Purification and Properties of the Colicin E3 Receptor of *Escherichia coli*

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SUMMARY

Colicin E3 receptor activity was extracted from the envelope fraction of colicin-sensitive *Escherichia coli* cells with Triton X-100 and EDTA, and was purified by ion exchange chromatography on DEAE-cellulose. The purified receptor fraction retained full receptor activity against both colicin E2 and E3, but it had little activity towards colicin E1 and almost no colicin K receptor activity.

A protein with a molecular weight of about 60,000 was detected in purified fractions by polyacrylamide gel electrophoresis. During the final stages of purification this protein was enriched in proportion to the specific activity of the colicin receptor. When excess colicin E3 was added to the purified receptor fraction and then precipitated with rabbit anticolicin, this 60,000 molecular weight protein was co-precipitated with the colicin, whereas other contaminating protein was only partially precipitated. In order to further confirm the relationship between this protein and colicin receptor activity, a culture of a colicin-sensitive strain was labeled with 3H-amino acids and mixed with a culture of a colicin-resistant mutant strain labeled with 14C-amino acids. When the purified receptor fraction obtained from this mixed culture was analyzed by gel electrophoresis, it was observed that the 60,000 molecular weight protein was labeled only with 3H, indicating that this protein is missing or altered in the colicin-resistant mutant. It was estimated that there are 220 copies of this protein per cell.

The purified receptor activity was inactivated by periodate, suggesting that a carbohydrate is also required for activity. A small amount of carbohydrate was present in the purified receptor fraction, and preliminary analysis indicated that glucose, galactose (and/or heptose), rhamnose, uronic acids, and amino sugars were present. The 60,000 molecular weight protein did not appear to contain any large amount of covalently linked carbohydrate. As yet, there is no evidence to confirm the identity of any specific carbohydrate which is essential for receptor activity.

Colicins are proteins produced by colicinogenic strains of enteric bacteria, and they are characterized by their ability to kill cells of a limited number of related strains and species. The first step in this killing process is the adsorption of the colicin to specific receptors present on the cell surface (1). The adsorption is followed by biochemical effects which are different for different types of colicin.

Initial studies on the killing process suggested that colicins were able to kill cells while they remained adsorbed on the cell surface (17), and this led Changeux and Thiery (2) to propose that colicins act by inducing a configurational change in the cell surface which was then propagated to the interior of the cell. However, recent studies by Boon (3) and by Bowman et al. (4) have shown that incubation of purified colicin E3 with 70 S ribosomes resulted in cleavage of the 16 S ribosomal RNA. Identical cleavage products of ribosomal RNA were observed *in vivo* when cells were treated with colicin E3. This suggests that colicin E3 may enter the cell, or at least penetrate to the cytoplasmic membrane. In either case the receptor may function not only in bringing the colicin into close contact with the cell but also in the process of penetration of the colicin.

In a previous study (5) we have shown that the colicin E3 receptor is localized in the cell wall fraction of the *Escherichia coli* envelope, and that colicin E3 receptor activity could be solubilized by Triton EDTA extraction. The present study describes the purification of colicin E3 receptor activity and an attempt to identify some of the components which are necessary in order for colicin binding to occur.

MATERIALS AND METHODS

**Bacterial Strains and Culture Media**—The bacterial strains and the culture conditions and media used in this study have been described previously (5).

**Colicin Induction and Purification**—Colicin E3 was induced and purified by the method of Herschman and Helinski (6), with the exception that colicin-containing fractions were concentrated using an Amicon concentrator with a Diaflo PM-10 membrane. The colicin E3 was purified through the CM-Sephadex step described by these authors. The final preparation had a specific activity of $2 \times 10^6$ units (5) per mg of protein, which is comparable in purity to that reported by Herschman and Helinski (6) who used a similar titration procedure. The purified colicin E3 was dialyzed against distilled water and lyophilized. The lyophilized colicin was used either for immunization of rabbits or was dissolved in colicin diluent (5) and stored frozen.
Colicins E1, E2, and K were also induced and partially purified by the procedure of Herschman and Helinski (6), except that these colicins were purified only as far as the ammonium sulfate precipitation step.

**Colicin and Colicin Receptor Assays**—The colicin and colicin receptor spot assays were carried out as previously described (5) with the exception that 2-fold dilutions of the colicin were used in both assays.

**Triton and Triton EDTA Extraction of Envelope Preparations**—E. coli cells were broken with a French pressure cell, and the crude envelope fraction was isolated as described by Schnaitman (7). The envelope fraction was extracted several times with 2% Triton X-100 in 0.01 M HEPES buffer, pH 7.2. The extractions were done at room temperature for 20 min followed by centrifugation in a Spincor type 42 rotor for 120 min at 30,000 rpm in the cold. The Triton-soluble supernatants were stored in the cold. The Triton-insoluble precipitate was then extracted several times with 2% Triton X-100 in HEPES buffer as above containing 5 mM EDTA. The extraction and centrifugation was carried out as described above except that centrifugation was carried out at 20°C to prevent reprecipitation of the solubilized receptor activity (5). The Triton-EDTA-insoluble fraction was suspended in buffer as above containing Triton X-100 and EDTA and was stored in the cold. Samples of each of the above fractions were precipitated by the addition of 2 volumes of cold 95% ethanol per volume of sample, and the precipitate was analyzed for protein, neutral sugar, phosphorus, and colicin neutralization. Samples for colicin neutralization were diluted to the same protein concentration, and Triton X-100, HEPES Buffer, and EDTA were added to give the same final concentrations as in the extraction procedure. The ethanol precipitation was necessary to remove Triton X-100, which interfered with the chemical determinations. The ethanolic precipitates retained full colicin receptor activity.

**Polyacrylamide Gel Electrophoresis**—Samples for electrophoresis were precipitated with cold ethanol as described above to remove Triton X-100 and lipid, and the precipitates were dissolved in 0.1 M sodium phosphate buffer, pH 7.2, containing 8 M urea, 1% SDS, 0.1% 2-mercaptoethanol, and 0.5 mM EDTA. The samples were then dialyzed overnight under an atmosphere of N₂ against the same solution except that the SDS concentration was 0.1%. Samples were heated in a boiling water bath for 5 min and then applied to gels 5 mm in diameter prepared as described by Maizel (8). Electrophoresis was carried out at 50 mA per gel for 5 to 7 hours. After electrophoresis the gels were stored frozen for radioactive counting or stained by the method of Swank and Munkres (9). Gels to be counted were sliced into sections 1.25 mm thick, and the slices were placed in vials containing 0.5 ml of a solution containing 9 parts of NCS solubilizer (Amersham-Searle, Inc., Des Plaines, Ill.) and 1 part of water and incubated at 50°C for 2 hours. Ten milliliters of toluene counting fluid were then added to each sample, and the samples were stored overnight in the cold prior to counting.

**Immunization**—Cn-Sephadex-purified colicin E3 in complete Freund's adjuvant (10) was used for the primary immunization. Each rabbit received a total of 6 mg of colicin protein as intradermal injections at several sites. Six weeks later each animal was challenged with 3 mg of colicin protein given intravenously without adjuvant. Nine days later the animals were bled. The blood was allowed to clot for 24 hours in the cold, and the serum obtained after low speed centrifugation was stored at -20°C.

The globulin fraction was obtained from the antisera by precipitation with 50% saturated ammonium sulfate. A single major precipitin line and one minor precipitin line were observed when the anticolicin globulin fraction was tested by immunodiffusion in agar against the colicin which was used for immunization. The major precipitin line was established to be the colicin-anticolicin complex by overlaying the immunodiffusion plate with soft agar seeded with a colicin-sensitive indicator strain of E. coli (5). The zone of inhibition of growth due to free colicin terminated at the major precipitin line.

**Colicin-Anticolicin Equivalence Point**—The globulin fraction of the anticolicin serum was dissolved in phosphate-buffered saline (Dulbecco's saline without Ca²⁺ and Mg²⁺, Grand Island Biological Company, Grand Island, N. Y.) at a concentration of 10.8 mg of protein per ml, and serial dilutions of this antibody preparation were added to equal volumes of colicin E3 solution containing 3.5 mg of protein per ml. The mixtures were then incubated for 1 hour at 37°C and then overnight at 0°C. The tubes were centrifuged, and the equivalence point was determined by measuring the protein content of the pellets and by titering the colicin activity in the supernatant. The equivalence point was taken as the ratio of antiserum protein to colicin protein at which the maximum amount of protein was found in the pellet and colicin activity was no longer detected in the supernatant. The equivalence point was determined to be 2.4 mg of globulin protein per mg of colicin protein. Due to limitations in the amount of purified receptor material it was not possible to repeat this equivalence titration in the presence of receptor material, and the same protein concentrations and ratio of colicin to anticolicin were employed in immune co-precipitation experiments.

**Chemical Analysis**—Protein determinations were performed by the method of Lowry et al. (11) with bovine serum albumin as a standard. Neutral sugar was determined by the anthrone method (12), and phosphorus was measured by the method of Goroni (13).

**Carbohydrate Analysis on Purified Receptor Fraction**—The receptor fraction purified by DEAE-cellulose chromatography was precipitated with ethanol as described above, and the precipitate was subjected to the following treatments:

1. Acid hydrolysis. The ethanol precipitate containing 50 μg of neutral sugar was suspended in 2 x HCl and heated for 3 hours at 100°C. This hydrolysis yields the monosaccharides present in the sample.

2. Tritiated borohydride labeling. The acid hydrolysate was dried and suspended in 0.1 M Tris buffer, pH 8.0, and 0.5 μCi of NaBH₄ was added to the suspended residue and allowed to stand at room temperature for 3 hours. Excess cold KH₂O₂ was then added and the sample was allowed to stand overnight in the cold.

3. High voltage electrophoresis. The borohydride-reduced samples were acidified, applied to Whatman No. 1 paper, and subjected to high voltage electrophoresis for 30 min at 3000 volts and 250 ma in a buffer consisting of 10 volumes of pyridine, 4 volumes of acetic acid, and 86 volumes of water (pH 5.8). The neutral and charged sugars which were resolved were detected by scanning strips of the electrophoresis paper for radioactivity and by staining with silver nitrate reagent.

4. Paper chromatography. Sugars resolved by high voltage electrophoresis were identified by cutting the labeled material with water and subjecting the eluted material to paper chromatography in two solvent systems. The solvent systems...
which were used were pyridine-ethyl acetate-water (20:72:2%) (organic phase) and isooamyl acetate-acetic acid-water (3:5:1).
The $R_f$ values of the unknown sugars were compared to authentic standards. The details of the sugar analysis are described by Volk et al. (14, 15).

**RESULTS**

Preparation of Envelope Material—E. coli K-12 C600 cells were grown on supplemented M-9 media (o) in 20- to 40-liter amounts. The cultures were labeled with 0.05 $\mu$Ci of L-[3H]leucine and 0.05 $\mu$Ci of L-[3H]tyrosine per ml of medium. In some experiments cultures of this organism labeled with [3H]-leucine and [3H]tyrosine were mixed after harvesting with cultures of the colicin-resistant mutant C600 R/E3 (5) grown in the same fashion on media labeled with 0.005 $\mu$Ci of L-[3H]leucine and 0.005 $\mu$Ci of L-[3H]tyrosine per ml. The cultures were then fractionated together. Cultures in which double labeling of protein $Z_i$ of L-[%]tyrosine per ml. The cultures were then fractionated together. Cultures in which double labeling of protein

$\text{Tris buffer, pH 7.2, containing 5 mM EDTA and 0.5% Triton X-100.}$

In some experiments, the receptor activity was purified up to 33-fold with respect to protein content by this column procedure, and all of the receptor activity applied to the column was recovered.

Gel Filtration of DEAE-I Purified Receptor—A portion of the receptor fraction from DEAE-column I was applied to a Bio-Gel A-5m column equilibrated with 0.05 M Tris buffer, pH 7.2, containing 5 mM EDTA and 0.5% Triton X-100. The column was eluted with the same solution. Fig. 2 illustrates the elution pattern from this column. This column offered no significant purification and indicated that the receptor activity obtained from DEAE-column I was quite inhomogeneous with respect to size. The receptor activity eluted as a broad peak, with the apparent molecular weight ranging from in the order of millions for the largest components to as low as 120,000 to 140,000 for the lowest molecular weight fraction with definite receptor activity.

DEAE-chromatography II—The receptor fraction obtained from DEAE column I was applied to a column (1 x 25 cm) of Whatman DE-52. The equilibration buffer was the same as for DEAE-column I except that the Triton X-100 concentration was 0.5%. The column was eluted with the same buffer containing 0.15 M NaCl and then with the same buffer containing 1 M NaCl and 0.25% Triton X-100. Fig. 3 illustrates the elution profile of this column. The receptor activity eluted as a sharp peak which coincided with a peak of [3H] labeled protein. Fig. 4 illustrates the over-all purification scheme employing both steps of DEAE-cellulose chromatography. Table I shows further data on the composition of the fractions which were obtained at the various stages of purification. The experiment shown in Fig. 4 and Table I was one in which the receptor ac-

![Fig. 1. DEAE-chromatography I.](http://www.jbc.org/)
Fig. 2. Gel filtration of the receptor fraction from DEAE-column I. The receptor fraction from DEAE-column I (Fig. 1) was applied to a Bio-Gel A-5m column which was equilibrated and eluted with buffer containing Triton X-100 and EDTA as described in the text. The bed volume was 90 ml, and 1-ml fractions were collected. The void volume (VO) was estimated as 38% of the bed volume. —, approximate molecular weight range of the fractions; O—O, 3H-labeled protein; the shaded area indicates colicin E3 receptor activity.

Fig. 3. DEAE-chromatography II. The receptor fraction from DEAE-column I was applied to a second DEAE-cellulose column as described in the text and eluted with a discontinuous NaCl gradient. Twenty-milliliter fractions were collected, and fractions with receptor activity were assayed by the neutralization test using 10-fold dilutions. This is the same preparation as shown in Figs. 1 and 4 and Table I. O—O, 3H-amino acid label; Δ—Δ, colicin receptor activity expressed as percentage of the total eluted activity. The solid line indicates the salt gradient.

activity was purified from a culture labeled with 3H-amino acids and [14C]glucose. The specific activity of the receptor fraction increased about 150-fold during the course of purification, and the purified receptor fraction was enriched about 300-fold with respect to the 3H-labeled protein recovered in the final fraction and about 1200-fold with respect to the [14C]glucose label recovered in the final fraction. In the best experiment to date, a purification of about twice that which is given above was obtained.

Table I also shows the yield of receptor activity at each step of the purification procedure. Virtually all of the receptor activity of the envelope fraction is recovered in the Triton-EDTA-soluble fraction. In each of the column chromatography steps all of the fractions containing significant amounts of receptor activity were pooled, and all of the activity applied to the column was recovered. This has been observed in a number of experiments. At least a month was required for the complete purification scheme and a gradual loss of receptor activity occurs over this time. This is illustrated in Table I where the receptor titer of “fresh” preparations (assayed as they were obtained, through the DEAE-column I step) is compared to the receptor titer of the same preparations assayed at the end of the purification procedure. The receptor activity of the envelope fraction is quite stable, but the fractions solubilized with Triton-EDTA declined in activity by about 50% by the end of the experiment.

As shown in Table I, there is a substantial amount of phosphorus present in the Triton-EDTA-soluble fraction of the envelope. This is associated primarily with non-phospholipid components such as lipopolysaccharide, since less than 15% of the phosphorus was extractable with chloroform-methanol. Extraction of the Triton-insoluble envelope fraction with Triton X-100 plus EDTA in the cold prior to solubilizing the receptor activity by Triton-EDTA extraction at room temperature reduced the phosphorus content of the solubilized receptor fraction more than 3-fold without affecting the receptor activity. However, this procedure offered little advantage in purification of the receptor activity and was not used routinely.

Gel Electrophoresis of Purified Colicin E3 Receptor Fraction—Electrophoresis of fractions obtained at various steps of the purification procedure was carried out in SDS-polyacrylamide gels as described under “Materials and Methods.” The gel pattern of the receptor fraction from DEAE-column II is characterized by the presence of two major 3H-labeled protein peaks (Fig. 5). The prominent faster migrating peak is the major protein peak observed in the E. coli cell wall (7) and is observed in all of the various fractions obtained at different steps of purification (Figs. 8 to 11). However, the slower migrating peak seen in Fig. 5 is present in a small amount in the receptor fraction from DEAE-column I and cannot be identified in any
Forty liters of culture labeled with \( ^{14} \text{C} \) glucose and \( ^{3} \text{H} \) amino acids as described in the text. The receptor titer was determined using the spot neutralization test (5) and 2-fold dilutions of the colicin. The units of receptor activity are as previously described (5). “Fresh” samples refers to samples which were assayed as they were obtained, and the total activity at the end of the experiment refers to the same samples assayed after the DEAE-column II step. About 1 month was required to complete the purification.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total label from ( ^{14} \text{C} ) glucose</th>
<th>Total neutral sugar</th>
<th>Total phosphorus</th>
<th>Colicin E3 receptor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>counts/min</td>
<td>mg</td>
<td>mg</td>
<td>mg/( % ) arbitrary units</td>
</tr>
<tr>
<td>Envelope</td>
<td>3045</td>
<td>( 1.6 \times 10^7 )</td>
<td>1549</td>
<td>173</td>
<td>9.0 ( \times 10^5 ) / 1.0 ( \times 10^6 ) / 100</td>
</tr>
<tr>
<td>Triton-EDTA-soluble</td>
<td>1181</td>
<td>( 3.9 \times 10^7 )</td>
<td>140</td>
<td>37.5</td>
<td>9.8 ( \times 10^5 ) / 5.1 ( \times 10^5 ) / 50</td>
</tr>
<tr>
<td>DEAE-column I</td>
<td>220 ( ^{a} )</td>
<td>( 2.8 \times 10^5 )</td>
<td>0.47 ( ^{a} )</td>
<td></td>
<td>1.0 ( \times 10^5 ) / 5.1 ( \times 10^5 ) / 50</td>
</tr>
<tr>
<td>DEAE-column II</td>
<td>13.9 ( ^{b} )</td>
<td>( 1.3 \times 10^6 )</td>
<td></td>
<td></td>
<td>1.0 ( \times 10^5 ) / 5.1 ( \times 10^5 ) / 50</td>
</tr>
</tbody>
</table>

\( ^{a} \) All samples were precipitated with ethanol prior to analysis.

\( ^{b} \) These amounts were calculated based upon the \( ^{3} \text{H} \) : protein and \( ^{14} \text{C} \) : neutral sugar ratios observed in the Triton-EDTA-soluble fraction.

The sample is the DEAE-column II purified receptor fraction from a culture labeled with \( ^{14} \text{C} \) glucose and \( ^{3} \text{H} \) amino acids as described in the text. The dashed line indicates \( ^{14} \text{C} \) counts and the solid line indicates \( ^{3} \text{H} \) counts. In order to facilitate comparison, the counts in this gel and in succeeding gels are expressed in terms of the percentage of total counts recovered from the gel. The top of the gel is to the left.

The receptor fraction obtained from DEAE-column II was incubated for 15 min at 37\(^{\circ} \) with a saturating amount of colicin E3, such that all of the receptor activity would be present in the form of a colicin-receptor complex. The globulin fraction obtained from anticolicin serum was then added in the optimum amount necessary for forming an antigen-antibody precipitate as determined from the colicin-anticolicin equivalence curve (see “Materials and Methods”). The antigen-antibody precipitate was sedimented by centrifugation and the pellet and supernatant were analyzed by SDS-polyacrylamide gel electrophoresis. The radioactivity profile of the immune supernatant (Fig. 6) was characterized by the absence of the slower migrating peak. In contrast, the radioactivity profile of the immune precipitate (Fig. 7) was characterized by the absence of the slower migrating peak. Extensive co-precipitation of the faster migrating protein also occurred. This suggests a tight binding between the two proteins, and it is possible that the faster migrating protein may be required for receptor activity. However, the presence of the faster migrating protein in the immune supernatant suggests that this protein does not by itself have receptor activity.

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FIG. 6. SDS gel electrophoresis of the supernatant obtained after immune precipitation of the purified receptor fraction. The DEAE-column II-purified preparation shown in Fig. 5 was used in this experiment, and a sample of this containing 0.5 mg of protein was precipitated with ethanol. The precipitate was resuspended in 0.25 ml of phosphate-buffered saline and added to 0.25 ml of phosphate-buffered saline containing 1.76 mg of colicin E3. This represented a 10-fold excess of colicin over colicin receptors. This mixture was incubated for 15 min, and then 0.25 ml of anticolicin globulin solution containing 4.2 mg of globulin protein in phosphate-buffered saline was added. This mixture was allowed to stand for 1 hour at 37° followed by overnight in the cold and was then centrifuged. The precipitate was then washed three times with phosphate-buffered saline, the washes were combined with the supernatant from the first centrifugation, and this material was prepared for gel electrophoresis as described under “Materials and Methods.” The dashed line represents 14C label from glucose, and the solid line represents 3H label from amino acids. About half of the total 3H of the receptor sample was recovered in the immune supernatant, the remainder of which was in the precipitate (Fig. 7).

The parent strain C600 was grown on medium containing 3H-labeled amino acids, and a parallel culture of the mutant strain C600 R/E3 was grown on medium containing 14C-labeled amino acids. After harvesting, the cultures were mixed and fractionated as described under “Materials and Methods,” and the colicin receptor activity was purified as described above and in Fig. 4. At various stages in the purification both active and inactive fractions were examined by SDS-polyacrylamide gel electrophoresis.

The radioactivity profiles of 14C and 3H are quite similar in the crude envelope fraction (Fig. 8) and likewise in the Triton-EDTA-soluble fraction (Fig. 9). The slower migrating protein described in the previous section is first seen in the radioactivity profile of the receptor fraction obtained from DEAE-column I (Fig. 10). This protein is strongly enriched in the receptor fraction obtained from DEAE-column II (Fig. 11), and in fact represents about 25% of the 3H-labeled protein present in this fraction. The region of the gel which contains this protein peak is devoid of any 14C-labeled protein from the resistant mutant. This experiment has been repeated several times with the same result and provides compelling evidence that the slower migrating protein is a specific and essential component of the colicin receptor.

Nothing is known about the specific nature of the genetic lesion in this mutant. However, this may not have a direct bearing on the presence or absence of this protein, since even a missense mutation might prevent this protein from being inserted into the outer layer of the cell envelope or from co-purifying with the active receptor components.

Much of the contaminating protein which is removed during DEAE-column purification is the faster migrating major protein peak described in the previous section, and other protein(s) with even lower molecular weight. This is seen in Fig. 12, which shows the radioactivity profile of the main, inactive protein peak eluted from DEAE-column I. The 14C and 3H profiles are similar in this fraction.

Receptor Activity for Other Colicins—Since the purification procedure was developed specifically for the purification of colicin E3 receptor activity, it was of interest to see whether the various fractions obtained during purification exhibited receptor activity for other colicins. These data are shown in Table II and indicate that the procedure selectively purifies receptor activity for colicins E2 and E3. Receptor activity for colicin...
FIG. 9. SDS gel electrophoresis pattern of the Triton-EDTA-soluble envelope fraction from the mixed culture shown in Fig. 8. The solid line represents $^{3}$H counts from the sensitive strain, and the dashed line represents $^{14}$C counts from the resistant strain.

FIG. 10. SDS gel electrophoresis pattern of the DEAE-column II-purified receptor fraction from the mixed culture shown in Fig. 8. Note the small peak labeled with $^{3}$H but not with $^{14}$C at about slice number 21. The solid line is $^{3}$H counts from the sensitive strain, and the dashed line is $^{14}$C counts from the resistant strain.

FIG. 11. SDS gel electrophoresis pattern of the DEAE-column II-purified receptor fraction from the mixed culture shown in Fig. 8. The solid line represents $^{3}$H counts from the sensitive strain, and the dashed line represents $^{14}$C counts from the resistant strain. The receptor-specific protein which is labeled with $^{3}$H but not $^{14}$C (slice number 21) represents 22% of the total $^{3}$H-labeled protein detected in the gel. In this experiment, the DEAE-column II-purified receptor fraction contained 0.17% of the protein present in the crude envelope fraction.

FIG. 12. SDS gel electrophoresis pattern of inactive protein from the mixed culture shown in Fig. 8. The sample applied to the gel is protein from the first major protein peak eluted at a low salt concentration from DEAE-column I (see Fig. 1). This fraction has no colicin receptor activity, and the receptor protein seen in Fig. 5 and 11 is not present in the gel. The solid line represents $^{3}$H counts from the sensitive strain, and the dashed line represents $^{14}$C counts from the resistant strain.

El was greatly reduced in the final purified fraction, and colicin K receptor activity was almost absent from this fraction.

**Molecular Weight of Specific Receptor Protein**—The molecular weight of the specific receptor protein identified previously by the gel electrophoresis and immune precipitation experiments was determined by the method of Shapiro et al. (16). Since it was difficult to visualize the specific receptor component on stained gels, the following modification was employed. A parallel comparison of stained gels of samples taken at various stages of purification indicated that the major protein peak which is the major contaminating protein in the purified receptor fraction (Fig. 11) and the major protein observed in the inactive fraction from DEAE-column II (Fig. 12) had exactly the same migration as the major peak observed in the starting envelope fraction (Fig. 8). This peak could be visualized easily in stained gels and radioactive profiles so it served as a useful reference point in estimating the molecular weight of the specific receptor protein. A sample containing this protein was mixed in various combinations with standard proteins with known molecular weights in the range of from 13,000 to 69,000. All samples were prepared and subjected to electrophoresis under the same conditions. The gels were stained, and the relative mobilities of the various standard proteins with respect to the major cell wall protein were calculated. A straight line was obtained when the log of the molecular weight of the standards was plotted versus the relative mobility. With this calibration curve the molecular weight of the specific receptor protein was calculated from the relative mobility of this protein with respect to the major contaminating protein in radioactivity profiles such as the one shown in Fig. 11. The molecular weight of the specific receptor protein was estimated to be 60,000.
Receptor activity was assayed using the spot neutralization assay and 2-fold dilutions of the colicin. Colicin El, E2, and K were crude ammonium sulfate precipitates. The titers of these colicins stocks were low, particularly colicin E2. Fractions to be tested for receptor activity were precipitated with ethanol and resuspended in colicin diluent to the same protein concentration.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Colicin receptor titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>arbitrary units</td>
</tr>
<tr>
<td>Triton-EDTA-soluble</td>
<td>64</td>
</tr>
<tr>
<td>DEAE-column I</td>
<td>16</td>
</tr>
<tr>
<td>DEAE-column II</td>
<td>4</td>
</tr>
</tbody>
</table>

The numbers above Scan A indicate the labeled peaks which were eluted for further analysis.

Since this experiment was done with a labeled sample, a control was done in which the sample was treated in an identical manner except that reduction was carried out only with unlabeled NaBH₄. No radioactive peaks were resolved by high voltage electrophoresis, indicating that the radioactivity came only from the H-labeled NaBH₄.

The radioactive peaks were eluted and subjected to paper chromatography. In the pyridine-based solvent system, charged sugars remain at the origin and neutral sugars migrate. In the isooamyl acetate solvent system, both neutral and charged sugars migrate.

When the neutral sugar peak (Peak 5, Fig. 18) was chromatographed in the pyridine solvent system, four radioactive peaks were observed. One of these stayed at the origin, indicating that it might be an overlapping charged sugar. The three peaks which migrated possessed Rf values similar to those of gluconol, rhamnositol, and galactitol (or heptitol). Identical results were obtained with the isooamyl acetate solvent system, and in addition the peak which remained at the origin appeared to migrate similar to an aldic acid.
The negatively charged peaks (Peaks 4 and 5) remained at the origin when chromatographed in the pyridine solvent system and moved in a manner similar to aldonic acids in the isomonyl acetate system. The sugar in Peak 5 was converted to neutral lactone by treatment with acid; upon high voltage electrophoresis this gave two peaks, one remaining at the origin indicative of a neutral lactone and one which migrated similar to the original sample. The identity of this sugar with 2-keto-3-deoxyoctonate which behaves in a similar fashion when treated with acid was ruled out by comparing the RP values of the unknown sugar to 2-keto-3-deoxyoctonate in a solvent system in which 2-keto-3-deoxyoctonate migrates. The neutral lactone resolved by high voltage electrophoresis was converted back to its original form by treatment with alkali, and regained its characteristic migration on high voltage electrophoresis.

In summary, this preliminary examination suggested the presence of the following sugars in the purified colicin receptor fraction: glucose, galactose or heptose, rhamnose, monosaccharides, amino monosaccharide, and amino disaccharide. This combination of sugars is not representative of any one specific class of polysaccharide antigen found in E. coli. However, glucose, galactose, heptose, rhamnose, and mono- and disaccharides of glucosamine are all components of E. coli K-12 lipopolysaccharide (17), and this would be quite consistent with the data described above. However, further experiments are clearly required in order to pin down the role of these sugars or of lipopolysaccharide in the functioning of the colicin E3 receptor.

**Discussion**

The receptor for colicin E3 is localized in the cell wall of E. coli (5), presumably in the outer lipoprotein membrane. The receptor activity can be solubilized by treatment of the cell wall with Triton-EDTA, and the solubilized preparation has the same specificity as in the intact cells. The present study demonstrates that this receptor activity may be substantially purified both with respect to protein and with respect to carbohydrate by DEAE-cellulose column chromatography in the presence of EDTA and Triton X-100.

The cell wall consists of a complex of lipoproteins, lipids, lipopolysaccharide, and other polysaccharides. All of these components are present to a certain degree in the Triton-EDTA-soluble fraction of the cell wall, and these molecules are all quite hydrophobic and exhibit a strong tendency to aggregate. Gel filtration of the receptor fraction at an intermediate stage of purification showed that this material was quite heterogeneous with respect to size, and much of the receptor activity was associated with high molecular weight aggregates. Fortunately for the purpose of this study, the components responsible for colicin receptor activity have a charge which is sufficiently different from the bulk of the cell wall material to permit further purification by ion exchange chromatography.

The final purified receptor fraction obtained from DEAE-column II is still not homogeneous with respect to protein, and still contains a small amount of carbohydrate. Thus the problem becomes one of attempting to identify in this preparation components which are specifically involved in colicin E3 receptor activity, and other components which are not specifically enriched in this fraction but which may also be required for functional receptor activity.

A protein with an apparent molecular weight of 60,000 and which cannot be detected in crude cell wall fractions is present in the final purified receptor fraction. Several lines of evidence strongly indicate that this protein is a specific component of the colicin E3 receptor. First, the amount of this protein as determined by polyacrylamide gel electrophoresis increases as the receptor activity. The amount of this protein increases from an amount which is below the limits of detection in the crude envelope fraction to as much as 25% of the total protein in a preparation in which the receptor activity was purified several hundred fold. In the final stages of the purification scheme, the amount of this protein in fractions with receptor activity increases in proportion to the specific activity of the receptor fractions. This protein was not present in several fractions obtained during the course of purification which lacked receptor activity, as for example in the first protein peak eluted from DEAE-column I (Fig. 12).

A second line of evidence comes from immune precipitation experiments. When an excess of colicin E3 was added to the receptor fraction and this was then precipitated by an equivalent amount of rabbit anticolon, the 60,000 molecular weight protein described above was detected in the immune precipitate but not in the supernatant after precipitation.

The most convincing evidence that this protein is a specific component of the colicin E8 receptor comes from experiments in which 3H-labeled cultures of a colicin-sensitive strain were mixed with 14C-labeled cultures of a colicin E-resistant mutant. When these mixed cultures were fractionated, this protein was labeled only with 3H, indicating that this protein is missing or altered in the resistant mutant.

The status of the other cell wall protein observed in the purified preparations is not so clear. The major structural protein (or proteins) of the cell wall (7) is still present in the purified receptor fraction, even after immune precipitation. We cannot exclude the possibility that this protein is required for functional colicin receptor activity. Since this protein is found in inactive fractions (Fig. 12) and in the supernatant after immune precipitation (Fig. 6), and since this protein is labeled with both 3H and 14C in the mixed-culture experiment (Fig. 12), it is not a component which is unique to the colicin receptor.

The situation with respect to the role of carbohydrate in the receptor activity is even more unclear at present. Both the Triton-EDTA-soluble fraction (3) and the purified receptor fraction are sensitive to periodate, suggesting a role for intact carbohydrate. Experiments in which cultures were double labeled with 3H-amino acids and 14C-glucose failed to provide any evidence for a large amount of carbohydrate covalently linked to the specific receptor protein, but the amount of 14C label incorporated and the specificity of this labeling were not good enough to rule out the presence of a small amount of sugar attached to the protein. The glucose label which was not bound to protein exhibited an apparent low molecular weight on gels (Fig. 5) as might be expected for lipopolysaccharide from a "rough" strain such as E. coli K-12, and a preliminary examination of the sugar composition of the purified receptor fraction also suggested that some of the carbohydrate associated with the receptor activity might be lipopolysaccharide or a similar polysaccharide. Lipopolysaccharide in itself does not have colicin E3 receptor activity (9).

It is tempting to speculate that carbohydrates or proteins other than the specific protein described above play a very specific role in modifying colicin E receptor function. This would help to explain certain paradoxes which have been observed. First, although the resistant mutant which was used in the experiment illustrated in Fig. 11 was resistant to colicins E1, E2, and E3, the purified receptor fraction was enriched only with respect to receptor activity for colicins E2 and E3 (Table
II). This suggests that there may be another component which is essential for E1 receptor activity which is lost or inactivated during purification. This is consistent with the observation of Maeda and Nomura (18) that colicins E2 and E3 share a common receptor. Specific modification as described above would explain the observation by Hamon and Peron (19) that mutants can be isolated which have lost the ability to bind colicins E2 and E3 while retaining the ability to bind colicin E1. The absence of colicin K receptor activity in the purified receptor fraction (Table II) is consistent with Fredericq’s early observation (1) that the receptors for colicin K and for the group E colicins are quite different.

On the basis of the purification data and the molecular weight, it is possible to calculate that there are about 220 copies of the specific receptor protein. If each of these actually represents a specific receptor site, this number is fairly close to the number of adsorption sites suggested by Reeves (20), who calculated on the basis of survival curve data that there were 30 to 90 sites for colicin E2 per cell. It is not consistent with the results of Maeda and Nomura (18), in which they determined that there were 2000 to 3000 radioactive colicin E2 molecules bound per cell. Perhaps the easiest way to resolve this discrepancy is to postulate two classes of binding sites for colicin E3 on the surface of the cell: specific, high affinity sites involved in the lethal action of the colicin which would be detected by killing and protection assays such as the ones employed in this study, and nonspecific sites of lower affinity which would be detected by the binding of radioactive colicin. In this context, it is worthwhile to mention recent studies of Konisky and Cowell (21) on the binding of radioactive colicin I. These authors found that colicin I-binding sites were heterogeneous with respect to the association constant of the colicin-receptor interaction, and they suggested that the most likely interpretation of their data was that there were two different classes of receptors with different association constants.

Bayer (22) has shown that certain regions of the cytoplasmic membrane are in close contact with the cell wall in intact cells, and upon plasmolysis these regions stay adhered. There are 200 to 400 of these regions per cell, and Bayer postulated that these sites may represent channels through which the penetration of viral nucleic acid could occur. The observation that there are about 200 molecules of the specific receptor protein per cell makes it appealing to think that this protein may in some way be related to the adhesion sites.

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