Proc. Nat. Acad. Sci. USA
Vol. 69, No. 3, pp. 549–552, March 1972

Inactivation of Ribosomes *In Vitro* by Colicin E_3 and Its Mechanism of Action

(30S and 50S ribosomal subunits/immunity factor/16S ribosomal RNA/colicins E_1, E_2, and K)

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Communicated by Norton D. Zinder, December 17, 1971

ABSTRACT **The incubation of purified ribosomes with colicin E_3 results in the cleavage of a terminal fragment from the 16S ribosomal RNA. The cleavage reaction requires three components: colicin E_3, the 30S ribosomal subunit, and the 50S ribosomal subunit. An immunity factor found in extracts derived from colicinogenic cells prevents the *in vitro* inactivation of ribosomes by colicin E_3. Evidence is presented suggesting that it does so by binding to the colicin molecule. The mode of action of colicin E_3 *in vivo* can be explained by the assumption that a small fraction of the adsorbed colicin penetrates into the cell and catalytically inactivates the ribosomes.**

The adsorption of colicin E_3 to sensitive bacteria causes a marked inhibition of protein synthesis, which results from inactivation of the ribosomes (1, 2).

In a recent communication, I have demonstrated that this effect can be reproduced *in vitro*: ribosomes extracted from bacteria are inactivated when incubated with purified colicin E_3 (3). Both the *in vivo* and the *in vitro* inactivation of ribosomes by E_3 appear to be due to the cleavage of a terminal fragment of the RNA of the 30S ribosomal subunit (4, 5, 3).

A direct interaction between colicin E_3 and the ribosomes is clearly difficult to reconcile with a model of colicin action that proposes that E_3 remains attached to the outer surface of the cell while causing the degradation of the ribosomes (5–7). It is, therefore, of some importance to show that the *in vitro* inactivation of ribosomes by E_3 involves only the colicin and the ribosomal components, and that other large components, such as contaminating fragments of the bacterial envelope, are not involved.

I shall describe here the results of experiments performed with purified ribosomes and ribosomal subunits. These results confirm that E_3 acts directly on the ribosomes.

MATERIALS AND METHODS

Bacterial strains: K56 and W3110 are sensitive to E_3. W3110 (E_3) is colicinogenic for E_3.

S-30 extracts for protein synthesis were prepared; assays of *in vitro* protein synthesis were performed as described (3). TM buffer is 50 mM Tris·HCl (pH 7.8)–30 mM ammonium chloride–10 mM magnesium acetate.

Unlabeled 50S ribosomal subunits were obtained by layering 50 A_{260} of S-30 on a 13-ml, 5–20% sucrose gradient in 50 mM Tris·HCl (pH 7.8)–30 mM ammonium chloride–1 mM magnesium acetate. The gradients were centrifuged for 4.5 hr at 4°C in a Beckman SW 40 rotor at 40,000 rpm.

Ribosomes with ^{3}H-labeled RNA were prepared as follows: W3110 was grown in the presence of [^{3}H]uracil treated with lysozyme and EDTA, and then lysed with Brij 58 in the presence of DNase (8). S-30 extracts were obtained by centrifugation of the lysate at 30,000 × *g* for 50 min to eliminate cell debris. Labeled 70S ribosomes and 30S subunits were obtained by incubation of the labeled S-30 for 20 min at 37°C with amino acids under conditions allowing protein synthesis, and layering the mixture on a 13-ml, 5–20% sucrose gradient in 5 mM Tris·HCl (pH 7.5)–10 mM magnesium acetate–60 mM potassium chloride. The gradients were centrifuged for 3 hr at 40,000 rpm in a Beckman SW 40 rotor. The 50S peak was badly resolved from the 70S peak. The central fractions of the 30S and 70S peak were collected.

RESULTS

Inactivation of 70S ribosomes

When S-30 lysates derived from W3110 are incubated with E_3, a small terminal fragment is cleaved from the 16S RNA, present in the 30S ribosomal subunits (3).

Labeled S-30 lysates were centrifuged to isolate 70S ribosomes. Incubation of the 70S fraction with E_3 resulted in the quantitative cleavage of the terminal fragment from the 16S ribosomal RNA (Fig. 1).

Inactivation of 30S ribosomal subunits

Labeled 30S ribosomal subunits were incubated with E_3: No significant cleavage of the 16S RNA was observed (Fig. 2).

The cleavage reaction occurred fully when 30S subunits were incubated with E_3 in the presence of unlabeled S-30 extract (conditions of Fig. 2, with 2 A_{260} units of S-30, derived from W3110). The addition of an equivalent amount of S-100 supernatant to the 30S subunits and E_3 did not result in the production of significant amount of fragment (data not shown).

When 30S subunits were incubated with E_3 in the presence of purified 50S subunits, a quantitative cleavage of the terminal RNA fragment was obtained (Fig. 3). It appears therefore that the cleavage reaction involves three components: the two ribosomal subunits and the E_3 molecule.

Further attempts to analyze the cleavage reaction

Conceivably, E_3 could irreversibly alter the 30S or the 50S subunit in such a way that a subsequent interaction between the two subunits, occurring in the absence of E_3, would result in the cleavage of the 16S RNA. E_3 does not seem to irreversibly "sensitize" the 30S subunit to the action of the 50S subunit: 30S subunits were treated with E_3 and separated

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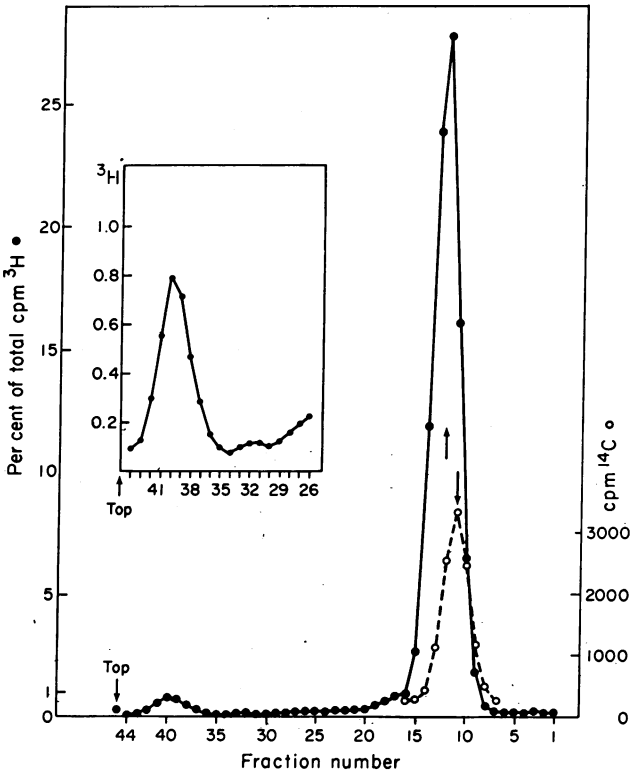

FIG. 1. Effect of E_3 on purified 70S ribosomes. ●, ^{3}H-labeled 70S ribosomes (0.6 A_{260}) were mixed with 50 μg of E_3 in 0.3 ml of TM buffer (see *Methods*), and incubated at 37°C for 60 min. The RNA present in the 30S subunit was isolated and centrifuged (3). The sedimentation pattern is shown here. O, marker 16S [^{14}C]RNA. The *inset* represents the upper part of the gradient by an enlarged scale, so as to indicate the amount of terminal-fragment released. The *arrows* indicate the median of the main peaks of [^{3}H]- and [^{14}C]RNA.

from the colicin by centrifugation; they were then incubated with 50S subunits. No cleavage of the 16S RNA was observed. The possibility that E_3 can irreversibly "activate" the 50S subunit is still open: a partial release of the specific RNA fragment was observed when 30S subunits were incubated with 50S subunits that had been purified from an S-30 extract treated with E_3 (data not shown).

The incubation of 16S ribosomal RNA with E_3 leaves the RNA intact (5, 3). When 16S ribosomal RNA was incubated with 50S subunits, either in the presence or in the absence of E_3, some nonspecific degradation occurred, but no unequivocal release of the specific fragment could be ascertained.

Binding of the immunity factor to E_3

The S-100 supernatant derived from the colicinogenic strain W3110(E_3) contains an "immunity factor" that suppresses the *in vitro* effects of colicin E_3 (3). When ribosomes are treated with E_3 in the presence of the immunity factor, they maintain their capacity to synthesize proteins (3), and there is no cleavage of the 16S RNA (unpublished result). The immunity factor does not inactivate E_3 irreversibly: we have observed that E_3 incubated with enough immunity factor to suppress its *in vitro* action retains its killing titer *in vivo*.

A priori, the immunity factor could prevent the inactivation of ribosomes either by binding to E_3 or by binding to the ribosomes. I have compared the inactivation of ribosomes incubated with increasing amounts of E_3 either in the presence or in the absence of immunity factor (Fig. 4).

Under the conditions of this experiment, the rate of inactivation of ribosomes is proportional to the concentration of active ribosomes (3), and probably also to the concentration of E_3. If the immunity factor binds to the ribosome to form a protected complex, this reduces by a certain factor n the concentration of ribosomes that can be inactivated. This can be compensated for by an n-fold increase in the concentration of E_3. The degree of inactivation observed at a given concentration of E_3 in the absence of "immunity factor" would therefore be the same as that observed at an n-fold higher concentration of E_3 in the presence of this factor. The two curves of Fig. 4 would then be parallel.

If, on the contrary, the immunity factor binds to E_3 to form an inactive complex, there should be a titration effect: the protection afforded by the immunity factor should drop abruptly when the concentration of E_3 reaches that of the immunity factor. The two curves shown in Fig. 4 would therefore converge. This is the result obtained.

I wish to point out that this experiment only suggests that the immunity factor binds to E_3, as the result obtained would also be generated by any nonspecific effect, due for instance to the very high concentration of E_3 used here.

DISCUSSION

The experiments presented above have not elucidated the detailed mechanism of the cleavage of the 16S ribosomal RNA.

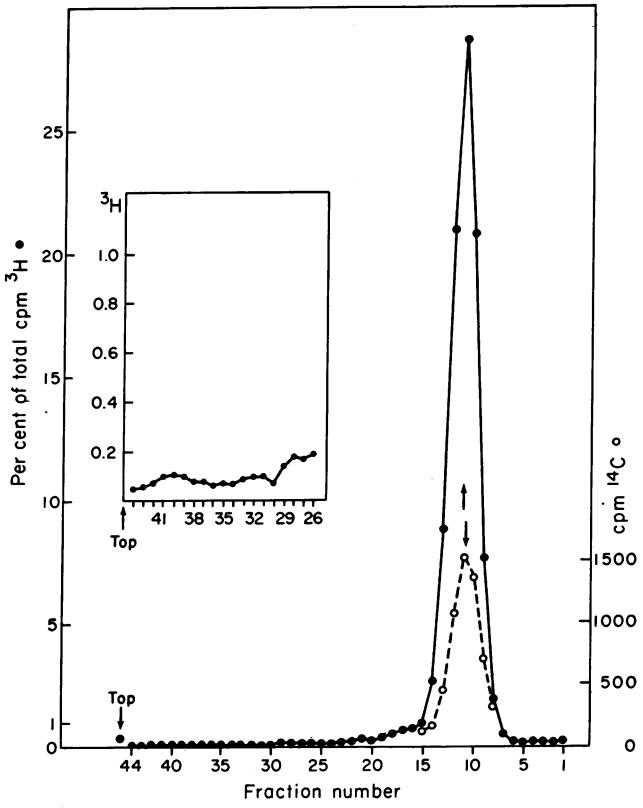

FIG. 2. Effect of E_3 on 30S subunits. ●, ^{3}H-labeled 30S subunits (0.08 A_{260}) were mixed with 80 μg of E_3 in 0.5 ml of TM buffer and incubated at 37°C for 60 min. The figure shows the sedimentation pattern of the RNA present in the 30S subunit (3). O, marker [^{14}C]RNA.

In particular, the responsible ribonuclease has not been located. However, a plausible picture of how E_3 inhibits cellular protein synthesis can be formed. The colicin E_3 molecule could catalyze the blocking of the translocation of ribosomes traveling on messenger RNA, so as to "freeze" the polysomes. This would be compatible with the observation that polysomes with nascent polypeptide chains are found in E_3-treated cells (16). This would also be an efficient way to halt protein synthesis, as the blocking of a single ribosome on the polysome could also block the progression of all the ribosomes traveling behind it.

Our understanding of the mechanism of action of colicin E_3 is derived from the following observations:

(*a*) E_3 molecules adsorb to a specific receptor on the surface of sensitive bacteria, and this adsorption is irreversible under normal ionic conditions (9, 6).

(*b*) E_3 kills sensitive bacteria according to a "one-hit killing curve", that is: each adsorption of a colicin molecule has a certain independent probability of provoking the "lethal event" that is sufficient to deprive the cell of its colony-forming ability (10, 1).

(*c*) Only for a small fraction of the E_3 molecules does adsorption result in a lethal event. It can be calculated from the molecular weight of E_3 that the number of colicin E_3 molecules that is required to cause an average of one killing-hit per bacterium is about 100 (unpublished result, 7, 11).

(*d*) It is possible to prevent the loss of colony-forming ability of cells treated with E_3 by incubating them with trypsin soon after the adsorption of the colicin ("trypsin rescue") (6).

(*e*) The adsorption of E_3 causes a complete inhibition of protein synthesis. The rapidity of this inhibition is highly dependent on the dose of E_3 (6).

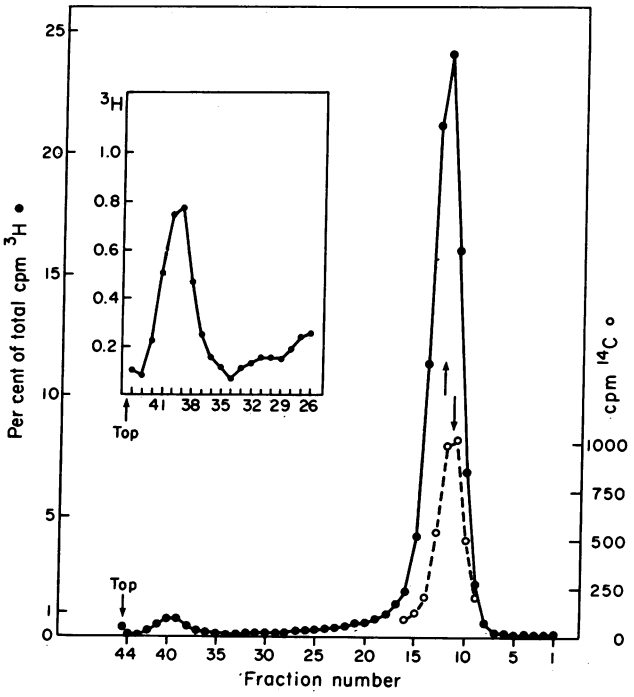

FIG. 3. Effect of E_3 on 30S subunits in the presence of 50S subunits. ●, ^3H-labeled 30S subunits (0.08 A_{260}) were mixed with 70 μg of E_3 and 0.5 A_{260} of purified 50S subunits in 0.4 ml of TM buffer. The mixture was incubated at 37°C for 60 min. The figure shows the sedimentation pattern of the RNA present in the 30S subunits (3). ○, marker [^{14}C]RNA.

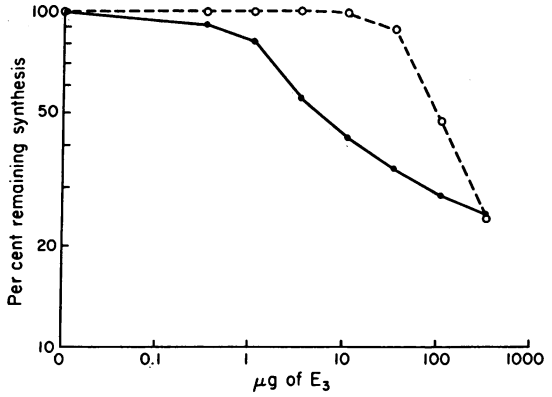

FIG. 4. ●, without immunity factor: 7.5 A_{260} of S-30 extract derived from K56 were mixed with the amounts of E_3 shown in the abscissa in 50 μl (of TM buffer) and incubated at 37°C for 10 min. 15-μl Aliquots were assayed (3) for protein synthesis directed by f2 RNA in 100-μl reaction mixtures. The reaction volume contained 25 μl of S-100 supernatant of W3110 (E_3) to minimize the effects of E_3 during the 10-min assay period. ○, with immunity factor: same experiment as above, performed with a S-30 extract derived from W3110 (E_3). The percent of remaining synthesis has been corrected for the inactivation that occurred during the incubation with f2 RNA. The inactivation figures shown here represent, therefore, the inactivation that occurred in the first incubation.

(*f*) Inhibition of protein synthesis is due to the inactivation of ribosomes, which results, most probably, from the cleavage of a terminal fragment from the RNA present in the 30S ribosomal subunit (2, 4, 5).

(*g*) The inactivation of ribosomes can be reproduced *in vitro*. This reaction requires three components: the E_3 molecule and the two ribosomal subunits (ref. 3 and above).

(*h*) Some bacterial mutants, called "tolerants", have lost their sensitivity to E_3, even though they still adsorb it (9, 12–14).

(*i*) Cells that are able to produce E_3 are immune to E_3. This immunity can, however, be overcome at high doses of E_3 (9). Cell extracts derived from colicinogenic cells contain an "immunity factor" that prevents the *in vitro* effects of E_3 (3).

These observations can be explained as follows: A small fraction of the colicin molecules that adsorb to the cell envelope penetrates into the cell. The penetration of these molecules does not necessarily occur immediately after their adsorption. Once inside the cell, a colicin molecule catalyzes the inactivation of the ribosomes by provoking the cleavage of the RNA contained in the 30S subunits. As a result, protein synthesis stops and the cell loses its colony-forming ability. Colicinogenic cells contain an "immunity factor" that probably binds to those colicin molecules that penetrate into the cell and prevents their action.

The entry of a single molecule of E_3 is sufficient to kill the bacterium: this is, thus, the "lethal event" implied by the "single-hit" killing curve. The larger the number of colicin molecules adsorbed to the surface of the cell, the larger the number of molecules that penetrate and the more rapid is the inhibition of protein synthesis. The addition of trypsin shortly after E_3 rescues those cells into which no colicin mole-

cule has yet penetrated by digestion of the colicin adsorbed on the cell envelope. Tolerant mutants could be mutants that do not allow the penetration of colicin molecules.

Can these conclusions be applied to the mode of action of the other colicins, notably E_2, E_1, and K? Nomura has proposed that colicins exert their lethal effects while remaining at the outer surface of the cells (1, 2, 5–7). In addition to his observations on the action of colicin E_3 Nomura based his model on two arguments. The first was that 95% of the E_2 molecules remained associated with the cellular envelope when the cells were broken (7). However, this finding can be explained even if colicins must penetrate to kill, as less than 1% of the adsorbed colicin generates a lethal event. This reservation was pointed out by Maeda and Nomura themselves (7). The second argument came from the observation that the macromolecular synthesis of cells treated with colicin K was significantly higher when trypsin was added after the addition of colicin than when it was not added (15). This effect of trypsin was interpreted as a reversal of the biochemical effects of colicin K. However, I think that the data presented do not unequivocally demonstrate a reversibility of the effects of colicin K, as opposed to a trypsin rescue of the type described and discussed above. To conclude: colicin E_2, E_1, and K may act from outside the cell, but there is no compelling evidence that they do so.

NOTE ADDED IN PROOF

Bowman, C. M., Sidikaro, J. & Nomura, M. (1971) *Nature New Biol.* **234**, 133–137 have independently confirmed the previously reported (3) *in vitro* effects of colicin E_3.

I thank Norton D. Zinder and Peter Model for numerous stimulating discussions. The experiments were performed in collaboration with Mrs. Karen Jakes. I am grateful to Mrs. Esther Lund for her editorial assistance. This work was supported by a grant to Norton D. Zinder from the National Science Foundation.

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