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Interaction of Colicins with Bacterial Cells

I. Studies with Radioactive Colicins[1]

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ABSTRACT

MAEDA, AKIO (Department of Genetics, University of Wisconsin, Madison), AND MASAYASU NOMURA. Interaction of colicins with bacterial cells. I. Studies with radioactive colicins. J. Bacteriol. 91:685–694. 1966.—By use of a preparation of radioactive colicin E2, the following conclusions, which had been obtained previously from indirect experiments, were tested directly: (i) colicin stays at the receptor site on the cell and acts from there; (ii) colicins E2 and E3 share the same receptor, although their mode of action is entirely different; (iii) colicinogenic cells adsorb homologous colicin, although they are resistant to that colicin. Our experimental results confirmed the above conclusions. When sensitive cells of *Escherichia coli* K-12 pretreated with radioactive E2 were disrupted with a French pressure cell, and fractionated by differential centrifugation, it was found that most of the radioactivity stayed with the cell envelope fraction. Trypsin removed a major part of radioactivity from cells pretreated with radioactive E2, as was expected from the previous experiments on trypsin reversal of colicin action. Furthermore, it was shown that the trypsin treatment causes a recovery of the capacity of E2-pretreated cells to adsorb further colicin E2. When cells were first treated with various amounts of nonradioactive E3, and then treated with radioactive E2, the amount of radioactive E2 adsorbed was found to be inversely related to the amount of nonradioactive E3 adsorbed first. It was also shown that E2-colicinogenic cells adsorb radioactive E2, and yet are resistant to this colicin.

Previous studies on the mode of action of various colicins have revealed two major features of colicin action (7, 8, 9). First, apparently a single colicin particle is enough to kill one sensitive *Escherichia coli* cell, and the colicin particle stays at the receptor site and acts from there. Second, different colicins exert different biochemical effects on sensitive cells; i.e., colicin E2 causes DNA degradation, colicin E3 inhibits only protein synthesis, and colicins K and E1 inhibit all macromolecular synthesis, presumably through inhibition of oxidative phosphorylation.

The conclusion that the colicin stays at the receptor site and affects the biochemical targets from there was first obtained indirectly from the reversibility of colicin action by treatment with trypsin (10). To test this conclusion more directly, we prepared radioactive colicins and studied their interaction with sensitive *E. coli* cells.

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The availability of these radioactive colicins enabled us to study several other questions. Colicins in the E group (E1, E2, and E3) have been thought to have a common receptor (1). Yet, their apparent modes of action are entirely different. The conclusion about the common receptor for the colicin E group came only from an analysis of mutants resistant to a colicin in the E group. The possibility has remained that the receptors are different from each other, but involve a common compound which the resistant mutants fail to synthesize. This problem, the identity of receptor for E group colicins, has also been studied conveniently by the use of radioactive purified colicins.

The third problem which has been studied with the radioactive colicin preparation is the problem of adsorption of colicin to the homologous colicinogenic immune cells. Immune cells are resistant to the killing action of the homologous colicins, but retain receptors, as evidenced by the sensitivity of these immune cells to other colicins or to phages in the same group (1). However, the

actual adsorption of colicin to immune cells was demonstrated unequivocally only by using heat-killed trypsin-treated cells (9). Our previous attempts to demonstrate the adsorption of colicin E2 to intact viable E2 colicinogenic cells did not give a clear answer, mainly because of the high endogenous colicin production by intact colicinogenic cells. The use of radioactive purified colicin has made the direct experimental test of this problem easier.

The experimental results obtained by use of radioactive colicins have generally confirmed the conclusions deduced previously through the indirect experiments.

MATERIALS AND METHODS

Organisms. Streptomycin - resistant derivative W3110Sm^r of *E. coli* K-12 strain W3110 was used as a strain sensitive to colicins E2 and E3. Colicin E2 factor [originally derived from the *Shigella sonnei* strain P9 of Fredericq, i.e., col E2-P9 according to the nomenclature of Lewis and Stocker (4)] was introduced into this sensitive strain from *Salmonella typhimurium* LT2 cys D36 (E2) by the method described by Stocker, Smith, and Ozeki (11). The resultant E2 colicinogenic strain, 52, was used as a colicinogenic strain for preparing both radioactive and nonradioactive colicin E2 and for an adsorption experiment. This strain is resistant (immune) to colicin E2, but sensitive to colicin E1, colicin E3, and phage BF23. Mutant strains ED11 and ED15 were isolated from the E2 colicinogenic strain 52 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These mutants are defective in colicin production, but retain the immunity to E2. *E. coli* strain CA38 which is colicinogenic for both E3 and I (2) was obtained from H. Ozeki and was used for preparing both radioactive and nonradioactive colicin E3. A mutant, ER343, derived from W3110Sm^r was used as a resistant strain which is resistant to E1, E2, E3, and phage BF23.

Chemicals. Trypsin and trypsin inhibitor (soybean) were obtained from Sigma Chemical Co., St. Louis, Mo.

Media. The following media were used. TB medium contained 1.3% tryptone (Difco) and 0.7% NaCl. Tris-S solution contained 0.1 M tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 7.4), 0.08 M NaCl, 0.02 M KCl, 6.4×10^{-4} M KH_2PO_4, 1.6×10^{-4} M Na_2SO_4, 10^{-3} M $MgCl_2$, 10^{-4} M $CaCl_2$, and 2×10^{-6} M $FeCl_3$. Tris-glucose-Casamino Acids medium was Tris-S fortified with glucose (0.2%) plus 0.5% Vitamin Free Casamino Acids (Difco). M9 consisted of 0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 10^{-4} M $MgSO_4$, 10^{-6} M $FeCl_3$, and 0.4% glucose. M9-Casamino Acids medium was M9 fortified with Casamino Acids (0.1%).

Assay of the killing activity of colicins. Two assay methods were used. The first method was a "spot test." A 0.01-ml amount of suitably diluted sample of colicin was spotted on the surface of a nutrient agar plate freshly seeded with 2×10^8 stationary-phase cells of W3110Sm^r in 2.5 ml of soft nutrient agar. The colicin titer (called "units" in this paper) was arbitrarily defined as the highest dilution which gave a clear inhibition zone. The second method was the determination of the number of "killing particles" present in the sample by measuring the fraction of survivors after incubation of washed cells of W3110Sm^r with various concentrations of colicins (7, 8). This second method was more accurate, and is referred to as the "standard method" in this paper. The number of "killing particles" thus estimated may or may not be equal to the actual number of active colicin molecules (see below). To avoid misunderstanding about this point, the term "killing units" will be used in this paper instead of "killing particles," which was used in our previous papers.

Preparation of radioactive colicins E2 and E3. Initial attempts to obtain a radioactive E2 with a high specific activity by the tritium gas exposure method failed because of breakdown of the E2 during tritiation. A biological method of preparation was therefore adopted. Cells of the E2 colicinogenic strain, 52, were grown to a concentration of 5×10^8 per milliliter in M9-Casamino Acids medium, harvested, washed once, suspended in M9 (5×10^8 per milliliter), and shaken for 15 min at 37 C. A 1-ml amount of this culture was mixed with 2 ml of M9 solution containing 1 mc of H^3-leucine (5,000 mc/mmole, New England Nuclear Corp., Boston, Mass.). Mitomycin C (0.2 μg/ml) was added to the culture, and the culture was shaken for 4 hr at 37 C. Cells were then collected by centrifugation. Colicin E2 was extracted from the cells and purified by the method developed by Helinski (*manuscript in preparation*). Carrier albumin (1 mg/ml) was added to prevent a possible inactivation of colicin in a dilute solution, whenever it was necessary. The last step of the purification was chromatography on a hydroxylapatite column. The elution pattern of the chromatography is shown in Fig. 1. The fractions containing E2 activity were pooled, dialyzed against water, and lyophilized. Colicin E2 thus prepared was stable in the cold in a lyophilized state for at least several months.

C^{14}-labeled E2 was prepared in the same way except that 0.1 mc of C^{14}-*Chlorella* protein acid hydrolysate (8.6 mc/mmole of carbon, a gift from the Institute of Applied Microbiology, University of Tokyo) was used instead of H^3-leucine.

H^3-labeled E3 was prepared in a similar way. *E. coli* strain CA38 was induced with mitomycin C to produce E3. The induced culture had a very high E3 activity, but very little colicin I activity. An elution pattern of the chromatography on a hydroxylapatite column is shown in Fig. 2. The fractions containing E3 were pooled, dialyzed against water, and lyophilized. This preparation was found to contain a significant amount of radioactive materials which adsorbed to a resistant strain, ER343, and, therefore, was treated with washed cells of strain ER343 to remove such radioactive materials. The resultant preparation was used within a few days.

Preparation of nonradioactive E2 and E3. Nonradioactive E2 was prepared on a larger scale by the method of Helinski (*in preparation*). The method gives a preparation which is pure as judged by ordinary

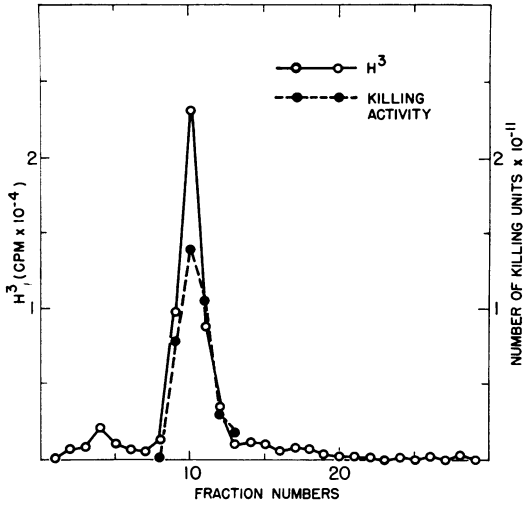

FIG. 1. *Chromatography of radioactive colicin E2 on hydroxylapatite. Samples of each fraction were analyzed for acid precipitable radioactivity, and for killing activity (by use of the standard method).*

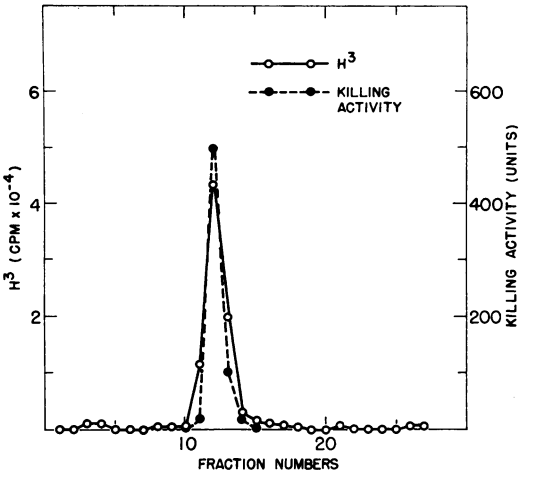

FIG. 2. *Chromatography of radioactive E3 on hydroxylapatite. Samples of each fraction were analyzed for acid-precipitable radioactivity and for killing activity (by use of the spot test).*

chemical and physicochemical criteria. Nonradioactive E3 was prepared in a similar way, but with the omission of column chromatography steps. The colicin E2 preparation used had a specific activity of 3×10^{13} to 20×10^{13} killing units per mg of protein and the colicin E3 preparation had a specific activity of 2×10^{13} to 6×10^{13} killing units per mg of protein.

Measurement of radioactivity. H^3-labeled proteins in solution were precipitated with 5% trichloroacetic acid, filtered on membrane filters (DW 2200, Millipore Filter Corp., Bedford, Mass.) or glass fiber filters (984 H, Reeve Angel Co., Clifton, N.J.), and washed with 5% trichloroacetic acid. Membrane filters were dried, and their radioactivity was measured with a Packard Tricarb liquid scintillation counter by use of a toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]-benzene system. When C^{14} labeling was used instead of H^3 labeling, the protein precipitate after trichloroacetic acid treatment was dissolved in 2 N NH_4OH and dried on an aluminum planchet, and the radioactivity was measured with a Nuclear-Chicago low-background gas-flow counter.

Measurement of adsorption of radioactive colicin to cells. Cells were grown in TB medium to 5×10^8 per milliliter, harvested, washed with Tris-S, and resuspended in Tris-S at a concentration of about 2×10^8 cells per milliliter. Radioactive colicin was added to the cell suspension and incubated. After the adsorption period, the cells were centrifuged (7,700 $\times$ *g* for 10 min), washed with Tris-S, and suspended in 5% trichloroacetic acid. The amount of radioactive colicin adsorbed to cells was determined by measuring the radioactivity of this trichloroacetic acid precipitate as described above.

RESULTS

Purity of radioactive colicine. The elution patterns of hydroxylapatite chromatography of both E2 and E3 (Fig. 1 and 2) showed that the peak of radioactivity curve coincided with the peak of the colicin activity curve. Therefore, the material was assumed to be reasonably pure. (Lack of exact proportionality between killing activity and radioactivity may be mostly due to the inaccuracy involved in the biological assay of the killing activity.) As an additional check on the purity, the adsorption of the radioactive material in the E2 preparations to both sensitive and resistant cells was tested. As shown in Table 1, about 80 to 90% of the total radioactivity was adsorbed to sensitive cells, whereas only a very small fraction (less than 6%) was adsorbed to resistant cells.

In view of the good chromatographic pattern of the radioactive material (Fig. 1), and the purity of the preparation obtained by the similar method (Helinski, *in preparation*), the unadsorbed radioactive material, which is 10 to 20% of the total radioactivity, might be a denatured colicin protein which cannot be adsorbed by the sensitive cells but retains chromatographic properties identical to those of active colicin. As already described, the radioactive E3 preparation obtained after hydroxylapatite chromatography contained a significant amount of radioactive materials which adsorbed to resistant cells. The preparation obtained after the adsorption with resistant cells showed that 60% of total radioactivity was adsorbed to sensitive cells, and only 5% to re-

TABLE 1. *Adsorption of radioactive colicins to sensitive (W3110Sm^r) and resistant bacteria (ER343)*

Prepn	Colicin (killing units/cell) used for test	H^3 adsorbed to		H^3 unadsorbed to		H^3 adsorbed†	
		W3110Sm^r	ER343	W3110Sm^r	ER343	W3110Sm^r	ER343
		count/min	*count/min*	*count/min*	*count/min*	%	%
H^3-E2 (prepn 1)*	22	525	19	144	620	80	3
H^3-E2 (prepn 2)	18	569	43	106	630	84 (87)	6
H^3-E3	18	669	57	480	1,080	59 (60)	5

* Data for preparation 1 are from Fig. 4.

† About 3% of total killing activity remained unadsorbed in the supernatant fluid. Corrections were made for these unadsorbed colicins, and corrected percentages are shown in parentheses.

sistant cells. Although the problem of a small fraction of radioactive contaminants was unsolved, especially with the preparation of radioactive E3, the fraction which was adsorbed by resistant cells was very small. Therefore, it was concluded that the colicin preparations could be used for the experiments described in this paper, which were concerned with the radioactive material adsorbed by sensitive cells.

Kinetics of adsorption. Radioactive colicin E2 was mixed with sensitive cells at a cellular concentration of 4×10^8 per milliliter, and the amount of radioactive material adsorbed at various times was measured. Figure 3 shows the results. Adsorption was found to be practically complete at 37 C within 10 min. However, adsorption was slower at 0 C. Accordingly, the incubation time selected to assure a complete adsorption was 45 min at 37 C and 2 hr at 0 C, in most of the experiments described in this paper.

Number of colicin particles that can be adsorbed by a sensitive cell. One can estimate the amount of colicin adsorbed to cells by first mixing various amounts of colicins with sensitive cells and then subtracting the amount of colicin left in the solution from the total amount initially added. By such a method Mayr-Harting (6) showed that under her experimental conditions a sensitive *E. coli* cell can adsorb a maximum of 11 killing units of E2. Under our experimental conditions, we found little killing activity left in the supernatant fluid up to a ratio of 20 to 30 killing units of E2 or E3 per bacterial cell (see Fig. 7). Therefore, the minimal number of units of colicins that can be adsorbed by a bacterium is about 20 to 30 for both E2 and E3 under the present experimental conditions.

However, the above method assumes that the sensitive cells do not have a mechanism which inactivates colicin and leaves it in the medium. If such a mechanism exists, the value would be higher than the real one. On the other hand, the accurate estimate of the amount of adsorbed colicin at the excess colicin concentration is very difficult, since the adsorbed amount, which can be estimated only by subtraction of the unadsorbed from the total, becomes a very small fraction of the total. Thus, a sensitive cell might adsorb more colicin particles at a higher concentration of colicin than those estimated by the method described above.

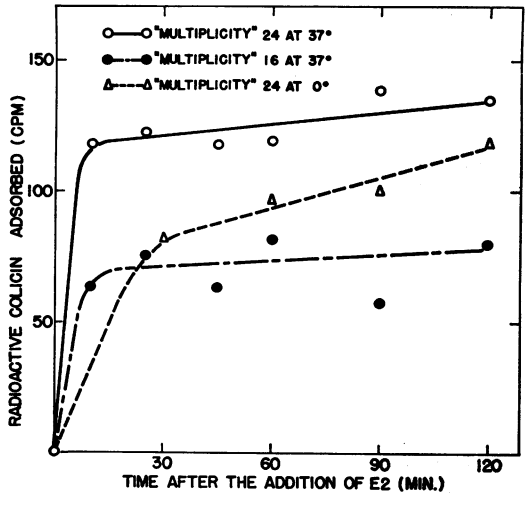

FIG. 3. *Kinetics of adsorption of radioactive colicin E2. Washed cells of W3110Sm^r were treated with E2 at "multiplicities" of 16 and 24 killing units per cell and were incubated at 37 and 0 C. At various times, samples were taken and immediately centrifuged in the cold. Cells were then washed, and their radioactivity was determined. The time necessary for the completion of the first centrifugation was less than 6 min.*

By the use of radioactive colicin, one can directly measure the amount of colicin adsorbed to a cell. Figure 4 shows an example of such experiments. Various amounts of radioactive colicin E2 were added to a constant number of cells, and the radioactivity fixed to cells was determined. It increased linearly with the increased amount of colicin, until the ratio of colicin killing units to bacterial cells became

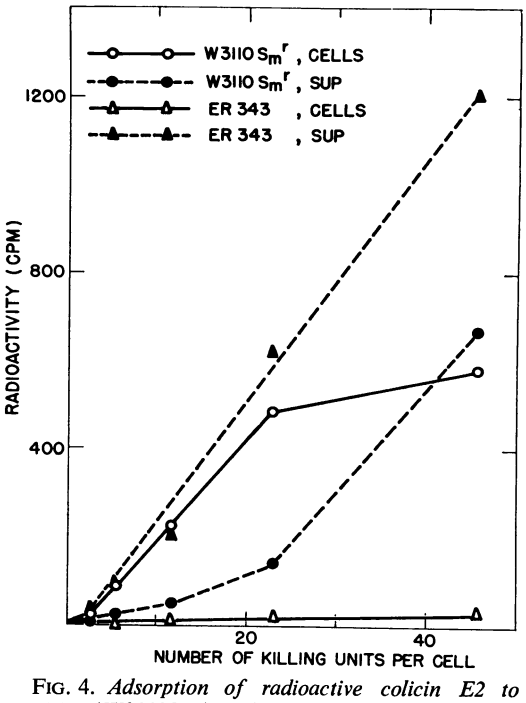

FIG. 4. *Adsorption of radioactive colicin E2 to sensitive (W3110Sm^r) and resistant bacteria (ER343). Various amounts of H^3-colicin E2 were mixed with W3110Sm^r and with ER343 and kept at 37 C for 45 min. The radioactivity in the supernatant fluid and that in the cell pellet were determined after centrifugation. The number of killing units added was calculated from the number of survivors at lower colicin concentrations measured simultaneously.*

about 20. Only a small increase in the fixed radioactivity was found after a further increase of input radioactive colicin. It was concluded that a sensitive *E. coli* cell can adsorb at least about 20 killing units of colicin E2 under the present experimental conditions.

The possibility mentioned above that more colicins are adsorbed at much higher concentrations has not been examined carefully yet, although some experiments suggested that this is the case (see Fig. 8a). This might indicate the existence of a second type of slow adsorption distinct from the first type (fast adsorption) discussed so far. However, the significance of these observations must await further experiments. Also, we noticed that the number of receptor sites [number of colicin particles (killing units) that can be adsorbed by a cell] obtained by both of the above methods varies somewhat depending on the experimental conditions, and this might explain the discrepancy between the above values of 20 to 30 and that of 11 obtained by Mayr-Harting (6).

As will be described below, one killing unit of E2 corresponds to about 100 molecules. The calculation is based on the assumption that our E2 preparation consists of pure E2 protein with a molecular weight of 60,000 (Helinski, *personal communication*). If so, the number of E2 molecules that can be adsorbed by a bacterium may be about 2×10^3 to 3×10^3.

Distribution of adsorbed radioactive colicin E2 after attachment to sensitive cells. The previous conclusion obtained indirectly, that the adsorbed colicin stays at the site of attachment, was tested directly by use of radioactive E2. Sensitive cells were treated with C^{14}-labeled E2 at 37 C for 60 min at a "multiplicity" of 1 (1 killing unit per cell). Excess cold colicin E2 (about 100 killing units per cell) was then added, and the mixture was incubated for an additional 5 min at 37 C to cover any unoccupied receptor with E2. Cells were then collected by centrifugation, washed, and disrupted with a French pressure cell. The disrupted cells were treated with deoxyribonuclease (20 μg/ml, for 3 hr at 0 C), the resultant preparation was fractionated by differential centrifugation, and the radioactivity in each fraction was measured. The results are shown in Table 2. It is clear that most of the radioactive material was with the cell envelope fraction (cell wall and cell membrane fragments), and very little free radioactive colicin (less than 1 or 2% of the total) was found in the soluble fraction. The small amount of radioactive colicin (8%) found in the ribosome fraction could be explained by the contamination of cell wall or membrane fragments in this fraction. Thus, the result agrees with the conclusion that colicin stays at the receptor site.

The treatment of C^{14}-E2-pretreated cells with the excess cold E2 before the cell disruption was done to cover any unoccupied receptor with the cold E2, and thus to prevent a possible reattachment of the hypothetical internal free C^{14}-E2 to the unoccupied receptor after the disruption of the cells. The experiment was also done without such cold E2 treatment, and essentially the same results were obtained; most of the radioactivity was found in the cell envelope fraction, and very little in the soluble fraction. The deoxyribonuclease treatment prior to the differential centrifugation degraded nearly all the deoxyribonucleic acid (DNA) to oligonucleotides which appeared in the soluble fraction after the differential centrifugation. Therefore, it was concluded that colicin E2 does not attach to DNA, a biochemical target. However, this experiment by itself does not exclude the possibility that a small fragment of DNA remains attached to the membrane, and

TABLE 2. *Distribution of adsorbed radioactive colicin E2*

Fraction*	Count/min†	Per cent
Unbroken cells and large fragments (3K5P)	7.9 ± 0.7	10
Cell wall and cell membrane fraction (12K30P)	61.5 ± 1.5	81
Ribosome fraction (40K240P)	5.9 ± 0.6	8
Soluble fraction (40K240S)	0.7 ± 0.4	1
Total	76.0	100

* A 200-ml amount of a washed-cell suspension of W3110Sm^r (2.5×10^8 per milliliter) was treated with C^{14}-labeled E2 at 37 C for 60 min at a "multiplicity" of 1 killing unit per cell, and then with an excess of cold E2. The distribution of the adsorbed radioactive E2 was examined among several fractions obtained by differential centrifugation of the disrupted cell preparation. Unbroken cells and large fragments were sedimented by centrifugation at 3,000 rev/min for 5 min (3K5P) by use of a Sorvall centrifuge SS-1 rotor. Cell wall and cell membrane fragments were next sedimented by centrifugation of the first supernatant fluid at 12,000 rev/min for 30 min (12K30P). The supernatant fluid was then centrifuged at 40,000 rev/min for 240 min by use of a Spinco model L centrifuge no. 40 rotor. The pellet (40K240P) obtained is a ribosome fraction and the supernatant (40K240S) is a soluble fraction.

† To measure the radioactivity, 50 mg of albumin was added to each fraction as a carrier, and the radioactive protein was precipitated with 5% trichloroacetic acid. The radioactivity of the precipitate was determined, and the background activity (3.0 ± 0.2 count/min) was subtracted from the observed values. The degree of self-absorption of the radioactivity was not significantly different among different fractions; therefore, the values are given in the table without such correction.

the radioactive E2 makes a complex with this DNA fragment.

Digestion of adsorbed radioactive E2 with trypsin after attachment to sensitive cells. Since the strongest evidence concerning the state of colicins after adsorption to sensitive cells is the reversibility of colicin action with trypsin treatment, our previous reasoning that the reversal is due to digestion of the colicin was tested with radioactive colicin E2.

Washed cells were treated with radioactive E2 at a multiplicity of 8 at 37 C for 5 min. The cells were then centrifuged, washed, resuspended, and divided into three tubes. Trypsin was then added to one tube (time zero). The second tube received the trypsin at 30 min, and the third tube served as a control. The addition of trypsin at the early time (5 min after E2 addition in this case) usually caused an increase of survivors from the order of 10^{-4} to the order of 10^{-1} of the original viable bacteria. This is understandable, since DNA degradation does not proceed very extensively by 5 min. As shown in Fig. 5, the trypsin gradually removed radioactive E2 from cells, and only 40% of total initial radioactivity remained after 3 hr of incubation. When trypsin was added at 30 min, the increase in survivors was usually rather low, since the irreversible breakdown of bacterial DNA took place by 30 min (see Fig. 6b). The transition from the reversible stage to the irreversible stage is not due to the penetration of E2 into the cytoplasm; the removal of radioactive colicin from treated cells was found to be as efficient as when trypsin was added at time zero. These results agree with the previous conclusion that trypsin reversal of colicin action is due to digestion of colicin protein itself.

Recovery of E2-adsorption capacity of E2-pretreated cells by trypsin treatment. If trypsin, in fact, removes a considerable part of the adsorbed colicin from the receptor site, it might be possible that the trypsin treatment causes a recovery of the

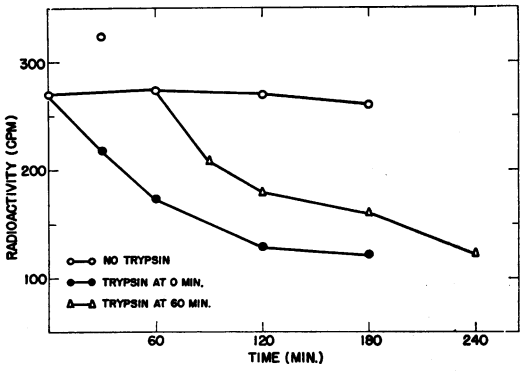

FIG. 5. *Digestion with trypsin of radioactive colicin E2 adsorbed to sensitive cells. Washed cells (2×10^8 per milliliter of strain W3110Sm^r were treated with radioactive E2 at a multiplicity of about 8 for 5 min at 37 C. Cells were then cooled by mixing with 2 volumes of cold buffer, centrifuged in the cold, washed, and resuspended in Tris-glucose-Casamino Acids medium containing chloramphenicol (CM, 50 μg/ml) at a concentration of 2×10^8 per milliliter. Radioactive cells were divided into three tubes and incubated at 37 C. The first and the second samples were treated with trypsin (500 μg/ml) at time zero and at 60 min, respectively. The third sample was not treated with trypsin. At times indicated, a 1-ml sample was withdrawn and diluted with 2 ml of Tris-S containing trypsin inhibitor (500 μg/ml). Cells were then separated from the medium by centrifugation, treated with trichloroacetic acid (5%), and collected on membrane filters. Their radioactivity was then measured.*

capacity of E2-pretreated cells to adsorb further colicin E2. This possibility was tested experimentally. Washed sensitive cells were first treated with an excess of nonradioactive colicin at 37 C for 5 min. Cells were then collected by centrifugation, washed, and resuspended in medium containing chloramphenicol (CM). Trypsin was added either at time zero or at 60 min. At intervals, samples were taken, and the capacity of the trypsin-treated cells to adsorb radioactive E2 was determined (after the treatment of cells with trypsin inhibitor), and compared with that of control cells without trypsin (Fig. 6 a). Simultaneously, colony formers were measured (Fig. 6 b). It is clear that as much as 50% of the total initial capacity was recovered by trypsin treatment in both cases (trypsin added at time zero and at 60 min). These experimental results show that the majority of E2 stays at the receptor site, and trypsin digests E2 and removes it from the receptor site. Thus, several experimental results support very strongly our previous conclusion that colicin stays at the receptor site and acts from there.

Competition between colicins E2 and E3 for a receptor site. As described in the introduction, colicins E2 and E3 have been thought to have a common receptor. This was tested directly by use of radioactive colicin. In the first experiment, sensitive cells were treated with various amounts of nonradioactive colicin E3 at 0 C for 60 min, and were then centrifuged. Unadsorbed colicin E3 was assayed by analyzing the killing activity of the supernatant fluid after centrifugation. Washed cells were then treated with an excess amount of radioactive E2, and the amount adsorbed to the cells was assayed (Fig. 7 a).

In the second experiment, cells were first treated with nonradioactive E2, and the adsorption of radioactive E3 was then examined in the same way (Fig. 7 b). Adsorption of the first colicin was performed at 0 C to minimize the possibility that the adsorbed colicin exerts some (unknown) biochemical effect on the membrane, causing a decreased efficiency of adsorption at other unoccupied receptor sites. However, preadsorption at 37 C instead of 0 C gave essentially the same results. As can be seen, the decrease in the amount of the second radioactive colicin adsorbed to the cells was found to be approximately proportional to the amount of the nonradioactive colicin added first, until the latter approached a saturation point, that is, 20 to 30 killing units per cell. The results show that the receptor for E2 and E3 are the same or are physically closely connected with each other. Thus, it is very likely that colicins E2 and E3 share a common receptor, and yet their apparent modes of action are entirely different. The specificity in biochemical action cannot be accounted for by the specificity in adsorption.

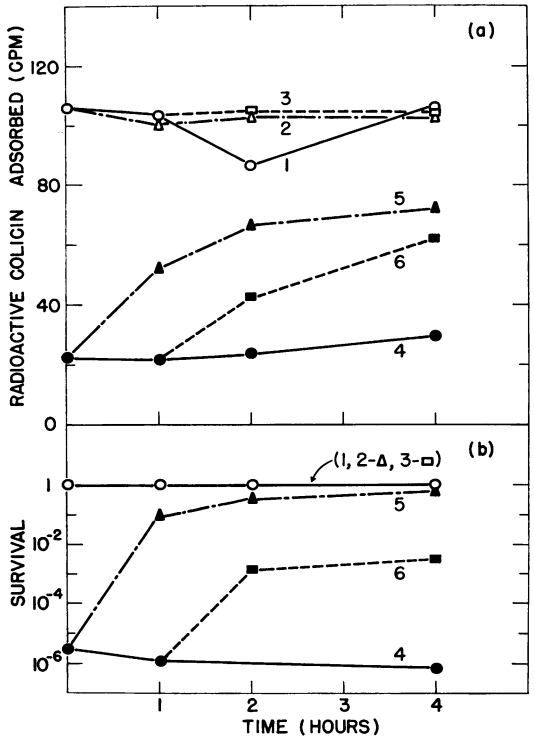

FIG. 6. *Restoration of E2-adsorption capacity of E2-pretreated cells by trypsin treatment. Washed cells (2×10^8 per milliliter) of W3110Sm^r were treated with excess nonradioactive colicin (about 2,000 killing units per cell) at 37 C for 5 min (curves 4, 5, 6). Control cells were not treated with E2 (curves 1, 2, 3). Cells were then collected by centrifugation, washed, resuspended in Tris-glucose-Casamino Acids medium containing CM (50 μg/ml), and were then incubated at 37 C. Trypsin (500 μg/ml) was added either at 0 min (curves 2, 5) or at 60 min (curves 3, 6). At intervals, both survivors (b) and E2-adsorption capacity (a) were determined. E2-adsorption capacity was measured as follows. Samples were centrifuged. Cells were washed and resuspended in buffer containing trypsin inhibitor (1 mg/ml). After 5 min at 37 C, cells were again centrifuged, washed, and resuspended in buffer (2×10^8 per milliliter). H^3-labeled colicin E2 was then added at the ratio of about 30 killing units to one cell and incubated at 0 C for 120 min. Radioactive colicins adsorbed to the cell were then determined by centrifuging cells and measuring their radioactivity.*

It was observed that a significant amount of radioactive material (about 20% of the total) was adsorbed even though the cells were pretreated with the excess cold colicin. The amount is too high to be due to a nonspecific adsorption of radioactive materials. This might be due to

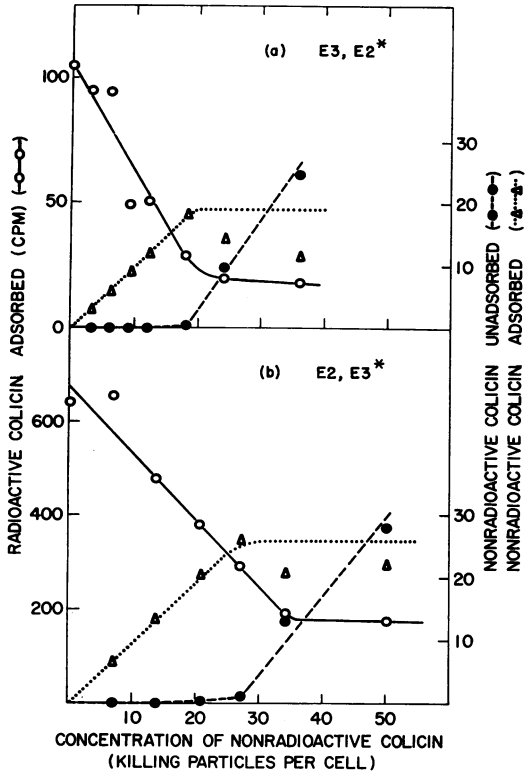

FIG. 7. *Competition between E2 and E3 for a receptor site. (a) Washed cells (2 × 10⁹ per milliliter) of W3110Sm^r suspended in Tris-S containing CM (50 μg/ml) were treated with various concentrations of nonradioactive E3 at 0 C for 60 min. Cells were then centrifuged, washed, and resuspended in Tris-S containing CM at a concentration of 2 × 10⁸ per milliliter. Unadsorbed E3 was determined by analyzing the killing activity of the first supernatant fluid by spot test and is expressed as killing units relative to the original number of cells. The resuspended cells were then treated with H³-labeled E2 at a "multiplicity" of about 30 and were kept at 0 C for 2 hr. The cells were then centrifuged and washed, and their radioactivity determined. (b) Cells were first treated with various concentrations of nonradioactive E2, and then with H³-labeled E3 at a "multiplicity" of about 30. Otherwise, the experimental procedure was the same as that described in (a).*

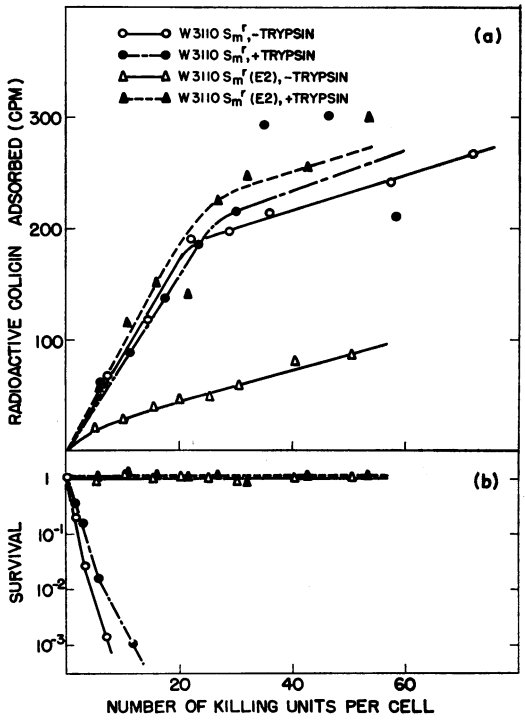

FIG. 8. *Adsorption of radioactive colicin E2 to sensitive (W3110Sm^r) and E2-colicinogenic [strain 52, i.e., W3110Sm^r (E2)] cells. Cells were grown in TB with or without added trypsin (250 μg/ml) to a cellular density of 5 × 10⁸ per milliliter. Cells were harvested, washed, and resuspended in Tris-S containing CM (100 μg/ml). Trypsin (500 μg/ml) was again added to the cells, which were grown in the presence of trypsin. After shaking at 37 C for 1 hr, cells were harvested, resuspended in Tris-S containing CM, and then treated with trypsin inhibitor (1 mg/ml) at 37 C for 5 min. Cells were then collected by centrifugation, washed, and resuspended in Tris-S containing CM at a density of 2 × 10⁸ per milliliter. Various amounts of H³-labeled E2 were added. Both the radioactivity adsorbed and the fraction of survivors were determined. The number of killing units shown at the abscissa was calculated from the fraction of survivors observed with sensitive cells grown in the absence of trypsin.*

the second type of slow adsorption observable at higher colicin concentrations (see above). Another possibility is that the excess second colicin displaces the preadsorbed first colicin from the receptor with a low but measurable efficiency. Future experiments should clarify this problem.

Adsorption of colicin E2 to E2-colicinogenic (immune) cells. As shown in Fig. 8, washed cells of E2 colicinogenic strain 52 [W3110Sm^r(E2)] adsorbed radioactive colicin E2. At near saturation concentrations (the ratio of E2 killing units to cells being 20 to 30) for noncolicinogenic cells, the amount was about one-fourth of that adsorbed by sensitive cells. Since resistant cells adsorbed less than 10% of the radioactive material in the preparation under the same conditions (see Table 1), the adsorbed radioactivity cannot be entirely explained by some noncolicin radioactive contaminants in the preparation or by nonspecific adsorption. Whereas the sensitive cells showed near saturation at the concentration of 20 to 30 E2 killing units per cell, as described

above, no such indication was observed with immune cells. This might suggest that the receptor sites of colicinogenic cells are covered by endogenously produced colicin particles and the additional adsorption of external radioactive colicin either corresponds to the second type of slow adsorption observable at higher colicin concentrations, or is due to the exchange with the preadsorbed colicin.

To minimize the adsorption of spontaneously induced colicins to the noninduced majority of cells during the growth, colicinogenic cells were grown in the presence of trypsin. The resultant cells were centrifuged, washed, and then treated with radioactive colicins. As shown in Fig. 8 a, such colicinogenic cells adsorbed radioactive colicin to the same extent as did control sensitive cells. Simultaneous assay of number of survivors (Fig. 8b), showed that such colicinogenic cells were resistant, whereas the sensitive cells both with and without trypsin pretreatment were killed. Thus, it was clearly established that E2-colicinogenic cells adsorb homologous colicin E2, and yet are resistant to this colicin.

The conclusion that the colicinogenic immune cells have the capacity to adsorb homologous colicin is also supported by experiments with the mutants ED11 and ED15 derived from the colicinogenic strain 52 which are defective in colicin production; the mutants produce very little colicin, but retain the immunity. The culture of these mutants contains no colicin, and, therefore, the adsorption of colicin E2 to these defective mutants could be tested more directly without using radioactive E2 and without trypsin pretreatment.

Cells of ED11 and ED15 were grown in the absence of trypsin, washed, and then mixed with nonradioactive E2 at a multiplicity of about 2. After the incubation at 37 C for 30 min, the cells were removed by centrifugation, and the colicin activity left in the supernatant fluid was assayed by the spot test. Less than 3% of the total activity initially added was found in the supernatant fluid. The adsorption of E2 to these mutants was also shown by use of radioactive E2. Thus, these defective colicinogenic cells adsorb E2, and yet they are resistant to E2.

DISCUSSION

The killing action of most colicins is a single-hit process (3, 7). This means that the killing does not require a cooperative function of several particles; that is, a single particle causes, *with a certain probability*, the death of a sensitive cell. The actual amount of protein corresponding to a single killing unit can be calculated from the experimental curves. The amount of colicin giving 37% survivor corresponds to a multiplicity of 1 killing unit of colicin per sensitive cell used. With the use of a molecular weight for E2 of 60,000, which Helinski obtained (*personal communication*), and assuming that our E2 preparation is pure, it is calculated that one killing unit corresponds to about 100 molecules. This value ranged between 40 and 300 depending on experimental conditions and preparations (Maeda and Nomura, *unpublished data*).

The deviation from the value one is either due to inactive particles contained in the preparation or to the low probability (1%) of a successful killing action per adsorbed particle, or to both. The second explanation includes the possibility of heterogeneity of receptors. Since the change of assay conditions (e.g., temperature, the physiological state of sensitive cells) was found to affect the slope of the killing curve, the second explanation accounts for at least a part of the deviation from one. As an extreme example, certain mutants show near complete resistance at 42 C and a sensitivity comparable to the wild-type strain at 30 C (Nomura, *unpublished data*). Since adsorption has been shown to be normal at 42 C, the probability of a successful killing action per adsorbed particle varies enormously with this mutant, that is, from 1% to less than 10^{-3}%, depending on the temperature.

If the second explanation is a major one, that is, if our radioactive E2 preparation consists mostly of more or less homogeneous potentially active molecules with a low probability (about 1%) of successful killing, the experiments described in the present paper give unequivocal answers to the questions asked as to the state of the active colicin molecules after the adsorption to cells, the relationship between the receptor sites for active E2 and E3 molecules, and the adsorption of active E2 molecules to the E2-colicinogenic immune cells. (It should be pointed out that the isotope experiment by itself does not exclude the possibility of penetration of a single "effective" molecule during the irreversible stage of killing. However, our previous demonstration that trypsin reverses colicin action, and that the reversal is not due to its action on cells, shows very strongly that colicin stays on the cell surface. The results of the present isotope experiments agree with the predictions from this conclusion and, therefore, strengthen our previous conclusion.) If, on the other hand, our E2 preparation consists of a minority (about 1%) of active molecules and a majority of "inactive" molecules, the present experiments cannot provide a definite proof of our previous conclusions obtained through the indirect experiments. However, the fact that the behavior of the bulk of

radioactive colicin agreed with that predicted from the results of the previous indirect experiments supports the previous conclusions very strongly, even though the problem of the possible majority of "inactive" molecules still exists.

The first major conclusion supported by the present study is that the colicin stays at the receptor site and acts from there. This implies that the primary action of colicin is on the cytoplasmic membrane. In this connection, the specificity of the biochemical action of different colicins suggests that bacterial cytoplasmic membrane is a mosaic, consisting of several specific parts, each of which has an important function, and that these functions can be inhibited individually by specific chemical stimuli initiated by specific colicins at the receptor site.

The second major conclusion supported by the present study is that there are some specific steps between adsorption and the target. Thus, the present study has established the following facts. (i) Colicins E2 and E3 share a common receptor, and yet their apparent modes of action are entirely different. The specificity in biochemical action cannot be accounted for by the specificity in adsorption. (ii) Immune cells adsorb a homologous colicin; that is, the immunity of colicinogenic cells does not depend on an alteration in the adsorption step. Since the immunity to E2 does not involve alteration in the properties of DNA, i.e., the final biochemical target of E2 (7), the altered component must participate in a step between adsorption and the target.

The significance of these conclusions has already been discussed in connection with the proposed mechanism of colicin action (8, 9; *see also* 5).

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