Behavior of Colicins E1, E2, and E3 Attached to Sephadex Beads†

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ABSTRACT: Colicins E1, E2, and E3 were covalently attached to Sephadex G-25 beads by cyanogen bromide activation. These immobilized colicins were still active in binding to specific receptors on sensitive and tolerant cells but not to resistant cells which lack such receptors. Bound colicin E3 also retained its ability to inhibit protein synthesis in vitro. Leakage of free colicin from these coated beads was negligible. Assays sensitive to free colicin activity of 1 part in 10^7 of the bound toxin failed to detect any soluble activity. The viability of different cell types bound specifically onto these colicin-Sephadex beads was assayed by using autoradiography based on labeled amino acid uptake. Immobilized E1 killed 90% of bound sensitive cells while less than 10% of sensitive cells bound to E2 and E3 were killed in this assay. These observations agree very well with previously suggested mechanisms which propose that E1, whose target site appears to be at the membrane level, can kill sensitive cells by binding to the cell surface, but that for E2 and E3 penetration of part or all of the molecule is necessary for killing action to be observed.

Colicins are proteins produced by colicinogenic strains of *Escherichia coli* and are capable of killing sensitive cells by specific modes of action. For colicins E2 and E3, the major sites of damage are DNA and ribosomes, respectively; lesions made in these targets subsequently lead to cell death. Nomura (1964) and Luria (1964) have postulated that after the binding of colicins to the cell surface receptors, the lethal effects are transmitted to the target sites through some conformational change of the membrane. Changeux and Thiery (1967) also proposed an allosteric model for the transmission mechanism in which they suggested that the effect was propagated to the target site through some conformational change of a nearest neighbor protein molecule. However, recently two groups, Boon (1971) and Bowman et al. (1971), simultaneously discovered that direct incubation of colicin E3 with ribosomes in vitro produced the same cleavage in the 16S rRNA as observed from ribosomes isolated from sensitive cells treated with colicin E3 in vivo. This startling finding implies that penetration of part or all of the E3 molecule may be necessary for it to exert its lethal effect on sensitive cells. These same workers discovered later that this specific in vitro cleavage requires that the 16S rRNA be present in intact 70S ribosomes (Boon, 1972). The fact that such a stringent conformation is necessary led Hendler to suspect that colicin E3 may not be the nuclease involved but that its binding to the surface receptors may activate a membrane-bound ribonuclease which tends to copurify with the ribosomes upon isolation (Hendler, 1974). According to this model, penetration of colicin E3 is not necessary for the killing action. In contrast Meyhack et al. (1973) found that ribosomes purified from ribonuclease deficient strains or ribosomes whose intrinsic nucleases had been inactivated or removed by washing were still susceptible to the cleavage action of colicin E3. The question of whether the actual entry of colicin E3 is necessary for sensitive cells to be killed remains uncertain.

The same dilemma exists for colicin E2. Almendinger and Hager (1972, 1973) found that periplasmic endonuclease I plus colicin E2 causes degradation of DNA in *E. coli* spheroplasts but neither by itself will do so. Thus colicin E2 may be activating Endo I or promoting its entry into the cell interior. However, Saxe (1975a,b) reported that colicin E2 even in its most purified form introduced one single strand scission in the λ DNA supercoil in vitro. By following the effects of colicin E2 on the supercoiled DNA of phage λ, Saxe found that the in vivo degradation of λ DNA supercoils in E2-treated cells followed the pattern observed for bacterial DNA. The effect of purified E2 on isolated λ supercoils in vitro may resemble the in vivo DNA degradation process implying a direct contact of colicin E2 with the bacterial genome.

Colicin E1 partially shares receptor sites with colicins E2 and E3. Penetration across the membrane is probably not necessary for E1 to exert its killing effect since the major lesion it produces appears to be at the membrane level (Fields and Luria, 1969). Evidence from fluorescence studies suggests that colicins E2 and E3 operate differently from E1. Addition of E1 to sensitive cells containing either of the dyes, 8-anilino-1-naphthalenesulfonate or *N*-phenyl-1-naphthylamine, caused a dramatic increase in fluorescence of the membrane bound probe whereas addition of excess colicin E2 or E3 failed to promote a similar effect (Phillips and Cramer, 1973a,b). This difference in response to E1 and E2 and E3, however, is not sufficient to show definitively whether or not E1 has to penetrate through the membrane in order to kill the sensitive cells.

In this paper we report on the properties of these colicins when they are attached to Sephadex beads thus preventing entry into the cell interior. Careful attention has been paid to eliminating both free colicin and leakage of bound material from the beads.

Materials and Methods

Bacterial Strains and Media. All strains of bacteria used

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received August 4, 1975.* This work was supported by funds of Yale University and initially by Grant GM-12006 and subsequently by Grant GM-21714 from the National Institutes of Health. A preliminary report of this work was presented at the 59th Meeting of the Federation of Societies for Experimental Biology, 1975, at Atlantic City, N.J., Abstract No. 2557.

in this study were obtained from the *E. coli* Genetic Stock Center of Yale University through the courtesy of Dr. Barbara Bachmann. The E colicins were prepared from W3110 (Col E1), W3110 (Col E2), and W3110 (Col E3) described by Herschman and Helinski (1967a,b). The sensitive strain used to assay the colicins was wild type K-12 (Bachmann, 1972). A592 (the tolerant strain), A586 (the strain resistant to E1), and A593 (resistant to E2 and E3) are all described by Nagel de Zwaig and Luria (1967).

Casamino acids and yeast supplemented salt medium as described by Jakes (1974) were used for cell growth. Cells used for [^{3}H]leucine incorporation were grown in leucine deficient medium as described below.

Materials. Sephadex G-25 beads (medium) were obtained from Pharmacia Fine Chemicals, cyanogen bromide was from Aldrich, [^{3}H]leucine and [^{14}C]phenylalanine were from New England Nuclear, and NTB-2 emulsion for autoradiographs was from Eastman Kodak.

Colicins. Colicins E1, E2, and E3 were purified to homogeneity essentially according to the method of Herschman and Helinski (1967a,b). As described by Konisky and Nomura (1967), the initial extraction of the colicins from whole cells was carried out with 1 M guanidine hydrochloride in place of 1 M NaCl. Their procedure of shaking overnight at 37 °C was also used in place of the homogenization in a Waring Blendor. These changes may have affected the amount of immunity factor extracted. Purification was carried out through both the DEAE-Sephadex and CM-Sephadex column steps. The final materials gave a single major band on sodium dodecyl sulfate gels with an apparent molecular weight of about 60 000. A faint band was seen occasionally near the position of the tracking dye and probably represented residual immunity factor. The amount of this faint fast moving band varied from preparation to preparation. The E3 preparation produced the specific cleavage fragment in rRNA, as shown by a band moving somewhat faster than the 4S RNA material in gel electrophoresis. These RNA gels showed no evidence of any other nucleolytic activity.

Later in the study the E3 immunity factor was purified according to the method of Jakes (1974).

Activation and Coupling of Colicins E1, E2, and E3 to Sephadex G-25 Beads. The method used was essentially that of Cuatrecasas (1970). All E type colicins were readily coupled onto CNBr activated beads. Sephadex G-25 was used instead of the commonly employed Sepharose in order to minimize entry of colicin molecules into the interior of the beads and thus to lower nonspecific binding. Thoroughly washed Sephadex G-25 beads were mixed with an equal volume of water. Finely divided cyanogen bromide, 100 mg/ml of packed Sephadex, was added all at once to the stirred suspension. Stirring had to be gentle to avoid breaking the beads. The pH of the suspension was raised to and maintained at pH 11 by continuously adding 5 M NaOH. Pieces of ice were dropped into the suspension to maintain the temperature at about 23 °C. When the pH became constant at 11, the suspension was transferred quickly to a Büchner funnel and washed with ice-cold 0.01 M potassium phosphate buffer (pH 7). Colicins E1, E2, and E3 (10 mg/ml) were added separately into aliquots of the packed Sephadex in a volume equal to that of the Sephadex. The suspensions were stirred gently for 5–6 h at 4 °C.

After coupling, the Sephadex beads (5 ml) were washed extensively as follows: wash 1, 1 l. of 0.01 M potassium phosphate buffer (pH 7) over a 2-h period; wash 2, 1 l. of 6 M urea over a 3-h period; wash 3, 1 l. of 0.01 M potassium phosphate buffer (pH 7); wash 4, the suspension was then transferred to a beaker containing 200 ml of 6 M guanidine hydrochloride and was stirred gently for 2 h; wash 5, the suspension was transferred back to the Büchner funnel and washed with 2 l. of 0.01 M potassium phosphate buffer. The whole washing procedure was carried out at 4 °C. This strenuous washing was necessary to reduce colicin leakage below the level of detectability.

The initial samples of E1, E2, and E3 in soluble form were placed in 6 M guanidine hydrochloride for 2 h. These solutions were then either dialyzed against the same potassium phosphate buffer used above or were diluted out directly in this buffer. No loss of colicin activity was noted in any of these soluble samples. It is assumed that whatever effects on activity, if any, the denaturing solvent may have, such effects are reversible on removal of the guanidine hydrochloride.

When 100 mg of CNBr/ml of packed Sephadex was used in the activation step, about 0.8–1.5 mg of colicin/ml of Sephadex was firmly coupled as estimated by amino acid analysis of the thoroughly washed beads. Coupling was done at pH 7 to minimize the number of attachment sites per molecule in order that perturbation of the structure of the bound protein would be minimal (Cuatrecasas, 1969). The amino acid analyses show that the bound colicins lost about 5–10% of their lysine residues. The specific activities of the free colicins, about 10^6 killing units/mg, in the coupling buffer remained constant before and after the coupling reaction indicating no inactivation of the soluble molecules due to exposure to the CNBr treated beads. Usually the activated beads looked more distorted than untreated beads, probably due to CNBr treatment and the rough washing procedure.

In Vivo Assays for Free Colicin Activity. Three assays were used: (1) a spot assay, (2) a survival assay, and (3) a radioactive amino acid incorporation assay.

In the spot assay, a nutrient agar plate was seeded with 10^8 sensitive cells and 10 μl each of serial tenfold dilutions of a stock colicin solution were spotted on the plate; the highest dilution of colicin which still gave a visible zone of inhibition of growth was used as a measure of killing units of the colicin.

In the survival assay, a fixed amount of cells, 5×10^8/ml, was incubated with different amounts of the colicin at 37 °C for 15–20 min, the cells were plated out and the number of colonies was counted.

In the incorporation assay, sensitive cells were grown up in a leucine deficient medium (all amino acids except leucine were added at 10 mM to the regular medium in place of the casein hydrolysate). Various concentrations of colicin were added to the log phase cells growing in a water bath shaker at 37 °C. Five minutes later [^{3}H]leucine was added and incorporation of [^{3}H]leucine was measured as a function of time by counting the Cl_3CCOOH insoluble precipitate.

In Vitro Amino Acid Incorporation Assay for Both Free and Bound Colicin E3. S30 and S100 extracts for protein synthesis have been described by Webster et al. (1967). Aliquots of free or bound colicins in a volume of 5–12 μl were added to tubes containing TM buffer (50 mM Tris-HCl (pH 7.8), 15 mM magnesium acetate, and 5 mM NH_4Cl), 3 mM ATP, 0.2 mM GTP, 10 mM phosphoenolpyruvate, 2.5 mM glutathione, 20 μg of phosphoenolpyruvate kinase, all amino acids except phenylalanine at 0.1 mM, and 5 A_{260}

Table I: In Vitro Activity of Sephadex-E3 Beads.

Sample Volume added to 50 μl of [^{14}C] Phenylalanine Incorporation Mixture (μl)	[^{14}C] Phenylalanine (cpm) in Cl_3CCOOH Insoluble Precipitate from Incorporation Mixture + the Following Samples			
	Soluble Colicin E3 (1 mg/ml)	Packed Beads of Sephadex-E3 Containing 1 mg of Colicin/ml of Beads	Packed Beads of Sephadex Coupled to Bovine Serum Albumin	Filtrate from Sephadex-E3 Beads[a]
0	2500	2600	2400	2800
3	1200	2000	2600	2710
5	700	1800	2400	2720
10	600	1600	2400	2780
15	600	1100	2300	2700
15[b]	2300	2450		

[a] After incubation of the Sephadex-E3 beads with ribosomes, the mixture was filtered and the filtrate concentrated and mixed with a fresh 50 μl of incorporation mixture to test for solubilized colicin. [b] Sample to which a slight excess of immunity factor was added to the incorporation mixture before addition of the colicin sample.

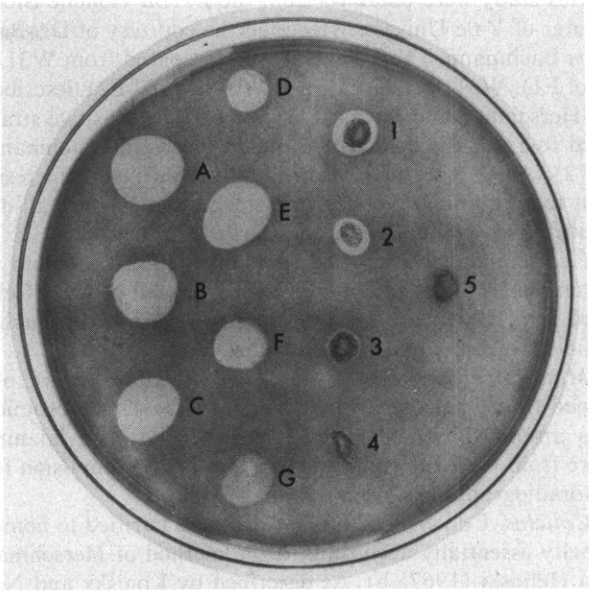

FIGURE 1: Tests for colicin leakage from bead samples. Colicin E3 was coupled to Sephadex beads as described in Materials and Methods. Aliquots of 10 μl of packed beads were removed at various steps and spotted on a lawn of sensitive cells. The numbers 1 through 5 correspond to samples taken after steps 1 through 5 of the washing procedure. Aliquots, 10 μl, of dilutions of a stock solution of the same colicin, 1 mg/ml, were also spotted. A through G represent serial 10× dilutions starting at 10^{-1} and going to 10^{-7}. It can be seen that there is no visible clear ring around sample 5 which thus presumably has less free diffusible colicin than is represented by spot G.

units of S30; the total volume was around 50 μl. After a 20-min incubation at 37 °C, 0.05 mM of [^{14}C]phenylalanine (405 mCi/mmol) and 5 μl of poly(U) (3 mg/ml) were added and the mixture was further incubated at 37 °C for 20 min. The mixture was precipitated with 10× the volume of ice-cold Cl_3CCOOH (5%) and the precipitate was filtered on Whatman 3 MM paper and counted in a liquid scintillation counter. The results of these incorporation assays are given in Table I. A volume of 12 μl of beads, containing about 12 μg of E3, gave about 50% inhibition. The considerably higher concentration of immobilized colicins required to give the same degree of inhibition as free colicin is probably due to the limited accessibility of the immobilized colicins to the ribosomes and possibly some chemical inactivation due to coupling. Beads coupled to either bovine serum albumin or ethanolamine did not inhibit protein synthesis. Immunity factor which inhibits the in vitro ribosomal inactivation activity of free colicin E3 also prevented immobilized E3 from inactivating the ribosomes.

With the bead-immobilized colicin samples, after incubation at 37 °C, an aliquot was removed, the beads were filtered, and the filtrate was concentrated by lyophilization. The residue was mixed with another 50 μl of incorporation mixture to test whether free colicin was released from the beads during the initial incubation step. No in vitro E3 activity could be detected in any of the filtrates, Table I last column. Thus the observed activity of the beads was due to the immobilized colicin.

Detection of Leakage from Colicin Loaded Beads. A suspension of the bead sample, 10 μl, was spotted on an agar plate seeded with a layer of sensitive cells. On the same plate, 10 μl each of tenfold dilutions of a known concentration of colicin were also spotted. An estimate of the amount of free colicin released from the beads was determined from the ring of inhibition of growth around the beads.

For most of our preparations of either E1, E2, or E3, less than 10^{-6} μg of colicin would still give a visible spot. For beads from the final wash step a 10-μl aliquot, containing a total of about 10 μg of colicin, did not give a detectable ring of inhibition of growth. Figure 1 shows the test for leakage of free E3. After the final wash the leakage must thus be less than 1 part in about 10^7. This maximum figure applies to the results obtained in separate experiments with beads coated with E1, and E2, as well as E3.

The spot test for solubilized colicin activity puts an upper limit on the amount of free colicin that exists in the bead preparations at the start of the cell attachment step discussed below. It does not completely rule out the possibility that attached *E. coli* cells may produce proteolytic enzymes which release active colicin fragments or other enzymes which destroy the Sephadex matrix to which the colicin is attached. Generation of free colicin activity in this manner was tested for by incubating beads saturated with sensitive cells with a known concentration of viable unattached sensitive cells. After a 2-h incubation period, the viability of the free sensitive cells was again assayed, any free colicin liberated from Sephadex by *E. coli* would have killed the sensitive cells and decreased the number of surviving cells in the mixture. The plating efficiency of the free sensitive cells was found to be the same before and after incubation with the loaded beads. Thus no soluble activity appeared to be generated by the interaction of Sephadex-bound colicin with sensitive cells.

Attachment of Cells to Coated Beads. Sephadex beads, coupled to either the appropriate colicin or bovine serum albumin as described above, were added to log phase sensitive cells and the mixture was incubated with shaking at 37 °C for 60 min. The beads were recovered from this mixture by centrifuging for 5 min in a clinical centrifuge. The beads were washed by resuspension and centrifugation from about 10 volumes of a fresh sample of the growth medium. This washing step was repeated until few or no cells could be seen on the bovine serum albumin coated control beads. Usually two or three washes were sufficient. Exactly the same procedure was followed with samples of tolerant or resistant cells. In each case with the colicin-coated beads, the

sensitive and tolerant cells showed substantial binding while the resistant strains showed little or no binding and were indistinguishable from the controls (compare, for example, Figure 2a and b).

Bead samples destined for use in autoradiography studies were grown and carried through the washing steps described above in a growth medium lacking leucine. They were then transferred to a flask with the same medium supplemented with [^{3}H]leucine. After another 30 min of incubation, incorporation was stopped by dilution in cold medium and the wash steps were repeated to remove the unabsorbed label.

For visualization in the light microscope the beads were spread on an acid-washed microscope slide and were fixed to the slide by drying slowly on a hot plate. No fixative was used. A 0.1% crystal violet solution (pH 7) was used for staining. The cells appear light blue against the whiter background of the beads. The very large size of the beads (150 μ) made photography difficult because of the shallow depth of focus of the microscope. Only a small part of the surface is correctly in focus at any one setting of the microscope. However, the entire top hemisphere of the bead is easily surveyed when operating the scope by hand and the cells were readily counted. Cells on the bottom half of the bead were effectively invisible and were not counted.

Assay for Survival of Cells Bound to Colicin-Coated Beads. Autoradiography. The incorporation of [^{3}H]leucine was used as evidence of protein biosynthesis. This in turn served to indicate cell viability. In principle the beads with attached cells which had been processed through the amino acid uptake procedure could be counted by standard scintillation procedures. However, the actual number of counts per bead would be very small due to the relatively small number of cells per bead and the large surface to volume ratio. The autoradiographic technique was chosen instead. Although more tedious, this procedure has the advantage of directly visualizing the distribution of dead and viable cells and, in principle, of giving at least some evidence of low incorporation levels if such a class of cells were to occur. (In fact the results appear to indicate that the cells are either normally active in incorporation or totally dead as far as this assay is concerned.) Previous work had already shown that the receptors on dead cells can bind colicins as well as living cells of the same strain (Shannon and Hedges, 1973).

Tests on [^{3}H]leucine incorporation in *E. coli* exposed to E1, E2, or E3 in soluble form showed that for all three colicins amino acid incorporation stopped after about 30 min of incubation in spite of the very different mechanisms of action involved. As noted above, all samples being checked for colicin action in this study were incubated for 60 min before the amino acid incorporation test was carried out.

To test for radioactivity, beads were plated on a microscope slide as described above. NTB-2 emulsion was melted in the dark in a 45 °C water bath. Distilled water was added to give a 1:1 dilution. The emulsion was poured into a dipping jar sitting in the 45 °C bath. The slides were dipped serially into the emulsion and were then placed in a slide holder in a vertical manner for an hour or until the slides were thoroughly dry. They were then put into a black plastic box with a few grains of silica gel and stored at 4 °C for exposure, 8–10 days. The slides were developed by dipping into D-19 solution for 2 min, 1% acetic acid for 30 s, Kodak fixer for 3 min, running distilled water for 30 min, dried, and stained with crystal violet as above. The silver grains indicating the presence of [^{3}H]leucine can usually be associated with a single cell or at most a small clump of cells. By looking at stained preparations of these autoradiographs under the light microscope, the cells with and without a silver grain cover can be distinguished and counted. The mean percentage survival can thus be estimated (Figure 2). Due to the huge size of the beads, even distribution of stain or emulsion is difficult to attain. Very often, high background is observed at the edge of the beads probably due to spreading and strain in the emulsion on drying. The thick emulsion also tends to flatten the beads so they look less spherical than the ones that had not been subjected to emulsion dipping. Sometimes the top of the beads tend to acquire more of the stain than other parts. These artifacts, however, do not prevent one from distinguishing dead cells from living ones.

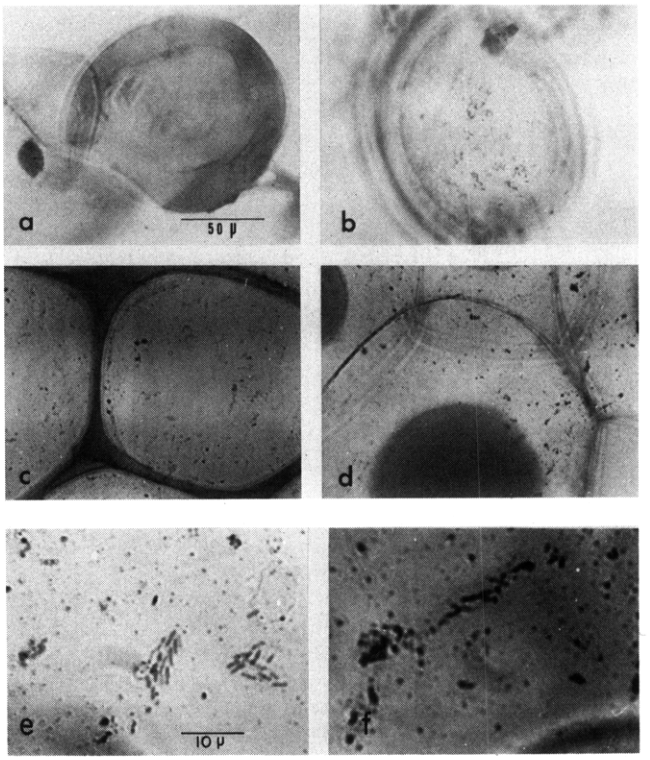

FIGURE 2: Photomicrographs of examples of stained *E. coli* cells on Sephadex beads. For a and b the beads were not covered with the autoradiographic emulsion and the beads are still roughly spherical. (a) Sephadex-bovine serum albumin bead after exposure to cells followed by washing and staining; (b) Sephadex-E1 bead after exposure to sensitive cells followed by same washing procedure as in a. For c–f the beads were coated with photographic emulsion, exposed, developed, and stained to visualize both cells and silver grains indicating [^{3}H]leucine uptake. (c) Sephadex-E1 beads with bound sensitive cells; (d) Sephadex-E3 beads with bound sensitive cells; (e) higher magnification showing a part of a Sephadex-E1 bead with attached heat killed sensitive cells; (f) a Sephadex-E2 bead with absorbed tolerant cells with the large number of silver grains showing clear viability. Panels a–d are all at the same magnification with the scale shown in a. Panels e and f have the higher magnification with the scale shown in e.

In Figure 2c is shown a bead with sensitive cells binding to colicin E1. Relatively few cells are covered with silver grains and the vast majority of the cells are dead. In Figure 2d parts of several beads are shown with sensitive cells bound to colicin E3. Here almost no cells are seen without silver grains and we conclude that they are largely viable.

In each experiment about 100–150 beads were examined and the numbers summed to produce the averages listed in Table II. The percent survival on E2 and E3 beads was

Table II: Binding and Killing Activities of Sephadex-Colicin Beads.

Sephadex-Colicin	Cells	Average No. of Cells Counted per Bead[a]	Average No. of Cells Covered with Silver Grains per Bead	Cell Survival (%)
Sephadex-E1	Sensitive	200	20	10
	Tolerant	150	140	93
	Resistant	20	18	90
Sephadex-E2	Sensitive	220	200	91
	Tolerant	160	155	97
	Resistant	25	23	92
Sephadex-E3	Sensitive	230	205	89
	Tolerant	160	155	97
	Resistant	27	25	93
Sephadex-E1[b]	Heat killed sensitive cells	205	20	10
Sephadex-E1[b]	Colicin saturated sensitive cells	30	28	92
Sephadex-bovine serum albumin	Sensitive	30	30	100

[a] Note that slightly less than ½ of the surface of each bead is visible in the microscope. Thus the total number of cells bound to each bead is about 2–2.5 times the value given in this column. [b] Essentially the same numbers were obtained in equivalent experiments with E2 and E3 beads.

about 90% while the E1 beads were 10% or less. Because of this very large difference no statistical analysis seemed necessary. All control runs with dead or inactivated cells indicated about 10% viability. This number is thus probably the background level obtained with this autoradiographic emulsion and the true percent survival with the E1 beads is undoubtedly much lower.

In order to confirm the validity of this technique, two control experiments were carried out in parallel. Tolerant cells, which are resistant to the killing effects of colicins but can still bind to them, were absorbed onto the three different colicin substituted beads and autoradiographs identical with those of sensitive cells were prepared. As an example, see Figure 2f. Practically all the cells were covered with silver grains indicating viability as expected. Conversely cells which have been killed either by heat treatment or by actinomycin D prior to absorption should show little or no incorporation and, in fact, were found to be essentially free of grains, Figure 2e.

Discussion

The release of free ligand into solvent after CNBr coupling to the solid matrix has been a problem in the application of affinity chromatography. A model study performed in ligands coupled to agarose and stored at 4 °C for 30 days suggested that about 0.1% of coupled alanine was released per day (March et al., 1973). Serious leakage problems have been observed with Sepharose-insulin (Takami and Topper, 1974) and Sepharose-growth hormone (Bolander and Fellows, 1975). In the present case, after going through the exhaustive washing procedure, the Sephadex-colicin beads showed no detectable leakage over a period of 2–3 weeks when stored at 4 °C. This extra stability may be due to multiple sites of attachment of the molecule to the polymer. Amino acid analysis showed that the three different colicins lost about 5–10% of their lysine residues after coupling. Thus more than one lysine per molecule is involved in binding to the bead matrix. Recently it has been shown that use of polymeric spacers such as polylysylalanine which can form multiple attachments can prevent leakage of the bound ligands. Stable attachment of other protein molecules like interferon to Sepharose beads by CNBr has been observed (Ankel et al., 1973). Furthermore, the fact that all three E colicins are monomers in solution makes the chances of noncovalent attachment to the beads much less than it may be for multimeric proteins.

The number of beads per milliliter of packed Sephadex sample was estimated by use of a hemocytometer. The average number was 2×10^5 beads/ml. From the known mass of bound colicin, about 1 mg/ml of beads, and a molecular weight of 60 000, the number of colicin molecules per bead can be estimated as about 5×10^{10}. With about 500 cells per bead, the total number of colicin molecules per bound *E. coli* cell is thus of the order of 10^8. The probable number of colicin molecules bound per cell to observe a clear killing effect in solution is between 200 and 1000 with the higher figure a closer estimate of the numbers observed in this study. On this basis the immobilized colicins appear to have a killing efficiency of 10^5 less than that of the soluble proteins. Looked at in another way the total area of one bead is about 70 000 μ^2. The maximum contact area of one *E. coli* cell is about 2 μ^2. If the colicin is uniformly coupled to the bead surface then each cell would cover about 10^6 colicin molecules. By this estimate the killing efficiency would be 10^3 lower than the solution value. Whichever number is correct the low ratio of effective activity may not be surprising in view of (1) the accessibility problem at the molecular level at the bead surface, (2) the probable random nature of the colicin coupling reaction, (3) the possible chemical inactivation of some material, and (4) the problems of geometrical distribution on the bead of potentially active colicins required for multiple interaction with the cell receptors.

The leakage detection is sufficiently sensitive that there cannot be more than about 5000 molecules per bead or 10 per cell in uncoupled form. Since, in our assay, about 1000 molecules per cell are required for killing in solution, this amount of free colicin is not likely to influence the observed results.

Colicins immobilized by the tight coupling to the bead matrix produced by the CNBr activation procedure are not long enough to penetrate through the *E. coli* double membrane. The receptors are known to be principally located in the outer membrane and thus the binding of sensitive cells to beads coated with the corresponding colicin is not surprising. The further action of the colicins, however, is now physically restricted to be at or close to this outer membrane. E1 inhibits active transport and oxidative phosphorylation and has a marked effect on the physical properties of the membrane (Fields and Luria, 1969). The killing action of the immobilized colicin found in this study indicates that this toxin does not need to move from the neighborhood of the surface receptor in order to exert its effects. This result implies, but does not prove, that there are intermediate effectors in the overall killing process.

The lack of killing by immobilized E2 and E3 is strong confirmation of the earlier work indicating that, in contrast to E1, these toxins actually must penetrate the inner membrane to exert their effects on DNA and ribosomes, respectively. The lack of killing also implies that the intact colicin molecule must move across the membrane. If proteolysis by periplasmic enzymes were involved, for example, a small ac-

tive fragment could easily have been released even from the immobilized colicin. (This possibility had to be seriously considered since one can produce such small fragments with in vitro activity in solution with a variety of proteolytic enzymes (C. Lau, unpublished results).) However, these results provide no indication of such a mechanism. The immobilization procedure seems to provide very clear answers to this positional aspect of colicin action. In this sense interpretation is perhaps more straightforward than the earlier work with trypsin rescue or the use of metabolic inhibition where killing is always seen with increasing incubation time.

Hopefully this work can be literally extended for E2 and E3 by putting the colicins on long covalently linked arms which would serve to measure the distance through the membrane that the toxins must move in order to exert their killing effects. With variable length arms it may be possible to halt the colicins at various stages of penetration and to survey the immediate protein neighbors other than the initial receptor in order to probe more deeply the process by which these macromolecules actually traverse the membrane without destroying it.

Acknowledgments

The authors thank Dr. John Cronan for suggesting the radioautographic procedure and for general advice and criticism, Dr. Joseph Gall for assistance in the radioautography, Dr. Paul Fletcher for help in the amino acid analysis, and Melody Lane and Johnnie Mouning for help in preparation of the manuscript.

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