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Highly Purified Colicin E3 Contains Immunity Protein

(polypeptide chains/activation of colicin E3)

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ABSTRACT **Colicin E3, even when highly purified, still contains about one molar equivalent of a second protein, "E3 immunity protein." The two proteins are bound together in a complex that can be dissociated only under strongly denaturing conditions, such as electrophoresis in sodium dodecyl sulfate–polyacrylamide gels or by gel filtration in 6 M guanidine·hydrochloride.**

Colicin and immunity protein were separated by preparative electrophoresis in sodium dodecyl sulfate–polyacrylamide gels. The immunity protein prepared in this way was shown to be functionally and immunologically identical to immunity protein purified from colicinogenic cells by other methods. Colicin E3 that had been freed of immunity protein was much more active than complexed colicin in inhibiting protein synthesis *in vitro*.

Colicin E3 is a protein antibiotic produced by certain strains of coliform bacteria, which kills other related bacterial strains (1). The colicin first attaches to specific receptors on the surface of sensitive cells (2, 3). Its ultimate effect is to inhibit protein synthesis by causing the cleavage of a fragment from the 3′ end of the 16S ribosomal RNA (4, 5). This colicin-induced cleavage can also be observed *in vitro* (6, 7), so it is likely that the colicin penetrates cells to reach its target. Ribosomes from cells treated with colicin E3 *in vivo*, as well as ribosomes so treated *in vitro*, show reduced activity in protein synthesis *in vitro* (6, 7).

Colicin E3 is produced by strains carrying a specific plasmid DNA, the Col E3 factor (2, 8). Cells carrying this plasmid are also immune to colicin E3 (2, 9). This immunity is distinct from the "resistance" to colicin exhibited by bacteria that lack colicin receptors on their cell surfaces. Immunity to colicin E3 is due to the presence of an acidic protein of 10,000 molecular weight (E3 immunity protein), which can be isolated only from cells carrying the Col E3 factor (10, 11). When added *in vitro* to mixtures of colicin E3 and ribosomes, immunity protein prevents the inactivation of the ribosomes by E3. We have previously reported that immunity protein can be readily purified to homogeneity, since it can be separated from the colicin on columns of DEAE–Sephadex. However, highly purified colicin E3 contains a component that migrates with immunity protein on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels (10). We will now show that this component of colicin E3 is immunity protein and that it is present in the colicin E3 in molar equivalent amounts.

MATERIALS AND METHODS

Colicin E3 and its immunity protein were purified from mitomycin C-induced cultures of W3110 (E3), as described (10, 12). Samples of purified colicin E3 were also generously provided by J. Sidikaro (Univ. of Wisconsin), D. Helinski (Univ. of Calif., San Diego), and S. Kerr (Univ. of Colorado, Boulder), for comparison with our own preparations. [^{3}H]Leucine-labeled colicin E3 was prepared from 1 liter of cells grown to a density of 5×10^8/ml in minimal medium (13) containing 200 μg/ml of casamino acids. [^{3}H]Leucine at 1 μCi/ml (Schwarz/Mann, 41 Ci/mmole), unlabeled leucine at 0.1 mM, and mitomycin C (Sigma) at 0.4 μg/ml were added simultaneously, and growth was continued for 1 hr. The colicin was then isolated as from unlabeled cells. The purification was only carried through one column, of DEAE–Sephadex (12).

Colicin and immunity protein were assayed by measuring their effects on f2 RNA-directed protein synthesis *in vitro*, as described (10). Colicin E3 activity *in vivo* was determined by spot-testing dilutions of colicin on an agar layer of sensitive cells (12).

Antisera to purified colicin E3 and to purified immunity protein were prepared from New Zealand red rabbits. Five hundred micrograms of protein in Freund's adjuvant were used as a primary subcutaneous injection. Each rabbit then was given three intravenous injections of about 500 μg of protein at monthly intervals. Antisera were prepared and stored frozen or at 4° in 0.01% merthiolate. Antigen–antibody reactions were analyzed by double-diffusion in 1.15% agarose dissolved in 0.15 M NaCl and spread on glass slides.

Five and 10% polyacrylamide–$NaDodSO_4$ phosphate gels were run as described by Weber and Osborn (14). Fifteen percent acrylamide–8 M urea–$NaDodSO_4$ slab gels with a stacking gel were run on the apparatus of Studier (15), by the method introduced by Laemmli (16) and adaptations described by Model and Zinder (17). Other gel systems used for analyzing colicin were 4 and 10% acrylamide in 0.25 M Tris·HCl (pH 8.9) and 4 and 10% acrylamide containing 8 M urea in 0.25 M Tris·HCl (pH 8.9). Gels were stained and destained as described (17). Radioactivity in gel bands of radioactive samples was determined by slicing frozen cylindrical gels into 1-mm horizontal slices and counting as described by Fromageot and Zinder (18).

Preparative $NaDodSO_4$–phosphate gels were run as described by Weber and Kuter (19). Colicin E3 (200–300 μg) could be run on a gel 1 cm in diameter. An extra gel was always run in parallel and briefly stained and destained to determine the positions of the protein bands. The corresponding positions in the unstained gels were cut out and macerated with a glass stirring rod in 2–3 volumes of 50 mM Tris-acetate (pH 7.8) containing 0.1% $NaDodSO_4$. The ground gels were left in the extracting buffer overnight, and then the gel parti-

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; colicin E3*, colicin E3 purified on polyacrylamide–$NaDodSO_4$–phosphate gels.

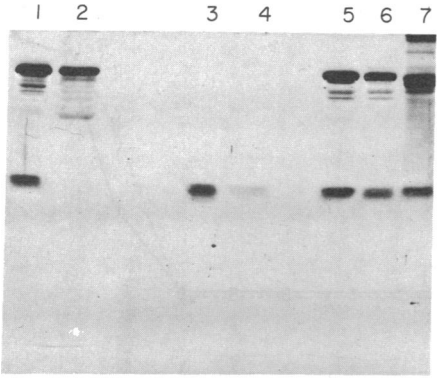

Fig. 1. Fifteen percent acrylamide–8 M urea–0.1% NaDodSO$_4$ slab gel electrophoretic analysis of colicin E3 and immunity protein. (*1*) Colicin E3 purified on carboxymethyl–Sephadex, starting material for samples *2* and *4*; (*2*) colicin E3* from NaDodSO$_4$ preparative gel; (*3*) purified immunity protein (10); (*4*) immunity protein extracted from NaDodSO$_4$ preparative gel of the colicin E3 in slot *1*; (*5*) colicin E3 from J. Sidikaro; (*6*) colicin E3 from D. Helinski; (*7*) colicin E3 from S. Kerr.

cles were removed by passing the mixture through a Millipore filter on a Swinnex apparatus (Millipore) attached to a syringe.

RESULTS

Highly Purified Colicin E3 Contains a Component that Migrates with Immunity Protein on NaDodSO$_4$–Acrylamide Gels. Slots 1, 5, 6, and 7 of Fig. 1 show a 15% acrylamide–8 M urea–NaDodSO$_4$ slab gel of purified colicin E3 from various sources, and slot 3 shows a sample of purified colicin E3 immunity protein. In addition to the high-molecular-weight colicin component and some minor contaminants in the colicin E3 samples, there is a prominent component in all of the colicin preparations that migrates at the position of immunity protein. These gels have good resolution in the low-molecular-weight region and are, therefore, ideally suited to the detection of any low-molecular-weight proteins in these preparations. On standard 10% acrylamide–NaDodSO$_4$ phosphate gels, immunity protein runs very close to the bromophenol blue marker dye, and the same component in colicin might well be ignored as a minor contaminant.

Identification of the Low-Molecular-Weight Component of Colicin E3. Antiserum prepared against purified immunity protein gives a precipitin band when reacted with purified colicin E3 on Ouchterlony double-diffusion plates (Fig. 2*A*). The band fuses without spurring with the band from the reaction of antiserum against immunity protein with purified immunity protein. This result indicates that there is a component of colicin that is identical to immunity protein. However, although colicin reacts with antiserum against immunity protein, the reverse reaction of immunity protein with antiserum against colicin E3 does not occur. The immunity protein bound to colicin E3 may be in a conformation different from free immunity protein, or, alternatively, there was not sufficient immunity protein in the colicin used to inject the rabbits to induce a measurable amount of antibody against immunity protein. Control reactions of antiserum against colicin E3 immunity protein with colicin E2 gave no precipitin band, although the colicins E3 and E2 crossreact immunologically (12) and purified E2 also contains a prominent low-molecular-weight component which is probably its own immunity protein (unpublished results).

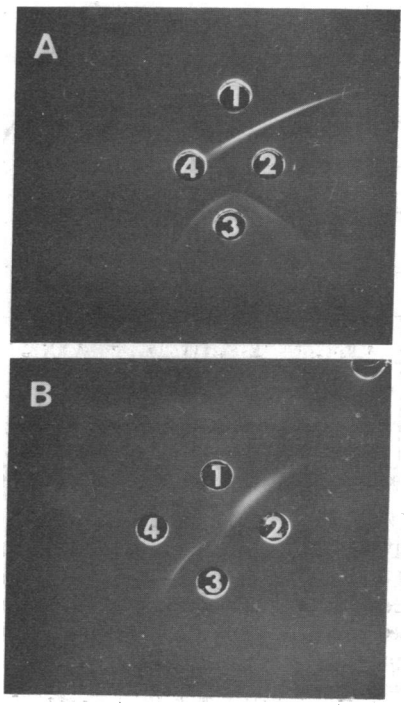

FIG. 2. Ouchterlony double diffusion in 1.15% agarose. (A) 1, Rabbit antiserum to colicin E3; 2, colicin E3 purified on carboxymethyl–Sephadex, 15 μl at 170 μg/ml; 3, antiserum to immunity protein; 4, immunity protein, 15 μl at 30 μg/ml. (B) 1, Antiserum to colicin E3; 2, colicin E3* from NaDodSO$_4$ preparative gel, 15 μl at 100 μg/ml; 3, antiserum to immunity protein; 4, immunity protein band extracted from NaDodSO$_4$ preparative gel of colicin E3, 15 μl at 10 μg/ml.

Since colicin E3 appeared to contain a component that was immunologically identical to its immunity protein, we attempted to separate the two preparatively, in order to determine whether immunity protein and the low-molecular-weight component in colicin were also functionally identical. The colicin used for these studies had been subjected to chromatography on DEAE–Sephadex and carboxymethyl–Sephadex, both of which should tend to separate molecules with as distinct ionic properties as colicin and immunity protein [colicin E3 has a pI of 6.6 (12) and immunity protein has a pI of 4.3 (10)]. It was, therefore, apparent that the immunity protein component was rather tightly bound to the colicin. Chromatography of purified colicin E3 on Sephadex G-75 in the presence of 1 M propionic acid, 1 mM dithiothreitol (20) also failed to yield colicin free of bound immunity protein. Electrophoresis on 10% polyacrylamide gels without NaDodSO$_4$ also did not separate immunity protein from colicin, even in the presence of 8 M urea. Thus, fairly strong denaturing conditions failed to separate the complex.

The dissociation does not require reducing conditions, however. The complex dissociated in NaDodSO$_4$ gels in the absence of mercaptoethanol or other reducing agents, so it is unlikely that the two components are held together by disulfide bonds.

Colicin was also separated from immunity protein by chromatography in 6 M guanidine·hydrochloride (21). How-

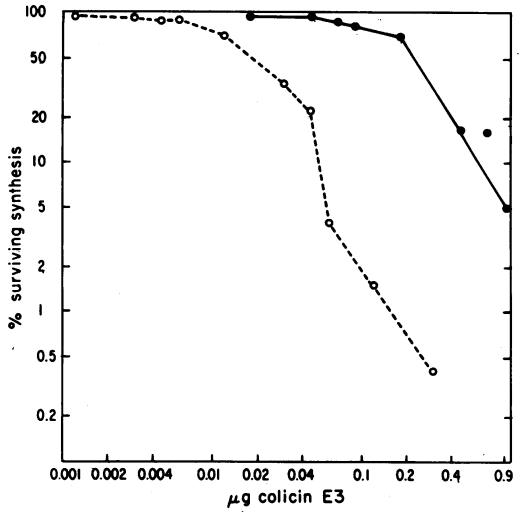

FIG. 3. Titration of activity of colicin E3* *in vitro* compared with that of colicin E3. Amounts of colicin or colicin* shown on abscissa were added to 100-μl reaction mixtures measuring f2 RNA-directed incorporation of [^{14}C]lysine *in vitro* into trichloroacetic acid-precipitable material (10). Results are expressed as the percentage of a control reaction to which no colicin was added. ●, colicin E3, purified on carboxymethyl-Sephadex, containing bound immunity protein and used as starting material for preparative $NaDodSO_4$ gels; ○, colicin E3*, extracted from preparative $NaDodSO_4$ gels and free of any detectable immunity protein.

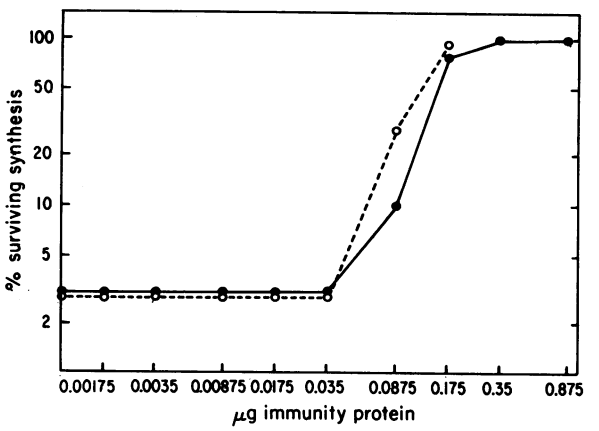

FIG. 4. Prevention by immunity protein of colicin E3-induced inhibition of f2 RNA-directed protein synthesis *in vitro*. The amounts of E3 immunity protein shown on the abscissa were added to 100 μl of *in vitro* protein-synthesizing reactions to which colicin E3 or E3* was then added. Results are expressed as the percentage of a control incorporation to which no colicin was added. ●, 1.8 μg of colicin E3, purified on carboxymethyl-Sephadex (containing bound immunity protein); ○, 0.12 μg of colicin E3*, extracted from preparative $NaDodSO_4$ gels. The amounts of colicins E3 and E3* used were chosen to be the minimum amount required for maximum inactivation of protein synthesis *in vitro*.

ever, we were unable to renature either protein after the chromatography, so the method proved unsuitable for preparative purposes. In addition, the two components of purified colicin E3 separate during diffusion at the low concentrations used on Ouchterlony double-diffusion slides. Thus, a mixture of antiserum against immunity protein with antiserum against colicin reacts with colicin to give two precipitin bands, one at the position of immunity protein and one at the position of colicin (results not shown).

TABLE 1. *In vitro activity of colicin E3* and immunity protein from preparative $NaDodSO_4$ gels of colicin E3*

E3, μg	E3*, μg	Immunity protein, μg	Immunity protein from gel of E3, μg	% Incorporation
0	0	0	0	100
4.8	0	0	0	1
4.8	0	0.87	0	94
4.8	0	0	0.25	80
0	0.1	0	0	2
0	0.25	0	0	0.5
0	0.25	0.87	0	85

The additions shown were made to 100-μl reaction mixtures measuring f2 RNA-directed incorporation of [^{14}C]lysine into protein *in vitro* (10). Appropriate controls (not included in Table) were done to determine that the effects seen in these experiments were not due to the presence of $NaDodSO_4$ in the gel-purified samples. $NaDodSO_4$ had no effect on the *in vitro* system at concentrations of 0.005% or below those used for these experiments. 100% incorporation was 61,191 cpm.

Colicin E3 that was free of any detectable immunity protein was prepared by preparative electrophoresis on both 5 and 10% polyacrylamide–$NaDodSO_4$–phosphate gels. (This gel-purified colicin will hereafter be referred to as colicin E3* or colicin*.) The two proteins, of molecular weights 60,000 and 10,000, resolved well on these gels; in fact, on the 5% gel, immunity protein migrated ahead of the bromophenol blue dye marker. When the colicin band extracted from a preparative gel was analyzed on a $NaDodSO_4$ slab gel, it appears to be free of immunity protein (Fig. 1, slot 2). This colicin E3* preparation was also free of material that reacted with antiserum against immunity protein (Fig. 2*B*). The immunity protein band extracted from these gels migrated upon electrophoresis like normal immunity protein (Fig. 1, slot 4), and reacted with antiserum against immunity protein (Fig. 2*B*).

Attempts to remove the $NaDodSO_4$ from the extracted proteins by standard procedures (19) were unsuccessful. Nevertheless, both the colicin E3* and the low-molecular-weight protein isolated by preparative gel electrophoresis had normal activity *in vitro* when stored in the buffer in which they were extracted. Colicin E3* was active in inhibiting f2-directed protein synthesis *in vitro*, and the smaller protein prevented the inactivation of ribosomes by colicin E3 (Table 1). Thus, by these criteria, the protein that is tightly bound to colicin E3 is normal, active immunity protein, indistinguishable from the free immunity protein that can be purified from colicinogenic cells.

Colicin E3 has Enhanced Activity In Vitro after Removal of Immunity Protein. Immunity protein has been shown to be active only in preventing the inactivation of ribosomes by colicin E3 *in vitro* (10). When added to colicin with colicin-sensitive cells, it has no effect on the colicin's ability to kill the cells. Presumably, there are no receptors for immunity protein on the cell surface, so that only the colicin is able to

penetrate to reach its target, the ribosomes. Since immunity protein has no effect on the level of colicin activity *in vivo*, but does inhibit its activity *in vitro*, it seemed likely that colicin E3* should have enhanced activity *in vitro* relative to its activity *in vivo*. When the inhibition of protein synthesis by colicin E3* *in vitro* was compared with that of colicin (that contains bound immunity protein), the gel-purified colicin E3* activity was at least ten times greater than the same amount of colicin E3 (Fig. 3). This same gel-purified colicin* is 50 to 500 times less active than the same amount of starting material, when assayed *in vivo* by the spot-test method (12). The implications of these findings will be discussed below.

While removal of the immunity protein from colicin enhanced its activity *in vitro*, it did not change the character of that activity, in that it was still preventable by purified immunity protein. Thus, the curves for the neutralization of colicin activity *in vitro* by added immunity protein are virtually identical for colicins E3 and E3* (Fig. 4). Although the amounts of colicin or colicin* needed to inhibit protein synthesis to a particular level were vastly different, the amount of immunity protein needed to relieve that level of inhibition was the same for both types of colicin.

Stoichiometry of Colicin-bound Immunity Protein. The molar ratio of immunity protein to colicin in purified colicin E3 was shown to be approximately one to one in colicin labeled *in vivo* with [^{3}H]leucine. The [^{3}H]colicin was purified on DEAE–Sephadex and then subjected to electrophoresis on $NaDodSO_4$ gels (Fig. 5). The number of counts in the colicin* and immunity protein peaks was integrated and normalized for the known fractional leucine contents of the two proteins (10, 12) and for the 6-fold difference in their molecular weights. By this criterion, there is about one molecule of immunity protein per molecule of colicin. Estimation of the amount of immunity protein in the colicin, as detected by the end-point of precipitation with antiserum against immunity protein on double-diffusion plates, gave a comparable figure. Interestingly, colicin E3 precipitates with antiserum against immunity protein at a lower concentration than with antiserum against colicin.

DISCUSSION

In these experiments, we have demonstrated that highly purified colicin E3 contains a stoichiometric amount of its immunity protein, bound in a complex that can only be dissociated under strongly denaturing conditions. These proteins are apparently held together only by weak forces, since neither proteolytic enzymes nor reducing agents are required for their separation. The bound immunity protein, when freed, does not differ, either immunologically or in activity, from the free immunity protein that can be isolated from colicinogenic cells. However, the bound immunity protein may be in a conformation different from free immunity protein, since colicin fails to stimulate measurable production of antibody that reacts with immunity protein. Removal of the bound immunity protein from the colicin greatly increases the activity of the colicin in its *in vitro* inactivation of ribosomes. The bound immunity protein thus appears to serve to inactivate the colicin in the complex.

The formation of a tight complex between colicin E3 and immunity protein seems to be an extremely efficient means by which colicinogenic cells protect themselves from the lethal effects of their own product. The immunity protein acts as a primitive antibody within the colicinogenic cell, probably binding specifically to the colicin as it is synthesized. Several moles of "free" immunity protein can be purified from colicinogenic cells per mole of colicin–immunity protein complex, so the cells appear to protect themselves by synthesizing an excess of immunity protein. Interestingly, we have never detected any "free" colicin at any stage of purification of the antibiotic.

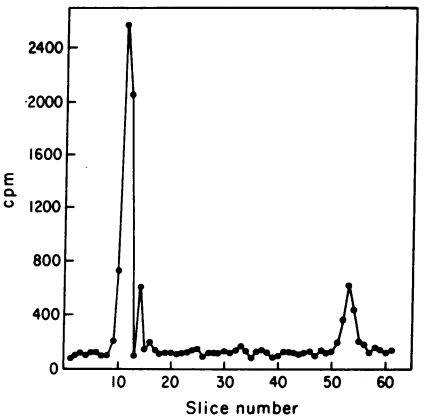

FIG. 5. $NaDodSO_4$–polyacrylamide gel pattern of colicin E3 synthesized *in vivo*. [^{3}H]Leucine-labeled colicin E3 was prepared as described in *Materials and Methods*. About 20,000 cpm of labeled colicin was applied to the gel.

Bound immunity protein is probably shed from colicin as the colicin attaches to the specific receptors on sensitive cells, or as it passes through the membrane. Thus, immunity protein can protect only those cells in which it is synthesized or ribosomes to which the colicin has been added *in vitro*. In fact, it is possible that the bound immunity protein may stabilize the colicin or facilitate its attachment to receptors on sensitive cells, since we found that the activity of a given amount of colicin E3* *in vivo* was greatly reduced relative to the same amount of colicin E3. If immunity protein does play a role in the attachment of colicin, then colicin would be somewhat analogous to diphtheria toxin, which blocks protein synthesis in eukaryotic cells by enzymatically inactivating the elongation factor, EF-2. Diphtheria toxin, like colicin, is one of the few proteins known to penetrate cells. To be active *in vitro*, the toxin must be proteolytically cleaved into two fragments. One of these fragments carries the enzymatic activity while the other, when attached, masks that activity *in vitro*, although it is necessary for the entrance of the enzymatic fragment into sensitive cells (22).

It is also possible that the reduced *in vivo* activity of colicin* eluted from $NaDodSO_4$ gels is simply a true reflection of the number of viable molecules that survive the rather harsh preparative procedure. If that is the case, then the actual *in vitro* activation of a colicin preparation upon removal of immunity protein is really several orders of magnitude, rather than the 10- to 20-fold activation calculated from the data shown in Fig. 3.

The significant activation of colicin E3 *in vitro* when it is freed of bound immunity protein may mean that the colicin activity observed *in vitro* before that separation is due either to a few percent of the colicin molecules that may already be free of immunity protein or to a low dissociation constant for the complex. This may also explain why different batches of highly purified colicin E3 (i.e., colicin–immunity protein

complex) have widely different activities *in vitro*, while their activities *in vivo* are almost the same for the same amounts of protein (unpublished observations). Different batches of the colicin may have slight differences in their bound immunity protein contents, and those small, undetectable differences could result in very different activities *in vitro*. Although gel-filtration of purified colicin E3 in propionic acid did not yield colicin that contained measurably less immunity protein, that colicin had a several-fold greater specific activity *in vitro*, while its activity *in vivo* remained constant (results not shown). The chromatography may have removed a few percent of the bound immunity protein and appreciably enhanced the activity *in vitro*.

We have described here the mechanism by which cells that produce colicin E3 protect themselves against the lethal antibiotic they are synthesizing. Colicin E2 is very closely related to colicin E3; it is also a protein of molecular weight 60,000, which shares the same cell surface receptor as colicin E3. The two proteins have similar physical properties (12), and share some common immunologic determinants. Colicin E2 produces its lethal effect by causing the breakdown of cellular DNA, but no assay has yet been described for this colicin *in vitro* (23). Nonetheless, it is fairly apparent that the cells colicinogenic for colicin E2 protect themselves in much the same way as do those carrying the related Col E3 factor. Highly purified preparations of colicin E2 also contain a low-molecular-weight protein (10), which has slightly different electrophoretic mobility and amino acid composition from the colicin E3 immunity protein. This protein is probably the colicin E2 immunity protein (unpublished results).

We have attempted to reconstitute the colicin*-immunity protein complex but have been unsuccessful thus far. Experiments of this kind have been hindered by the fact that, although colicin E3* is active in the 0.1% $NaDodSO_4$ buffer in which it is extracted, we have been unable to remove the $NaDodSO_4$ from those preparations without also losing the protein.

One possibility for the synthesis of a complex of colicin and immunity protein would be for the two to be synthesized as a single molecule and then cleaved to form a one-to-one complex. This is unlikely, since cells bearing the Col E3 factor are immune to addition of fairly high concentrations of colicin E3 (2, 24) and free immunity protein can be purified from such cells without induction of colicin (10). In addition, preliminary experiments indicate that after induction, the syntheses of colicin and immunity protein are regulated separately, with an excess of immunity protein being made at any given time.

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