Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2421–2425, October 1971

Inactivation of Ribosomes *In Vitro* by Colicin E_3

(*E. coli*/immune strains/S30/16S RNA)

THIERRY BOON

The Rockefeller University, New York, N.Y. 10021

Communicated by Norton D. Zinder, July 15, 1971

ABSTRACT Cell-free protein synthesizing extracts incubated with purified preparations of colicin E_3 showed a marked decrease of their protein synthesizing activity. Incubation of ribosomes prepared from S-30 extracts with E_3 resulted in their inactivation. Their 16S RNA was found to lose a terminal fragment, similar in size to the fragment released upon *in vivo* treatment with E_3. Extracts derived from colicinogenic strains are resistant to the action of E_3 *in vitro*.

Colicin E_3 is a protein antibiotic produced by strains of coliform bacteria that carry a specific episome (1). It is lethal for many strains of *Escherichia coli*.

The adsorption of colicin E_3 by sensitive bacteria results in a marked inhibition of protein synthesis, without a corresponding inhibition of RNA and DNA synthesis (2). Ribosomes isolated from cells treated with E_3 are unable to function in an *in vitro* protein-synthesizing system; their defective character has been localized in the "30 S" subunit (3). It has recently been demonstrated that in cells killed by E_3, the 16S RNA present in the 30S ribosomal subunit loses a fragment of about 50 nucleotides from its 3'-end (4, 5).

The killing of bacteria by colicins, as defined by the loss of their colony-forming ability, is partially reversible by trypsin treatment (6). It was also shown with colicin E_2 that most of the adsorbed colicin remains associated with the cell membrane (7). These and other considerations prompted Nomura to propose that colicins act by inducing a reversible change at the receptor site; this change would trigger a transmission process that would result in the destruction of the ultimate biochemical target (6, 7).

Consistent with such a model was the possibility that the adsorption of E_3 to the bacterial membrane would cause the release of molecules that would directly inactivate the ribosomes. To explore this possibility, I added to a protein synthesizing extract the sonicates of cells that had been treated with a large amount of E_3 and subsequently washed. This addition resulted in a marked inhibition of the protein synthesis directed by f_2 RNA. However, the size of the inhibitory molecule was about the same as that of colicin E_3, and control experiments showed that even small amounts of the crude colicin preparation caused a similar inhibition of *in vitro* protein synthesis. This inhibitory effect did not disappear upon further purification of the colicin.

I wish to describe here the inhibition of *in vitro* protein synthesis by highly purified preparations of colicin E_3. Evidence will be presented that shows that ribosomes treated with E_3 *in vitro* show alterations that are similar to those of ribosomes obtained from E_3-treated cells.

MATERIALS AND METHODS

Bacterial Strains. Extracts were made from the sensitive strains W3110, K56, and K289. The two last strains are derivatives of S26 from A. Garen. The colicinogenic strain W3110 (E_3), obtained from D. Helinski, was used for the production of E_3.

Preparation of Purified E_3. E_3 was produced and purified precisely as described by Herschman and Helinski (9). The protein found in the colicin peak from DEAE-cellulose chromatography was eluted entirely as a single peak upon carboxymethyl-cellulose chromatography. The material present in this peak gave a single band on polyacrylamide gel electrophoresis. We also used preparations of E_3 generously provided by Dr. Sylvia Kerr and Dr. Helinski. These preparations gave the same results as our preparations.

Preparation of "S-30" Extracts, Ribosomes, and Ribosomal Subunits. "S-30" extracts for protein synthesis were prepared according to Webster *et al.* (10). Ribosomes were prepared from S-30 extracts by centrifugation at 35,000 rpm for 4 hr in small tubes containing 0.6 ml in the SW 39 Spinco rotor. The top half of the supernatant was used for "supernatant factors". The ribosomes were resuspended in TM buffer: Tris·HCl 50 mM–ammonium chloride 30 mM–magnesium acetate 10 mM. Ribosomal subunits were prepared by layering 300 A_{260} units of S-30 extracts on top of a 10–25% sucrose gradient in Tris·HCl 20 mM (pH 7.5)–magnesium acetate 0.1 mM. Centrifugation at 27,000 rpm was for 11 hr at 4°C in the SW 27 rotor. The material present in the 30S and 50S peaks was concentrated by ethanol precipitation (11).

In Vitro Incorporations. Incorporations stimulated by f_2 RNA were performed in a "lysine assay mixture" of 100 μl of TM buffer containing: ATP 3 mM, GTP 0.2 mM, phosphoenolpyruvate 10 mM, glutathione 5 mM, all amino acids except lysine 0.1 mM, lysine 0.05 mM. Incubations were performed with 20 μg of f_2 RNA (10) and 0.5 μCi of [^{14}C]lysine (300 Ci/mol).

Incorporations stimulated by poly(U) were performed in a "phenylalanine assay mixture", identical to the lysine mixture, except that the magnesium acetate was 17 mM and lysine and phenylalanine were 0.1 mM (unless otherwise mentioned). Incubations were performed with 10 μg of poly(U) and 0.5 μCi of [^{14}C]phenylalanine (455 Ci/mol).

The reactions were stopped with 3 ml of 5% trichloroacetic acid. The reaction mixtures were heated at 90°C for 10 min, filtered on glass-fiber filters, and rinsed with trichloroacetic acid.

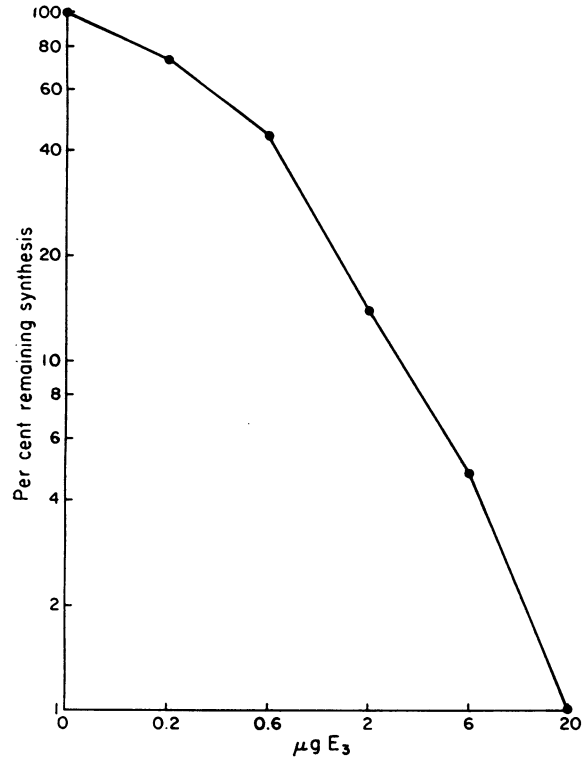

FIG. 1. Effect of E_3 on incorporation. 100 μl of "assay mixture" containing 2.5 A_{260} of "S-30" derived from strain K56 were incubated for 20 min at 37°C with the amounts of E_3 indicated in the abscissa. After 20 min, f_2 RNA was added; 10 min later, [^{14}C]-lysine was added. The reaction was stopped with Cl_3CCOOH 15 min later. 100% is equivalent to 50 pmol of incorporated lysine.

Preparation of Radioactive Ribosomes. W3110 was grown in the presence of [^{3}H]- or [^{14}C]uracil. The cells were concentrated and lysed by lysozyme–EDTA treatment, followed by treatment with Brij-58 and DNase (12). The lysates were spun 20 min at 30,000 × *g* to eliminate cell debris. The supernatants were centrifuged for 90 min at 200,000 × *g*. The ribosome pellets were resuspended in TM buffer.

Examination of the RNA Present in the 30S Subunits. 30S subunits were isolated by layering 0.1 ml of radioactive ribosomes on top of a 5–20% sucrose gradient in Tris·HCl 50 mM (pH 7.8)–NH_4Cl 30 mM–magnesium acetate 1 mM.

TABLE 1. *Effect of colicin E_3 on S-30 extract*

Experiment	Incorporation of [^{14}C]lysine (f_2 RNA)
S-30 untreated	7033 (100%)
S-30 treated with E_3	247 (3.4%)
	Incorporation of [^{14}C]phenylalanine [poly(U)]
S-30 untreated	2926 (100%)
S-30 treated with E_3	1222 (43%)

2.5 A_{260} of an S-30 extract, derived from strain K289, was diluted in the assay mixtures described in *Methods*. The mixtures were incubated at 37°C, for 20 min, with or without 4 μg of E_3. The RNA [f_2 or poly(U)] was then added; 10 min later the labeled amino acid was added. The reaction was stopped 15 min later.

TABLE 2. *Incorporation by ribosomes treated with E_3*

Experiment	[^{14}C]Lysine incorporation (cpm)
S-30 not treated with E_3, ribosomes pelleted once:	4770
S-30 treated with E_3, ribosomes pelleted once:	229 (4.8%)
S-30 not treated with E_3, ribosomes pelleted twice:	2313
S-30 treated with E_3, ribosomes pelleted twice:	195 (8.4%)
Ribosomes pelleted once and not treated with E_3:	2366
Ribosomes pelleted once and treated with E_3:	58 (2.5%)
Ribosomes pelleted twice and not treated with E_3:	1112
Ribosomes pelleted twice and treated with E_3:	40 (3.6%)

1: A S-30 extract derived from K56 (350 A_{260} units in 0.7 ml) was incubated at 37°C for 20 min, with or without 60 μg of E_3. The ribosomes were then pelleted, resuspended in TM buffer, and assayed.

2: The ribosomes described in *1* were passed through one more cycle of pelleting and resuspension and assayed.

3: Ribosomes were pelleted once from the S-30 extract, resuspended in TM buffer (300 A_{260} units in 0.7 ml) and incubated for 20 min at 37°C with 60 μg of E_3; they were then pelleted, resuspended, and assayed.

4: Ribosomes were pelleted from the S-30 extract, resuspended, and passed through one more cycle of pelleting and resuspension; they were then treated like the ribosomes described in *3* and assayed.

Assay: Ribosomes (4 A_{260} units) were diluted in 100 μl of "lysine assay mixture" with 0.6 A_{260} unit of untreated supernatant factors. After 10 min of incubation at 37°C, 20 μg of f_2 RNA was added; 10 min later, [^{14}C]lysine was added; 15 min later the reaction was stopped with Cl_3CCOOH. 1000 cpm are equivalent to the incorporation of 10 pmol of lysine.

After centrifugation for 5 hr at 40,000 rpm and 4°C in a SW 40 rotor, the fractions containing the 30S peak were extracted with 1 volume of phenol. The aqueous layer was made 0.1 M in potassium acetate (pH 5) and the RNA was precipitated with 3 volumes of ethanol. The RNA was resuspended in TM buffer, layered on top at a 5–20% sucrose gradient in TM buffer, and centrifuged for 10 hr at 40,000 rpm and 4°C. The fractions were collected and counted in Bray's solution.

RESULTS

Inhibition of S-30 extracts by E_3

S-30 protein-synthesizing extracts were incubated with purified E_3 and then assayed for protein synthesis directed by f_2 RNA and poly(U). The addition of E_3 markedly inhibited the incorporation stimulated by f_2 RNA, as shown in Fig. 1. The incorporation stimulated by poly(U) was always considerably less inhibited than that stimulated by f_2 RNA. An example is shown in Table 1.

Similar results were obtained with the three independently obtained preparations of E_3 mentioned in *"Methods"*. My colicin preparation formed a single band on polyacrylamide gel electrophoresis. This clearly did not exclude the possibility that a minor impurity accounted for the inhibition. However, this impurity would have to have about the same molecular weight as E_3, since the colicin activity (measured by bacterial

killing) and the activity inhibiting protein synthesis were found to sediment at the same rate.

Defectiveness of ribosomes treated with E_3

Ribosomes isolated from E_3-treated S-30 extracts were found to be defective, as shown in Table 2. The defective character of these ribosome preparations appears not to be due to the presence of residual colicin or supernatant factors. The amount of E_3 remaining in the ribosomal preparation was much smaller than the amount required for the observed inhibition. Moreover, as is shown in Table 2, an additional cycle of pelleting and resuspension of the ribosomes does not alter significantly the degree of the inhibition.

The results shown in Table 2 also indicate that ribosomal fractions free of supernatant factors are sensitive to E_3. Here again, the extent of the inhibition does not decrease significantly if the ribosomes are given additional cycles of pelleting and resuspension before E_3 treatment.

Analogy between the ribosomes treated with E_3 *in vivo* and those treated *in vitro*

Konisky and Nomura (3) assayed ribosomal subunits extracted from normal and E_3-treated cells. The results indicated that the E_3-treated cells contain defective 30S subunits. I performed a similar experiment with ribosomal subunits isolated from a S-30 extract treated with E_3. The results shown in Table 3 suggest that the 30S subunit is also defective after treatment of ribosomes with E_3 *in vitro*.

In agreement with the results of Senior *et al.* (4) and Ikemura *et al.* (5), I found that the RNA contained in the 30S ribosomal subunits, isolated from E_3-treated W3110 cells, has lost a terminal fragment. This result is shown in Fig. 2; a small fragment is obtained and the main RNA peak is located almost one fraction behind that of the marker 16S RNA. Neither of these two features are found with RNA extracted from untreated cells.

Examination of the RNA extracted from ribosomes treated with E_3 *in vitro*, in the presence of W3110 supernatant factors, revealed the formation in the 30S subunits of a terminal fragment of a size similar to that of the fragment obtained after *in vivo* treatment. This result and its control are shown in Figs. 3 and 4. From the amount of fragment obtained and the

TABLE 3. *Incorporation by ribosomal subunits from control and E_3-treated S-30*

30S	50S	[^{14}C] Phenylalanine, cpm
Control	—	120
—	Control	330
E_3	—	106
—	E_3	177
Control	Control	5420
E_3	E_3	2709
Control	E_3	4113
E_3	Control	2156

An S-30 extract derived from K56 (300 A_{260} units in 0.6 ml) was incubated at 37°C for 70 min with ("E_3") or without ("control") 150 μg of E_3. 30S and 50S ribosomal subunits were then obtained as described in *Methods*. The subunits (350 A_{260}) were assayed in the poly(U)-directed system, in the presence of 0.05 mM phenylalanine. There was no preincubation; poly(U) and [^{14}C]phenylalanine were added and the reaction was stopped 20 min later.

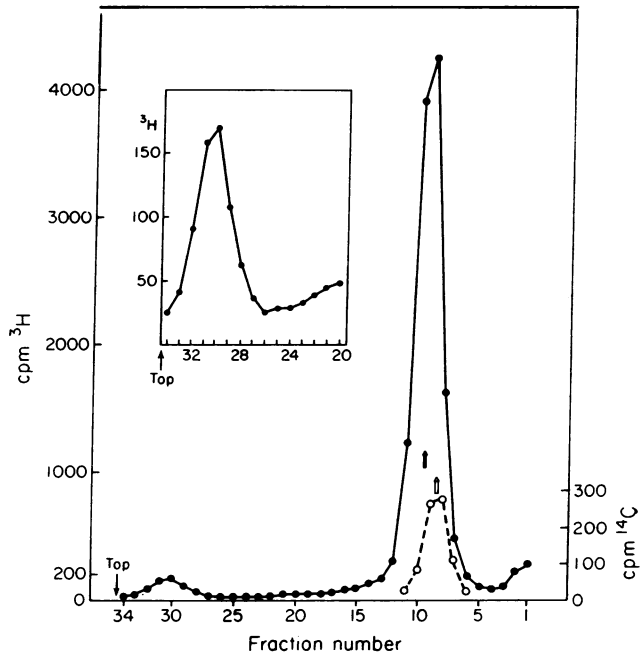

FIG. 2. Effect of E_3 on RNA *in vivo*. ●(^{3}H): cells were labeled with [^{3}H]uracil and treated with E_3 at the end of the labeling period. Then, ribosomes were isolated and the RNA present in the 30S subunits was isolated and centrifuged.

O: ^{14}C-marker 16S RNA. The *inset* represents the counts present in the top fractions of the gradient on a larger scale. The *filled arrow* indicates the median of the ^{3}H peak, the *hollow arrow* that of the ^{14}C peak.

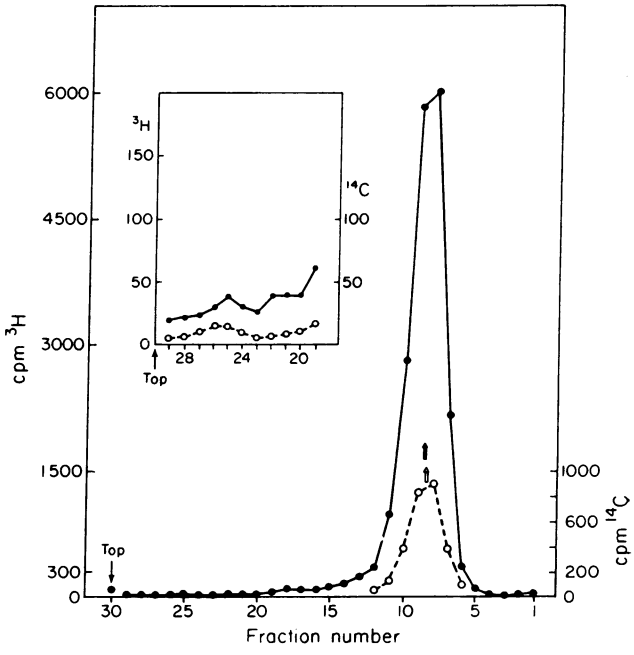

FIG. 3. Isolation of control RNA from *E. coli*. ●(^{3}H): cells were labeled with [^{3}H]uracil and their ribosomes were isolated. The ribosomes (150 μg in 80 μl of TM buffer) were incubated at 37°C for 45 min with 4 A_{260} units of supernatant factors from W3110. The RNA present in the 30S subunits was isolated and centrifuged.

O: ^{14}C-marker 16S RNA. The *filled arrow* indicates the median of the ^{3}H peak, the *hollow arrow* that of the ^{14}C peak.

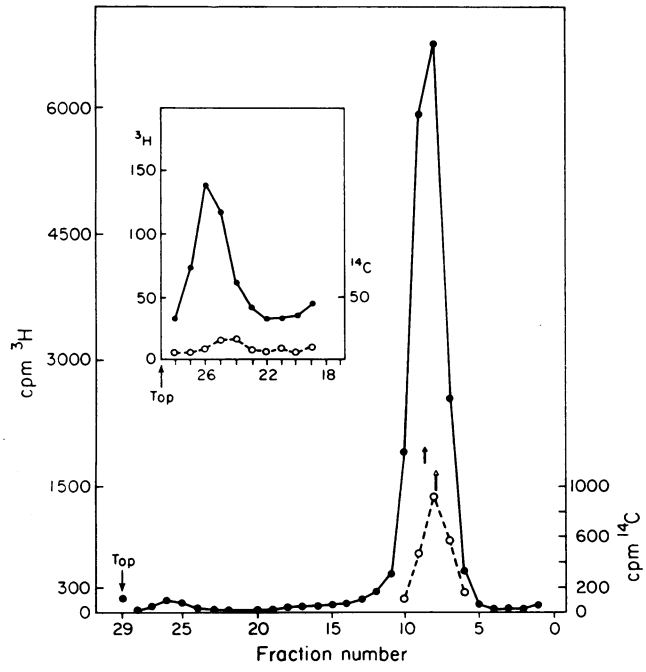

FIG. 4. Effect of E_3 on ribosomes *in vitro*. ●(^{3}H): cells were labeled with [^{3}H]uracil. The ribosomes (150 μg in 80 μl of TM buffer) were incubated at 37°C for 45 min with 4 A_{260} of supernatant factors of W3110 and with 7 μg of E_3. The RNA present in the 30S subunits was isolated and centrifuged.

O(^{14}C): marker 16S RNA. The *full arrow* indicates the median of the ^{3}H peak, the *hollow arrow* that of the ^{14}C peak.

displacement of the main peak relative to the marker RNA, it appears that the amount of RNA cleaved in this *in vitro* experiment was about 50% of that cleaved *in vivo*.

The same result was obtained in an experiment where ribosomes were incubated with E_3 in the absence of supernatant factors. The incubation of purified 16S RNA with E_3 did not result in a detectable yield of fragment.

Reduced sensitivity of an S-30 extract derived from an immune strain

Bacteria carrying a colicinogenic factor are largely resistant to the corresponding colicin. This property is called immunity. An S-30 extract derived from W3110 (E_3) is much less sensitive to E_3 than extracts prepared from sensitive strains.

TABLE 4. *Effect of colicin E_3 on S-30 from an immune strain*

Experiment	[^{14}C]Lysine incorporated (cpm)
S-30 from sensitive strain: control	6203
E_3 treated	208 (3.3%)
S-30 from immune strains: control	8896
E_3 treated	6999 (78%)

The S-30 extracts were derived from K289 (*sensitive*) and W3110(E_3) (*immune*). The S-30 extracts (20 A_{260} units in 50 μl) were incubated at 37°C with 2 μg of E_3. After 20 min, 5 μl of extract were added to a "lysine assay mixture"; f_2 RNA and [^{14}C]lysine were added immediately and the reaction was allowed to proceed for 25 min.

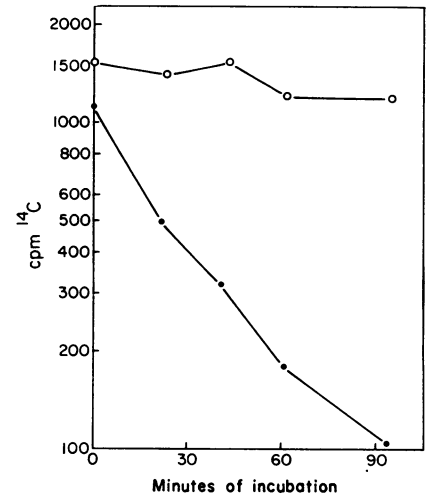

FIG. 5. Effect of E_3 on incorporation by ribosomes treated *in vitro*. Ribosomes were pelleted once from a S-30 extract derived from strain K289 and resuspended in TM buffer (300 A_{260} U/ml). They were incubated at 37°C with or without E_3 (30 μg/ml). Aliquots of 5 μl were taken at the times shown in the abscissa and added to a lysine assay mixture and [^{14}C]lysine. The reaction was stopped after 15 min. O: Incorporation by ribosomes incubated without E_3. ●: Incorporation by ribosomes incubated with E_3.

This result, shown in Table 4, is obtained when an undiluted S-30 extract is incubated with E_3. However, the ribosomes isolated from immune strains were as sensitive as those derived from sensitive strains when incubated in the absence of supernatant factors (Table 5).

The immunity appears therefore to be linked to a supernatant factor that is nondialyzable.

Enzymatic action of E_3

Ribosomes incubated with small amounts of E_3 for a prolonged period were extensively inactivated. In the experiments described in Table 6, the ratio of ribosomes to colicin molecules was as high as 600. This implies that colicin acts catalytically to inactivate ribosomes *in vitro*. One could argue that our preparations of ribosomes contain only a very small minority of functional ribosomes and that E_3 selectively inactivates those. The *in vitro* production of an RNA fragment in at least 50% of the ribosomes suggests, however, that this is not the case. Moreover, ribosomes incubated with E_3 show an exponential decrease of their protein synthesizing activity as a function of time (Fig. 5). This is the expected result if the rate of the inactivation reaction is proportional to the con-

TABLE 5. *Effect of colicin E_3 on ribosomes from an immune strain*

Experiment	[^{14}C]Lysine incorporated
Ribosomes from sensitive strain: control	689
E_3 treated	28
Ribosomes from immune strain: control	668
E_3 treated	16

Ribosomes derived from W3110 (*sensitive*) and W3110 (*immune*) were treated and assayed as described in Table 2, number 4.

TABLE 6. *Effect of colicin E_3 on different concentrations of ribosomes*

Experiment	[^{14}C]Lysine incorporated
Ribosomes at concentration 1x: control	1208
E_3 treated	176 (14%)
Ribosomes at concentration 2x: control	1220
E_3 treated	177 (14%)

Ribosomes derived from K289 were pelleted from a S-30 extract and resuspended in TM buffer at a concentration of 33 mg/ml (1x) and 66 mg/ml (2x). They were incubated at 37°C, with 2.5 μg of E_3 for 120 min. They were then pelleted, resuspended, and assayed as described in Table 2.

centration of ribosomes. Although the concentration of ribosomes in weight per volume is large in our experiments, their molar concentration is only of the order of 10^{-8} M, a value that is much lower than the K_m of most enzymes.

DISCUSSION

The experiments described above show that the *in vivo* effects of colicin E_3 on ribosomes (3–5) can be obtained *in vitro*. E_3 treatment results in the release of a terminal fragment from the 16S RNA both *in vivo* and *in vitro*. Protein synthesizing extracts derived from immune cells are resistant to E_3. These two facts strongly indicate that the *in vitro* inactivation of ribosomes caused by our preparations of E_3 is a specific effect of the colicin.

The *in vitro* inactivation of ribosomes by E_3 does not appear to require the presence of supernatant factors. However, these experiments do not exclude the possibility that some other component present in the ribosomal fraction is required for inactivation. Further experiments on purified ribosomal subunits should clarify the nature of the specific target of colicin action. In view of the simple kinetics observed and in view of the fact that inactivation is obtained with widely different concentrations of ribosomal fractions, the simplest interpretation of our results is that the colicin molecules interact directly with the ribosomes.

The results described above suggest the possibility that the killing of bacteria by E_3 involves the penetration into the cell of a colicin molecule that inactivates the ribosomes enzymatically.

I thank Robert Webster for introducing me to *in vitro* protein synthesis, and am very grateful to Norton D. Zinder and Peter Model for their invaluable advice. Supported in part by a grant from the National Science Foundation to Norton D. Zinder.

1. Nomura, M., *Annu. Rev. Microbiol.*, **21**, 257 (1967).
2. Nomura, M., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 315 (1963).
3. Konisky, J., and M. Nomura, *J. Mol. Biol.*, **26**, 181 (1967).
4. Senior, B. W., and I. B. Holland, *Proc. Nat. Acad. Sci. USA*, **68**, 959 (1971).
5. Bowman, C. M , J. E. Dahlberg, T. Ikemura, J. Konisky, and M. Nomura, *Proc. Nat. Acad. Sci. USA*, **68**, 964 (1971).
6. Nomura, M., *Proc. Nat. Acad. Sci. USA*, **52**, 1514 (1964).
7. Maeda, A., and M. Nomura, *J. Bacteriol.*, **91**, 685 (1966).
8. Luria, S., *Ann. Inst. Pasteur Paris*, **107**, 67 (1964).
9. Herschman, H. R., and D. R. Helinski, *J. Biol. Chem.*, **242**, 5360 (1967).
10. Webster, R. E., D. L. Engelhardt, W. Konigsberg, and N. D. Zinder, *J. Mol. Biol.*, **29**, 27 (1967).
11. Falvey, A. K., and T. Staehelin, *J. Mol. Biol.*, **53**, 1 (1970).
12. Phillips, L. A., B. Hotham-Iglewski, and R. M. Franklin, *J. Mol. Biol.*, **40**, 279 (1969).