Reconstitution of Colicin E₂-Induced Deoxyribonucleic Acid Degradation in Spheroplast Preparations

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Received for publication 5 March 1973

Spheroplasts are insensitive to colicin E₂ and do not show deoxyribonucleic acid (DNA) degradation even in the presence of massive amounts of E₂. However, when both endonuclease I and E₂ were present, spheroplast DNA was degraded by an endonucleolytic activity which gave rise primarily to double-strand DNA cleavages, producing fragments having an average molecular weight of 9 × 10⁴. Pancreatic ribonuclease could substitute for colicin E₂ in the reconstitution system, but pancreatic deoxyribonuclease could not replace endonuclease I. However, colicin E₂ could not activate transfer ribonuclease acid-inhibited endonuclease I in an in vitro system where pancreatic ribonuclease caused full stimulation.

Colicin E₂ has been shown to kill sensitive bacteria by a single-hit mechanism (38, 39, 45, 53). The primary result of colicin E₂ adsorption to bacteria appears to be the degradation of the bacterial chromosomal deoxyribonucleic acid (DNA), first by an endonucleolytic attack which is then followed by a second stage of exonucleolytic degradation (38, 42, 48). The degradation of DNA ultimately leads to the cessation of all macromolecular synthesis and to cell death (38).

Colicin E₂ does not appear to possess deoxyribonuclease activity (4, 5, 38, 48; R. Almendinger, Ph.D.thesis, Univ. of Illinois at Urbana-Champaign, 1972), and recent studies from this laboratory suggested that at least one cellular enzyme is involved in the E₂ killing process (2). Endonuclease I has been implicated by experiments which show that cells made deficient in endonuclease I either by osmotic shock or spheroplast formation or through mutation become more resistant to both colicin-directed killing and DNA degradation. This paper reports the reconstitution of colicin-induced DNA degradation in spheroplasts incubated in the presence of both purified endonuclease I and colicin E₂.

MATERIALS AND METHODS

Cultivation of bacteria. M. Nomura supplied both the colicinogenic Escherichia coli strain, W3110Sm⁻ (E₁), and the colicin-sensitive E. coli strain, W3110Sm⁻. The colicinogenic bacteria were grown on M-9 containing Casamino Acids (23), and the sensitive bacteria were grown on a tris(hydroxymethyl)aminomethane (Tris) - glycerol - Casamino Acids medium (40). If the cells were to be labeled with ³²P, the latter medium was supplemented with 0.25 mCi of H₃²PO₄ per ml when the cultures had reached 10⁹ cells per ml, and the cultures were subsequently incubated for two more generations.

E. coli B used for the preparation of endonuclease I was obtained in bulk quantities from the Grain Processing Co.

Purification and assay of enzymes and colicin. Colicin E₂ was purified by the method of Herschman and Helinski (23). The assay for killing units followed the procedure of Nomura (38, 39).

Endonuclease I was purified as described by Lehman (26) except that a 0.75 M NaCl-0.01 M potassium phosphate (pH 7.0) wash of E. coli B cells instead of a whole cell lysate was used as the starting material. Stage I represents the original 0.75 M NaCl wash of whole cells. Stage II represents the enzyme fraction after protamine precipitation, autolysis, ammonium sulfate precipitation, and dialysis. Stage III represents the purified enzyme obtained after diethylaminoethyl (DEAE)-Sephadex and DEAE-cellulose column chromatography. For the determination of total endonuclease I occurring in the cells, a crude cell sonic extract was prepared by treating 7 g of cells in 20 ml of 0.01 M phosphate buffer (pH 7.4) for 90 s (three consecutive 30-s bursts) in a Branson sonic oscillator followed by centrifugation at 15,000 × g for 10 min to remove the debris. The assay for endonuclease I activity employed ³²P-polydeoxyadenosine-ribozyme (poly dAT) as substrate (41). Specific
activities are defined as micromoles of nucleoside phosphate released per 30 min per milligram of protein at 37 °C.

Ribonuclease I was assayed with transfer ribonucleic acid (tRNA) as substrate (26). Specific activities are defined as micromoles of nucleoside phosphate released per 30 min per milligram of protein at 37 °C.

Protein concentrations were determined by the method of Groves et al. (21).

Formation and incubation of spheroplasts. The 32P-labeled cells were converted to spheroplasts by the method of Maeda and Nomura (40). The spheroplasts were gently suspended at a level of 10^6 cells per ml in 30 mM Tris-hydrochloride–5 mM potassium phosphate buffer (pH 7.4), 1.3 mM adenosine triphosphate (ATP), 1 mM MgCl₂, 0.1 mM CaCl₂, 0.5% glucose, and 20% sucrose, and were subsequently incubated at 37 °C with the other additions indicated in each experiment.

Sucrose gradient centrifugation. After incubation, the spheroplasts were gently lysed by a 10-fold dilution either in a cold solution of 0.2 M disodium ethylenediaminetetraacetate (Na₂EDTA)–0.6 M NaCl (pH 8) for the neutral gradients or in the same solution containing 0.25 M NaOH for the alkaline gradients. A sample of lysed spheroplasts representing 10^7 cells was layered on the appropriate sucrose gradient (48, 50); 5.0-ml total volume), and the gradients were centrifuged in an SW 39 rotor at 35,000 rpm for 90 min at 20 °C. Samples having a volume of 0.17 ml were collected from each gradient.

Potassium hydroxide was added to each sample to a final concentration of 1 N, and the samples were then incubated for 16 h at room temperature to hydrolyze the RNA. The acid-soluble material was then removed by the filter-paper technique of Bollum (6, 32). The radioactivity in each fraction was measured by counting in a scintillation solution consisting of 4 g of 2,5-bis-3-(5-t-butylbenzoxazolyl) thiophene per liter of toluene (BBOT-toluene; 48).

In vitro DNA degradation. 32P-labeled E. coli DNA was prepared according to Marmur (33, 34). Samples of 1 μg of 32P-DNA in 0.1 ml of 0.03 M Tris-5 mM potassium phosphate buffer (pH 7.4), 1.3 mM ATP, 1 mM MgCl₂, and 0.1 mM CaCl₂ were incubated at 37 °C for 30 min with enzyme, and the molecular weight of the DNA was analyzed by alkaline sucrose gradient centrifugation. The radioactivity associated with each fraction was measured directly in BBOT-toluene–Triton X-100 (2:1, vol/vol).

Materials. Chloramphenicol, crystalline bovine serum albumin, grade I egg white lysozyme, pancreatic deoxyribonuclease, type VI horse heart cytochrome C, and type I-A pancreatic ribonuclease were purchased from Sigma Chemical Co. Miles Laboratory supplied the H₄-poly dAT. H₄PO₄ was purchased from International Chemical and Nuclear Corp.

RESULTS

DNA degradation induced in colicin E₂-treated cells. DNA degradation by endonucleases can be readily detected in DNA samples by sedimentation analysis with the use of sucrose gradients (17, 32, 50). Both single- and double-strand nicks in DNA can be detected when the samples are analyzed in sucrose gradients at both alkaline and neutral pH. The average molecular weight of the DNA appearing at the various peak positions in the sucrose gradients can be calculated by use of empirical equations developed in several laboratories (1, 12, 55). This technique was used to detect the endonuclease activity in colicin E₂-treated cells and spheroplasts.

When intact, sensitive E. coli cells were incubated with saturating amounts of E₂, chromosomal DNA was degraded in approximately 15 min to fragments having an average molecular weight of 3 × 10⁶ (Fig. 1). Other laboratories have obtained results similar to these. Thus, there is general agreement that the initial breakdown of DNA in whole cells treated with E₂ is brought about by an endonuclease activity which gives rise primarily to double-strand DNA cleavages (42, 48). When the exposure of the intact cells to E₂ was extended to longer periods of time, no further

![Fig. 1. DNA degradation in normal cells treated with colicin E₂. 32P-labeled E. coli W3110Sm' was incubated at 4 °C for 30 min with purified colicin E₂ (25,000 killing units per cell). The cells were resuspended in Penassay broth containing 0.005 M MgCl₂ and incubated at 37 °C for 0 min (curve A, •), 15 min (curve B, ●), and 30 min (curve C, ○). The cells were then converted to spheroplasts, and the DNA was analyzed on alkaline sucrose gradients.](http://aac.asm.org/DownloadedFrom/10.1128/AAC.03698-16/fig1.png)
endonuclease activity could be detected (compare curve A with curve B, Fig. 1). Instead, upon prolonged exposure of the cells to E₂, an exonuclease activity developed, and the amount of DNA appearing in the $3 \times 10^8$ dalton position in the gradient gradually decreased until it represented only 20% of the original DNA (Fig. 1, curve C).

Reconstitution of DNA degradation in colicin E₂-treated spheroplasts. Colicin E₂ has been shown to be ineffective in causing DNA degradation in spheroplast preparations regardless of whether the colicin was adsorbed to the cells before or after spheroplast formation (40; Almendinger, Ph.D. thesis, Univ. of Illinois, 1972). Neither endonuclease nor exonuclease activity could be detected toward spheroplast DNA, even when excessively high concentrations of colicin were added to the spheroplasts. However, when E₂ and a crude preparation of endonuclease I (purified 28-fold) were both present in the spheroplast reconstitution experiments, endonucleolytic breakdown of spheroplast DNA could be detected (Fig. 2, curve C). In a 45-min exposure of spheroplasts to both E₂ and endonuclease I, the single-strand molecular weight of the spheroplast DNA shifted from an average value of $1.4 \times 10^8$ to $2 \times 10^7$. Endonuclease alone (curve A) or colicin alone (curve B) did not promote degradation of the spheroplast DNA.

These results were independent of the method or time of addition of E₂ to the spheroplast preparation. Similar results were obtained in the reconstitution experiments when E₂ was adsorbed to the cells prior to spheroplast formation, when the E₂ was adsorbed after spheroplast formation and the excess E₂ was washed away, or when the E₂ was merely added at saturating levels just before incubation of the spheroplasts with endonuclease I.

Colicin E₂-induced DNA degradation with pure endonuclease I. The endonuclease I preparations used in the reconstitution experiments reported in Fig. 2 had been purified only 28-fold over the crude cell sonic extract and, even though no other deoxyribonuclease activity could be detected, the possibility remained that some other enzyme or enzymes were involved in reconstitution. To distinguish between these possibilities, the partially purified endonuclease I preparation was carried through the entire Lehman purification procedure (26), and material from each purification step was monitored to determine whether the reconstitution factor had co-purified with endonuclease I. The overall endonuclease I purification procedure consisted of protamine precipitation of the enzyme, an autolysis step, ammonium sulfate precipitation, chromatography on DEAE-cellulose, and finally, chromatography on carboxymethyl (CM)-cellulose. Table 1 summarizes the purification achieved for the endonuclease I preparation. Ribonuclease I is the major contaminant in most preparations of highly purified endonuclease I (26). Thus, for comparative purposes, the ribonuclease I activity at each purification stage is also recorded in Table 1. The enzyme levels indicated for the CM-cellulose chromatography step are given for a single 5-ml fraction which showed the highest activity for endonuclease I. Ribonuclease activity was undetectable in this fraction. The ambiguity in the specific activities listed for the stage III preparation arises from the fact that extremely small amounts of protein were present in this fraction. The Lehman purification procedure produced an endonuclease I preparation which was greater than 1,700-fold purified and which possessed a specific activity higher than that reported for any previous endonuclease I preparation. Most pertinent, as indicated in Fig. 3, the spheroplast reconstitution factor did co-purify with endonuclease I activity throughout the purification range. Figure 3, column A, shows the alkaline sucrose sedimentation profile of spheroplast DNA after incubation with colicin E₂ alone for 20, 30, and 45 min. In accord with
previous results, colicin \( E_2 \) alone did not affect the molecular-weight profile of spheroplast DNA; control experiments carried out in the absence of \( E_2 \) matched the DNA profiles shown in column A. Columns B, C, and D show the alkaline sucrose gradient profiles of spheroplast DNA when the spheroplasts were incubated with colicin \( E_2 \) plus endonuclease I which had been purified 17-fold (B), 109-fold (C), and greater than 1,700-fold (D). All incubation mixtures which contained both endonuclease I and colicin \( E_2 \) showed DNA degradation in the spheroplast reconstitution assay, regardless of the endonuclease purity. In fact, DNA degradation was slightly more rapid with the highly purified enzyme than with the less pure endonuclease I preparation, although equivalent amounts of endonuclease I were used in both cases. Therefore, it seems reasonable to assume that the factor required in addition to \( E_2 \) for spheroplast DNA degradation is indeed endonuclease I.

The rate of DNA degradation in the spheroplast reconstitution experiments was rather insensitive over wide ranges of both colicin and endonuclease I concentration. Colicin could be added at levels between 300 and 10,000 killing units per spheroplast without affecting the overall degradation rate. At a level of 100 killing units of \( E_2 \) per spheroplast, the rate of DNA hydrolysis was slightly slower, and with only 4 killing units of \( E_2 \) per spheroplast the rate of DNA degradation was very much slower. Endonuclease I levels could be varied over an 8,000-fold concentration range (1.5 \( \times 10^{-11} \) to 1.2 \( \times 10^{-7} \) units per spheroplast) with only a twofold change in the rate of DNA degradation. These enzyme levels are approximately equivalent to a range of endonuclease I concentrations from 0.5 to 400 times the endonuclease I content of one \( E. coli \) cell. The ultimate molecular weight of the DNA fragments produced in the reconstitution experiments also was unaffected by this range of endonuclease concentrations, provided the reconstitution experiments were incubated for a

Table 1. Purification of endonuclease I

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Endonuclease I specific activity</th>
<th>Ribonuclease I specific activity</th>
<th>Ribonuclease/ deoxyribonuclease</th>
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<tr>
<td>Whole cell sonic extract</td>
<td>0.08</td>
<td>4.2</td>
<td>51</td>
</tr>
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<tr>
<td>Stage III</td>
<td>&gt;143</td>
<td>&lt;30</td>
<td>&lt;0.2</td>
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</tbody>
</table>

Fig. 3. Identification of endonuclease I as the requirement for colicin \( E_2 \)-induced DNA degradation in spheroplasts. \( ^{32} \)P-labeled spheroplasts were incubated with endonuclease I from each of the purification steps described in Table 1 at a level of 15 units of endonuclease per ml. Colicin was added at 3 \( \times 10^{13} \) killing units per ml. The samples were incubated at 37°C for 20, 30, and 45 min with colicin alone (column A), colicin plus stage I endonuclease I (column B), colicin plus stage II endonuclease (column C), and colicin plus stage III endonuclease (column D). The DNA was analyzed on alkaline sucrose gradients.
sufficiently long period of time. The smallest DNA fragments obtained in the spheroplast preparations during incubation with the endonuclease I and colicin E₂ had molecular-weight averages of $9 \times 10^4$. This result could be obtained both with the highly purified endonuclease I and with the crude RNA-inhibited enzyme.

Attempts to optimize the conditions for the reconstitution have shown that degradation of spheroplast DNA could occur under a wide variety of conditions. The optimal pH for reconstitution was 7.0 to 8.0. At pH 6.5 the reconstitution process was completely inhibited, and at pH 8.5 the rate of DNA hydrolysis was substantially decreased. Potassium chloride and CaCl₂ did not affect the rate of DNA degradation, although Mg²⁺ appeared necessary. The requirement for the latter ion was difficult to test because Mg²⁺ was also required to maintain the integrity of the spheroplast preparations. ATP and glucose were not necessary for reconstitution although they were routinely added. In fact, at high concentrations, ATP became inhibitory. When ATP was present in the reconstitution system at a concentration of 13 mM, DNA degradation in the spheroplasts was inhibited completely. This inhibition by ATP could possibly be due either to direct chelation of Mg²⁺ or to an indirect effect such as release of the DNA from the spheroplast membrane (4, 5).

Occasionally, when the spheroplasts had been extensively washed with cold Tris buffer, purified endonuclease I would catalyze DNA degradation in a small portion of the spheroplasts in the absence of E₂. However, the same small portion of spheroplast DNA was also sensitive to pancreatic deoxyribonuclease. Since colicin E₂ always induced the remaining spheroplasts to respond to endonuclease I but not to pancreatic deoxyribonuclease, it seems reasonable to assume that this occasional partial sensitivity of spheroplasts to endonuclease I alone was due to the loss of permeability barriers produced by the treatment with cold Tris buffer. Other investigators have made similar observations on whole cells treated with cold Tris buffer (22, 28–31, 36, 46, 58, 60).

We have attempted to determine whether single- or double-strand DNA cleavage occurred in the reconstitution experiments. Unfortunately, lysis of the spheroplast preparations in the neutral medium released only approximately 50% of the DNA from the membrane complex; thus, the results were not deemed to be completely reliable. However, if this 50% fraction of DNA released from the spheroplasts was a representative sample of the entire DNA population, the endonuclease activity in the spheroplast reconstitution experiments yielded double-strand cleavages (Table 2). Double-strand cleavage is also the major activity which occurs in whole cells treated with colicin E₂ (42, 48). The double-strand DNA cleavages continued to appear up to 45 min after the addition of colicin and endonuclease I to the spheroplast preparations. No exonuclease activity was ever detected in the reconstitution experiments, and this may be a result of the overall slowness of DNA degradation in spheroplasts as opposed to whole-cell experiments. The spheroplasts often began to lyse after 45 min of incubation, making it impossible to carry out long-term incubations to detect a slow exonuclease activity. Alternatively, there may have been a factor lacking in the reconstitution experiments which is necessary for the second-stage exonucleolytic attack observed in intact cells.

**Pancreatic ribonuclease as an inducer of endonuclease I activity in the spheroplast system.** It has been demonstrated previously (15, 26, 27, 37, 41) that endonuclease I is released from *E. coli* cells as an inhibited RNA complex. When the endonuclease I-RNA complex is incubated with pure DNA, the inhibited enzyme does not catalyze the hydrolysis of DNA to acid-soluble products. However, under certain conditions, the inhibited enzyme will cause a few internal single-strand cleavages (20). When the enzyme is freed from the inhibitory RNA by treatment with pancreatic ribonuclease, endonuclease I produces double-strand cleavages in substrate DNA (35, 55) and also shows an appreciable exonucleolytic activity (44). Thus, RNA-free endonuclease I is capable of degrading

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>DNA molecular wt</th>
<th>DNA molecular wt</th>
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<tbody>
<tr>
<td></td>
<td>Double-strand</td>
<td>Single-strand</td>
</tr>
<tr>
<td>0</td>
<td>$220 \times 10^4$</td>
<td>$87 \times 10^4$</td>
</tr>
<tr>
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</tr>
<tr>
<td>45</td>
<td>$64 \times 10^4$</td>
<td>$30 \times 10^4$</td>
</tr>
</tbody>
</table>

*Colicin and endonuclease I levels were the same as in Fig. 2. Molecular weights were determined by neutral and alkaline sucrose gradient centrifugation.

This value is probably not correct, as the DNA was almost sedimented to the bottom of the tube.
high-molecular-weight DNA to acid-soluble products.

We tested the ability of pancreatic ribonuclease to stimulate DNA degradation in spheroplasts treated with endonuclease I. The results showed that, indeed, DNA degradation did occur in these spheroplasts, and, in fact, pancreatic ribonuclease could replace E2 in the reconstitution system (Fig. 4). Endonuclease I plus ribonuclease in the spheroplast reconstitution assay promoted DNA degradation as revealed by a shift in the spheroplast DNA molecular weight to $2 \times 10^7$ (curves C and D) during a 30-min incubation. In a similar time period and with similar amounts of colicin and endonuclease I, the average molecular weight of the spheroplast DNA fell to $4 \times 10^7$ (curve B). The ribonuclease effect occurred in the presence (curve D) and absence (curve C) of E2. Ribonuclease alone, pretreated to destroy contaminating pancreatic deoxyribonuclease by heating at 80 C for 10 min in 0.15 M NaCl, pH 5.0, did not promote degradation of spheroplast DNA.

**Effects of colicin E2 on in vitro DNA degradation by endonuclease I.** Since pancreatic ribonuclease could replace colicin E2 in the spheroplast reconstitution system, we tested the possibility that colicin E2 might function as a ribonuclease for the removal of inhibitory RNA from endonuclease I, and thereby activate the endonuclease I for DNA degradation in the spheroplast reconstitution experiments. Table 3 shows the results of incubations designed to determine the in vitro effect of colicin E2 on the formation of acid-soluble material from $^3$H-poly dAT as catalyzed by partially purified endonuclease I inhibited with tRNA. The endonuclease I preparation contained no other deoxyribonuclease activity, as can be seen by the lack of DNA degradation when tRNA was added to the system to inhibit endonuclease I completely. However, considerable deoxyribonuclease activity (approximately 40% of maximal) was seen in the absence of the added tRNA, indicating that the endonuclease preparation was only partially inhibited by endogenous RNA. The addition of colicin E2 to the partially inhibited enzyme did not alter this level of activity. However, the addition of pancreatic ribonuclease to the partially inhibited endonuclease was effective in increasing the rate of formation of acid-soluble material.

Since the increase in the rate of formation of acid-soluble products presumably depended on the exonuclease activity of the enzyme, the endonucleolytic activity of the enzyme was also checked for possible stimulation by colicin E2. Figure 5 shows the results of experiments in which E. coli $^{35}$P-DNA was used as substrate for

![Figure 4. Pancreatic ribonuclease stimulation of DNA degradation in spheroplasts by crude endonuclease I. The various incubations contained, per ml, 10$^9$ $^{32}$P-labeled spheroplasts plus the following: 5 units of stage I endonuclease (curve A, ●), 200 µg ($4 \times 10^3$ killing units) of colicin E2 and 5 units of endonuclease (curve B, O), 200 µg of pancreatic ribonuclease and 5 units of endonuclease (curve C, △), or 5 units of endonuclease, 200 µg of colicin E2, and 200 µg of ribonuclease (curve D, ▽). Incubation time was for 30 min, and the DNA was analyzed on alkaline sucrose gradients.](image-url)}
FIG. 5. Colicin effects on in vitro degradation of E. coli DNA by endonuclease I. Where indicated the basic endonuclease I assay with E. coli DNA was supplemented with 4 x 10^{12} killing units (20 μg) of colicin E_2 and 20 μg of pancreatic ribonuclease (curve A, ●), 0.35 units of stage I endonuclease I and 10 μg of tRNA (curve B, ○), endonuclease I, tRNA, and colicin E_2 (curve C, △), and endonuclease I, tRNA, and ribonuclease (curve D, Δ). Alkaline sucrose gradients were used to determine molecular-weight changes in the DNA.

tests with the partially inhibited endonuclease preparation and colicin E_2. Curve A, a control, represents the DNA profile obtained on an alkaline sucrose gradient after incubation of the ^3P-DNA with colicin and pancreatic ribonuclease. This sample, which had the same profile as did unincubated samples of the DNA, had an average molecular weight of 10^7 at the peak maximum. The reaction mixtures containing endonuclease I supplemented with tRNA showed a shift in the DNA profile to a species having an average molecular weight of 2.3 x 10^4 after 30 min of incubation (curve C). Based on the other in vitro experiments, the tRNA-inhibited endonuclease I used in this experiment should have been fully inhibited with respect to the formation of acid-soluble products. The addition of colicin E_2 to the tRNA-inhibited endonuclease I reaction mixture did not affect the endonuclease activity (curve B). However, the addition of pancreatic ribonuclease to the inhibited enzyme did increase the extent of degradation (curve D). The addition of pancreatic ribonuclease to the reaction mixtures yielded a DNA having an average molecular weight of less than 3 x 10^4.

Similar incubations carried out for shorter time periods (15 instead of 30 min) showed the same pattern. In 15-min incubations, the inhibited endonuclease I alone produced a DNA species having an average molecular weight of 5 x 10^4. The addition of colicin E_2 caused no further degradation. However, the addition of ribonuclease to the inhibited preparation activated the endonuclease to produce DNA fragments with a molecular weight of less than 10^6.

These experiments appear to indicate that the colicin itself does not possess a ribonuclease activity capable of degrading tRNA complexed to endonuclease I. These experiments, of course, do not rule out the possibility that colicin E_2, in the presence of an additional factor or factors not supplied in the incubation mixtures, acquires ribonuclease activity or that E_2 requires a special substrate for ribonuclease activity.

The requirement for colicin E_2 in the degradation of spheroplast DNA by highly purified tRNA-free endonuclease I also suggests that the colicin is not acting as a ribonuclease to remove inhibitory tRNA from the endonuclease I. Whereas the stage I endonuclease I preparation was fairly well inhibited by endogenous RNA (Table 4), the more purified enzyme had lost most of its inhibitory RNA (80 to 90% uninhibited), and yet this purified enzyme still required colicin for reconstitution.

**DISCUSSION**

Although it is now fairly certain that endonuclease I is involved in the E_2-induced degradation of chromosomal DNA, the exact mecha-

<table>
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<th>Purification stage</th>
<th>Deoxyribonuclease activity (units/mg of protein)</th>
<th>Percent uninhibited</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>tRNA, cytochrome c</td>
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</tr>
<tr>
<td>Whole cell sonic extract</td>
<td>2.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Stage I</td>
<td>0</td>
<td>136</td>
</tr>
<tr>
<td>Stage II</td>
<td>0</td>
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</tr>
<tr>
<td>Stage III</td>
<td>0</td>
<td>&gt;4,430</td>
</tr>
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</table>

* Incubation conditions were as for the endonuclease I assay except that cytochrome c (50 μg per assay) was added (30) to stabilize the pure enzyme in those incubations which did not contain ribonuclease.
nism and site of colicin action remain ambiguous. As discussed above, colicin E₈ does not appear to be acting as a ribonuclease for removing inhibitory RNA from endonuclease I, since no such activation could be detected under in vitro conditions. Alternatively, the hypothesis that E₈ might stabilize endonuclease I in solution also appears unlikely. Highly purified endonuclease I is very unstable at 37°C. Both cytochrome c and ribonuclease have been used to stabilize the enzyme under these conditions (16, 26). However, when cytochrome c was added to the spheroplast reconstitution experiments at levels similar to the amounts of colicin and ribonuclease used for reconstitution, no effect of cytochrome c on reconstitution could be detected. Similarly, bovine serum albumin could not replace colicin E₈ in the reconstitution. Therefore, it seems unlikely that the role of E₈ in the reconstitution system could be merely one of stabilizing the endonuclease I.

A recent report (49) indicated that colicin E₂ may bind directly to DNA and cause local melting of the double-strand helix. However, this effect occurs only when the DNA used in the experiment has been purified by phenol extraction. Furthermore, it has been shown that traces of phenol must still be present to obtain the maximal effect. When the number of colicin molecules bound to the phenol-treated DNA is used to calculate theoretical Tₘ changes according to the von Hippel formulation (59), one finds that the maximal allowable calculated change in Tₘ is much less than the experimental value measured by Ringrose. Therefore, it seems that the E₂ effect on DNA melting must be an artifact, and not a direct effect produced by colicin E₂ on native DNA.

What then might be the actual role of E₂ in promoting DNA degradation in a normal cell? Originally, we hypothesized that E₂ was responsible for the transport of endonuclease I from periplasmic space either onto the membrane or into the cell. In light of the data presented here, we must also consider the possibility that the colicin is acting either as an activator or modifier for intracellular endonuclease activity or that E₂ is a highly specific ribonuclease for a unique RNA species which protects chromosomal DNA from nuclease attack. Colicin E₂ does not possess a broad-spectrum ribonuclease activity like, for example, pancreatic ribonuclease. E₂ is not capable of modifying RNA-inhibited endonuclease I activity, at least not in the absence of the internal cell environment. It is possible that colicin E₂ could induce another cellular protein to remove inhibitory RNA from endonuclease I. Alternatively, a second possibility would be to insist that E₂ be attached to the cell membrane or require some other cofactor or cell constituent before it could modify endonuclease I activity.

There are several examples known where the endonuclease I activity in the cell is modified by the presence of a second protein. When cells are actively making DNA, a protein is present which protects single-strand DNA from degradation by endonuclease I (57). This protein, which is absent in resting cells, has been suggested as a control element for endonuclease I during recombination.

The T phages also seem to code for a protein which modifies periplasmic endonuclease I activity (3, 18). After phage infection and phage gene expression, E. coli endonuclease I becomes activated in a manner which allows it to degrade the DNA of any superinfecting phage. This degradation does not occur in phage-infected endonuclease I-deficient cells.

Finally, the cellular enzymes ribonuclease I and ribonuclease II also seem to be able to activate endonuclease I in cells treated with tolulene or Tris-EDTA (13, 14, 61). In this case, endonuclease I alone causes a limited degradation of the DNA present in the cell.

A modifier hypothesis for colicin E₈ is interesting in light of several studies done recently with colicin E₈. Colicin E₈ has been shown to inhibit protein synthesis by causing a single nick in the 16S RNA of the ribosome (10, 52). This activity occurs both in vivo with sensitive cells treated with colicin and in vitro with ribosomes incubated directly with colicin E₈ (7, 11). A recent report indicated that a supernatant factor may be required for the in vitro degradation with ribosome preparations (43). Colicin E₈ is very similar both in physical parameters and in amino acid composition to E₈, although the ultimate biochemical effects of the two colicins on the cell are quite different (19, 23, 24). By analogy with E₈, E₈ may also be a modifier protein for some cellular ribonuclease which is loosely bound to ribosomes in some preparations and is found in the supernatant fluid in others. E₈ action on this hypothetical ribonuclease could cause the ribonuclease to produce the specific cleavage in the 16S ribosomal RNA, and thus inhibit protein synthesis.

The studies reported here on colicin E₈ may also have implications for the events occurring in the cell after T₄ phage infection. Normal T₄ phage expression causes host-cell DNA
degradation within a few minutes after the phage infects sensitive cells (25). This degradation closely resembles the colicin-induced DNA degradation. T4-induced degradation begins as an endonucleolytic activity which stops when the DNA fragments reach an average molecular weight of $10^4$. An exonuclease activity then takes over in a second stage and completes the degradation of host DNA to acid-soluble products. Three T4 genes have been identified which appear to be involved in the overall degradation process. The endonuclease activity is controlled by the denA gene (51), and the exonuclease activity by genes 46 and 47 (25). When phage containing a defect in any of these three genes is used to infect cells, no phage-directed acid-solubilization of host DNA occurs. If these cells which have been infected with defective T4 are also treated with colicin E$_2$, the E$_2$-induced DNA degradation is likewise inhibited (56). The inhibition of E$_2$-induced DNA degradation by T4 mutants requires that the defective T4 be added in an appropriate time sequence with respect to the binding of E$_2$ to the cells. That is, the T4 denA mutant must be added to the cells 5 to 10 min before colicin E$_2$ because, under ordinary conditions, the E$_2$-induced endonucleolytic activity begins approximately 2 min after colicin E$_2$ addition. On the other hand, T4 phage defective in gene 46 or 47 can be added 5 to 10 min after the colicin since the E$_2$-induced exonuclease activity does not occur until 15 min after colicin adsorption. Chloramphenicol inhibits the effect of the defective T4 mutants, and E$_2$-induced DNA degradation proceeds in the presence of chloramphenicol. This observation suggests that the T4 gene products of genes denA, 46, and 47 are not themselves nucleases but rather that these genes code for proteins which are regulators or modifiers of E. coli deoxyribonucleases. When defective modifier (regulator) proteins are produced, they could actually inhibit interaction of both colicin E$_2$ and normal phage products with E. coli nucleases. Studies with T4 mutants indicate that E. coli endonuclease I is not involved in T4-induced host chromosomal degradation, but that an enzyme very similar to endonuclease I may be involved (47). Phage T4-induced endonuclease II activity controlled by denA has been implicated in host DNA degradation. However, the mutant studies do not distinguish between control or structural gene products for the denA locus. As noted above, it seems reasonable to interpret the denA gene product as a control protein in the E$_2$ inhibition studies. Thus, it may be profitable to reinvestigate the possible involvement of E. coli endonuclease I in T4-induced DNA degradation.

An attractive hypothesis which would unite all E colicin activity under a single mechanism would be the theory that all E-type colicins are ribonucleases which have a high substrate specificity with respect to RNA structures. In the colicin-ribonuclease hypothesis, the 16S RNA component of the 30S ribosomal subunit would be the unique substrate for colicin E$_2$. Previous work has established that E$_2$ is not a general ribonuclease (7, 10, 11, 43). E$_2$ cannot promote cleavage of the ribosomal 16S RNA molecule in vitro when the RNA is separated from its parent 30S particle. In fact, current evidence indicates that the 30 and 50S ribosomal particles must be combined in the form of a functional 70S ribosome before the E$_2$-mediated attack on 16S RNA can occur (8, 9). Thus, if E$_2$ is actually a ribonuclease, it has a very special substrate requirement. Extension of this hypothesis to colicins E$_1$ and E$_2$ suggests that special RNA structures might be substrates for these proteins. In searching for unique and special RNA substrates for colicins E$_1$ and E$_2$, RNA species peculiar to membranes and DNA complexes deserve attention. Stonington and Pettijohn (54) have reported the isolation of a compact, folded chromosomal DNA-RNA-protein complex which expands into a high-viscosity, extended form when treated with traces of pancreatic ribonuclease. A ribonuclease-sensitive structure of this type would be an ideal candidate for the role of a unique substrate for colicin E$_2$. The fact that pancreatic ribonuclease will substitute for E$_2$ in the spheroplast DNA degradation studies reported in this paper would be compatible with this hypothesis, since any general ribonuclease should attack the DNA-RNA complex and produce the high-viscosity DNA species. In addition to providing a unique RNA structure for hypothetical E$_2$-ribonuclease action, the ribonuclease-sensitive, folded chromosome hypothesis would also explain the requirement for endonuclease I in the spheroplast reconstitution system. The opening of the folded complex via ribonuclease action would only be the initiating step in a series of degradative reactions. The role of E$_2$ would be labilization of chromosomal DNA for endonuclease I attack. Clearly, further work is required to decide effectively between alternate hypotheses; however, the spheroplast reconstitution system should provide a valuable
aid in the elucidation of the mechanism of colicin action.

LITERATURE CITED