therefore, are apparently required for the reaction. SDS (0.001%) prevented the reaction.

Increasing the reaction time increases the loss of supercoils (Table II). As in in vivo experiments (Saxe, 1975) the loss of supercoils with time approaches a simple exponential function.

Glycerol Gradient Fractionation of Colicin E2. A colicin

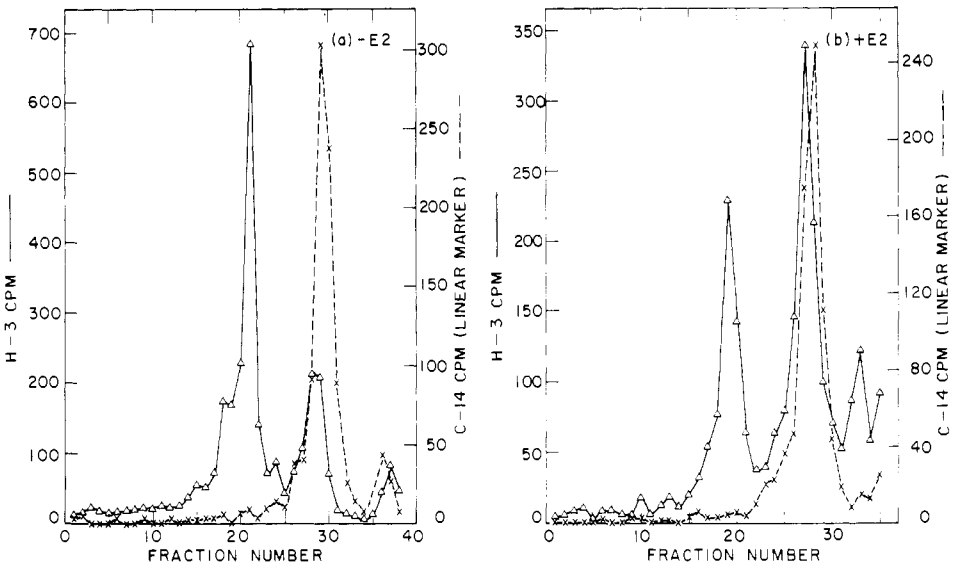

FIGURE 1: Action of a colicin E2 preparation on supercoiled λ DNA in vitro. The in vitro reaction mixture contained: supercoiled λ DNA (the kind gift of M. Gefter) (25 μg/ml) and E2 preparation I (12.5 μg/ml) in a total volume of 50 μl. (The DNA concentration was determined from the OD_{260} and might represent an overestimate due to contaminating RNA.) The mixtures were incubated at 37° for 2 hr, and then stored at 4° prior to gradient analysis at pH 12.1. Note that the supercoils were not denatured by the alkaline conditions.

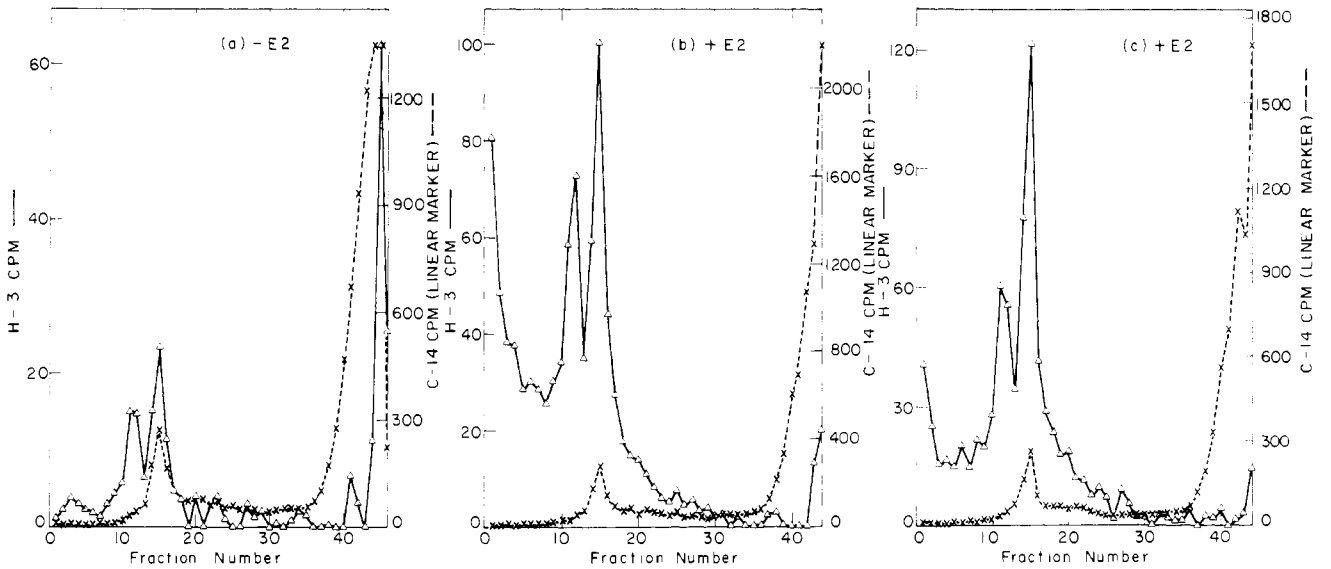

FIGURE 2: Alkaline sucrose gradients of λ DNA molecules after incubation with colicin E2. Procedure as described in Table II, part D. The in vitro reaction mixture contained supercoiled λ DNA (1.8 μg/ml) and colicin E2 preparation III (fraction 99) (30 μg/ml) in a total volume of 44 μl. They were incubated at 37° for 160 (a and b) or 380 (c) min. Alkaline sucrose gradient centrifugation was as described in Experimental Procedure section. ^{14}C-Labeled λ DNA marker was prepared from λ phage by treatment with SLS (Botstein, 1968). The counts at the top of the gradient represent ^{14}C-labeled acid-soluble counts in the crude phage preparation.

E2 preparation, preparation IV, was fractionated on a glycerol gradient and selected fractions were tested for both cell-killing activity and for nucleolytic attack on supercoils. The two activities sedimented identically (Figure 3). A similar result was obtained using colicin E2, preparation Ia.

Role of Endonuclease I in the in Vitro Action and Heat Inactivation of Colicin E2. Endonuclease I is an enzyme located in the periplasmic space of the cell; gentle treatments release it complexed with inhibitory tRNA. The complex, in the presence of 0.5 *M* NaCl, introduces one single-strand scission in supercoiled molecules of Col E1 DNA (Goebel and Helinski, 1970). Stripped of the inhibitory RNA by RNase treatment, endonuclease I produces double-strand breaks in bacterial DNA yielding terminal fragments of seven to eight nucleotides. Almendinger and Hager (1972, 1973) have proposed that endonuclease I may be responsible for colicin E2 attack on DNA in vivo. Since endonuclease I might be present as an unrecognized impurity in our colicin E2 preparations, tests were performed to examine a possible role of endonuclease I in the in vitro action of colicin E2. Colicin E2 preparation II was purified from strain 1100 (Col E2) deficient in endonuclease I activity (0.1–0.3% residual level; Dürwald and Hoffman-Berling, 1968). This preparation exhibited in vitro activity comparable to preparation I from a wild type strain (see Table II). tRNA (120 μg/ml) did not inhibit the reaction and RNase (20 μg/ml) added to destroy inhibitory RNA did not enhance the action of E2 in vitro.

Three colicin E2 preparations as well as a control preparation of endonuclease I purified according to the procedure of deWaard and Lehman (1966) and obtained from Dr. T. Rajhbhandary were assayed for endonuclease I activity

Table II: Characteristics of the in Vitro Nucleolytic Reaction.[a]

DNA (μg/ml)	E2 (μg/ml)	Slowly Sedimenting DNA (%)	DNA Cut (μg/ml)
	A. Dependence on Colicin Concentration		
13	Preparation I, 5	7	
13	10	29	
13	20	51	
	B. Dependence on DNA Concentration		
13	Preparation I, 10	29	3.8
26	10	24	6.2
52	10	24	12.5
	C. Effect of Colicin E2 Prepared from an Endonuclease I Deficient Strain		
26	Preparation II, 23[b]	25	
26	46[b]	39	
	D. Effect of Highly Purified Colicin E2[c]		
2	Preparation III, 9 (fraction 101)	18	
2	18	29	
	E. Dependence on the Reaction Time		
26	Preparation I, 10	8	(1 hr)
26	10	24	(2 hr)
26	10	51	(4 hr)

[a] Procedure as described in Figure 1 except that the samples were analyzed on neutral sucrose gradients. The values are the percents of the available counts in the supercoil peak that were converted to slowly sedimenting counts. [b] The killing titer of this colicin preparation had decreased fourfold during storage. [c] The λ DNA supercoils were further purified on neutral preparative gradients and the supercoil-containing fractions pooled. The reaction mixtures also contained approximately 15% sucrose and 1 m*M* EDTA. They were incubated at 37° for 3 hr.

Table III: Tests for Endonuclease I in Colicin E2 Preparations.[a]

Additions (μg/ml)	Acid-Soluble (cpm) − tRNA	Acid-Soluble (cpm) + tRNA
None	134	130
Endonuclease I (0.25)	2313	117
E2 preparation III, fraction 101 (93)	115	127
E2 preparation IV (250)	122	113
E2 preparation V (375)	171	125

[a] Procedure for the endonuclease I assay, performed with the aid of M. Silberklang, as described in Experimental Procedure.

using uniformly labeled [^{3}H]poly[d(A-T)] as a substrate. The samples were tested at concentrations of 93 to 375 μg of protein per ml. As shown in Table III, in no case was any activity detected except in the endonuclease I control. The assay is sensitive to less than 0.025 μg/ml of endonuclease I.

Colicin E2 and the preparation of endonuclease I were tested for nucleolytic action on λ DNA supercoils. At a final concentration of 0.05 μg/ml, endonuclease I caused only partial breakage (18%) of λ supercoils, and this reaction was inhibited by tRNA (120 μg/ml). Colicin E2, preparation III (fraction 98) or V, 30 μg/ml, induced the breakdown of approximately 25% of the supercoils to slowly sedimenting forms in the presence or absence of tRNA. Colicin E2 preparation V, at 375 μg of protein per ml, contained no detectable endonuclease I (less than 0.025 μg/ml; see Table III). Colicin E2, preparation III or V, added together with endonuclease I appeared to increase considerably the activity of endonuclease I.

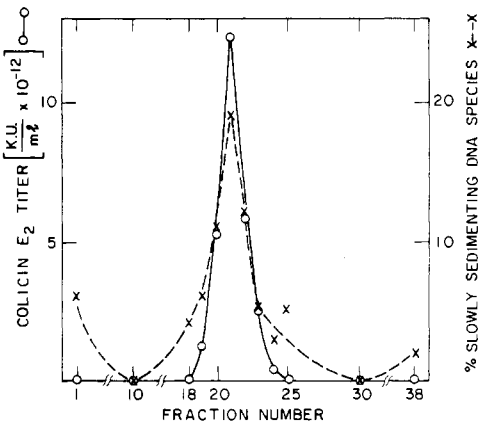

FIGURE 3: Glycerol gradient fractionation of colicin E2. A 0.05-ml sample of a purified colicin preparation, preparation IV, was layered on a 4-ml glycerol gradient (10–30%) containing Tris-HCl (pH 8.0) (50 m*M*), KCl (10 m*M*) and EDTA (1 m*M*). The gradient was centrifuged 24 hr at 55 krpm, 4°, in an IEC SB405 rotor. Fractions of about 0.1 ml were collected from the bottom of the tube. Supercoil-attacking activity was assayed on selected fractions using λ DNA supercoils as described in Table II, part D. Twenty microliters of each fraction was mixed with 25 μl of λ DNA supercoils (1 μg/ml) and incubated at 37° for 4.5 hr. The reaction mixtures were sedimented on neutral sucrose density gradients. The percent of slowly sedimenting λ DNA (fraction of the counts under the slowly sedimenting peak minus the fraction in an untreated control) was calculated for each gradient. The cell-killing activity in the gradient fractions was tested on aliquots diluted in cold 0.85% saline plus BSA (2 mg/ml).

Table IV: Heat Inactivation of Colicin E2.[a]

Time of Heating at 65° (min)	Slowly Sedimenting DNA (%)	E2 (KU/ml)
0	43	1.5×10^{13}
1	32	7.4×10^{12}
2	29	4.5×10^{12}
6	27	3.2×10^{12}
10	26	2.3×10^{12}

[a] Procedure as in Table II, part D. The reaction mixture contained in a volume of 55 μl: supercoiled λ DNA (2.4 μg/ml) and colicin E2 preparation III (30 μg/ml). The colicin samples had been heated for various times. Incubation was for 380 min.

Heat inactivation for the two activities of several colicin E2 preparations, breakage of DNA supercoils and killing of bacteria, proceeded at roughly comparable rates, as shown in Table IV. For two colicin preparations (IV and V), however, incubation at 70° resulted in an increased nucleolytic activity. For example, a sample of preparation V heated for 10 min caused more extensive breakdown of λ DNA supercoils, including two-strand breaks. This nucleolytic reaction was partially inhibited by tRNA (120 μg/ml) which prevented the production of linear molecules by two-strand breaks. A likely explanation is that preparations IV and V contained a masked form of endonuclease I, either free or bound to the colicin. No comparable findings were made with other purified colicin E2 preparations.

Molecular Action of Colicin E2. When reaction mixtures of λ DNA supercoils with colicin E2 were run in alkaline gradients to separate single-stranded circles from linear molecules, it was apparent that both forms were present, as shown in Figure 2. This suggests that at least a large part of the damage to supercoils consists of single-strand breaks. The ratio of linear to circular species increased after pro-

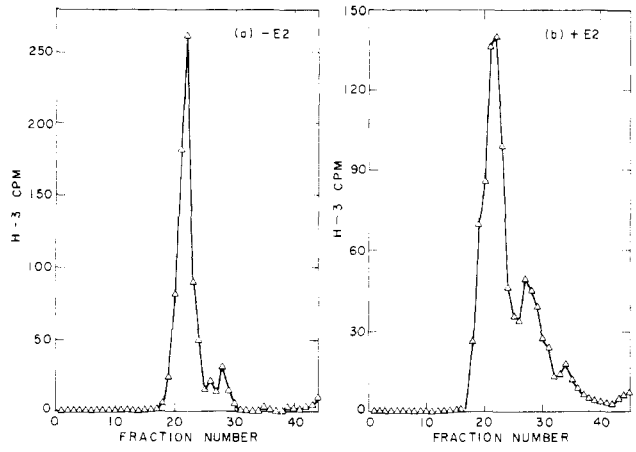

FIGURE 4: Change in SV40 DNA in an in vitro mixture with colicin E2. Procedure as in Table II. The in vitro reaction mixtures contained: supercoiled SV40 DNA (the gift of G. Fareed) (2.3 μg/ml) and colicin E2 preparation I (70 μg/ml) in a total volume of 45 μl. The killing titer of colicin E2 preparation I had decreased fivefold during storage. The mixtures were incubated at 37° for 3 hr. The samples were centrifuged on neutral sucrose gradients at 38,000 rpm, 15°, for 5 hr.

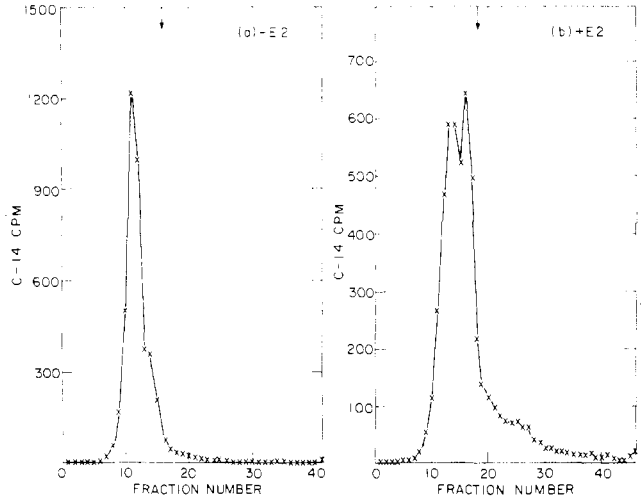

FIGURE 5: Action of colicin E2 on single-stranded DNA of phage fd. Procedure as in Table II, part D. The in vitro reaction mixture contained: ^{14}C-labeled fd DNA (kindly provided by I. Molineux) (2.9 μg/ml) and colicin E2 preparation III (fraction 98) (30 μg/ml) in a total volume of 55 μl. Incubation was at 37° for 3 hr; 0.1% SLS instead of SDS was added to stop the reaction. The in vitro reaction mixtures were analyzed on alkaline gradients containing 10 to 30% sucrose. A mixture of [^{3}H]SV40 forms I, II, and III DNA (the gift of R. Weinberg) was added to the samples as a marker before layering on the gradients. The arrow represents the position of single-stranded linear SV40 DNA.

longed incubation. No fragments less than λ DNA in size were produced. Since the reaction has not yet been carried past completion it is not clear whether colicin E2 preparations can put more than one break into λ DNA supercoils. Preliminary studies with polynucleotide kinase and alkaline phosphatase indicate that two-thirds of the 5′-end groups on λ DNA supercoils made available by colicin E2 action are phosphorylated. The 5′-phosphate nucleotides appear to represent a nonrandom mixture of the four nucleotides (L. Saxe and A. Panet, unpublished results).

Experiments with linear λ DNA as substrate gave negative results. Reaction mixtures were analyzed on 5–20% alkaline sucrose density gradients instead of 10–30%. No evidence of breaks could be consistently detected after treatment with colicin E2 preparation I, 50 or 100 μg/ml. It is possible, but by no means certain, that the endonucleolytic activity in colicin E2 preparations attacks preferentially circular double-stranded DNA.

Action of Colicin E2 Preparations on Other DNA Molecules. Experiments with DNA supercoils extracted from SV40 virus particles gave results similar to those with λ DNA supercoils. In addition to uncoiled circular molecules, small pieces of DNA were produced as seen both in neutral (Figure 4) and alkaline gradients, indicating that the colicin E2 preparation produced two-strand breaks in addition to one or more one-strand breaks in double-stranded SV40 superhelical DNA.

The action of a colicin E2 preparation was also tested on single-stranded DNA extracted from fd phage. Treatment with E2 resulted in the production of linear molecules and of some fragments, as shown in Figure 5. No acid-soluble counts were detected. These results suggest that colicin E2 causes one or more endonucleolytic breaks in fd DNA.

Discussion

The main finding presented here is that preparations of colicin E2, even the most purified, introduce one single-strand scission into λ DNA supercoils. This activity cannot be separated from the cell-killing activity of colicin E2 by glycerol gradient fractionation. The present results do not completely exclude a role of some nuclease that happens to be copurified and to share other properties with colicin E2. This seems unlikely, however. Preliminary tests with a preparation of a protein purified from E2-colicinogenic bacteria by K. Jakes and presumed to be the E2-immunity protein (IP-E2; Jakes et al., 1974) support the identification of the endonuclease with colicin E2 since IP-E2 inhibits the in vitro attack on λ DNA supercoils, whereas IP-E3, the immunity substance of colicin E3, does not inhibit the action of E2. Although these results appear to be confirmatory, further experiments are required since IP-E2 and IP-E3 contain nuclease activity and in some experiments partially inactivated the cell killing activity of colicin E2.

A protein band that is probably IP-E2 is present in SDS-acrylamide gels for all preparations of the colicin, as described under Experimental Procedure. A similar band found accompanying colicins E2 and E3 was reported by Jakes et al. (1974). The presence of an inhibiting protein tightly bound to colicin E2 might make most of the molecules inactive and explain the relatively low specific activity of colicin E2 in the nuclease assay. It has not yet been possible to obtain active colicin E2 free of the presumed IP-E2. The relatively low specific activity of colicin E2 in the nuclease assay (protein to DNA ratio of 1:5 or 1:1) may also be due to low specific activity in vivo (more than 50 to 100 molecules per killing unit), instability of the colicin molecules in dilute solution, or subsaturating concentrations of DNA.

Among the *E. coli* nucleases that might contaminate colicin E2, endonuclease I has been examined carefully because of the role attributed to it in colicin E2 action (Almendinger and Hager, 1972, 1973). A role of an endonuclease I impurity in the in vitro reaction of E2 preparations is doubtful, because the reaction is not inhibited by tRNA and because colicin E2 prepared from endonuclease I deficient bacteria is fully active on λ DNA supercoils. Yet colicin E2 preparations IV and V when heated gave an increased nucleolytic activity, which was inhibited by tRNA. This raises the possibility that there is some endonuclease I hidden in

the colicin preparations. It is not possible to eliminate this possibility until preparations of much higher purity are obtained.

If the colicin E2 molecule has the nucleolytic activity described in this paper, this may be the basis of the in vivo action. Many aspects would remain to be explained, however, including the entry into the bacterial cell and the kinetics of DNA attack in vivo. The in vitro action on λ DNA supercoils as well as on supercoiled SV40 DNA may be a good model for its in vivo action since the bacterial DNA is supposed to be mostly in supercoiled form, associated with RNA and proteins (Worcel and Burgi, 1972). Whether colicin E2 can break linear DNA molecules is still uncertain. Since colicin E2 did attack single-stranded fd DNA molecules, it might recognize single-stranded regions of superhelical DNA, as is the case with the S1 nuclease from *Aspergillus oryzae* (Beard et al., 1973) and with the single-strand specific endonuclease from *Neurospora crassa* (Kato et al., 1973).

The existence of an in vitro activity of colicin E2 on DNA, if substantiated, would make its mode of action less dissimilar from that of the closely related protein, colicin E3. This colicin causes a specific endonucleolytic break in the 16S ribosomal RNA both in vitro and in vivo (Boon, 1971, 1972; Bowman et al., 1971), although some details of the process remain uncertain. Again for colicin E3, the process of colicin access to the ribosomes in vivo requires clarification.

Acknowledgments

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