Purification and Characterization of Colicin E₂ and Colicin E₃*

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SUMMARY

Colicin E₂ and colicin E₃ were purified from Escherichia coli W3110(ColE₁) and W3110(ColE₃), respectively, by salt extraction, ammonium sulfate precipitation, and ion exchange chromatography on diethylaminoethyl Sephadex and carboxymethyl Sephadex. Polyacrylamide gel electrophoresis, ultracentrifugal characterization, immunochemical analysis, and isoelectric density gradient electrophoresis indicated that both protein preparations were homogeneous. Colicin E₂, however, was shown to exist under certain conditions in two active, electrophoretically distinguishable forms, which were interconvertible.

Biochemical analysis showed that both colicin E₂ and colicin E₃ are simple proteins composed exclusively of amino acids. Both proteins have sedimentation coefficients of 4.0 S and molecular weights of approximately 60,000. Amino acid and immunochemical analyses of colicins E₂ and E₃ suggest that there are regions of similar structure in the two proteins as well as regions unique to each protein. These observations are consistent with the specificity for the same receptor site, the differing modes of killing action, and the differing responses to immunity exhibited by these two colicins.

Colicins E₁, E₂, and E₃ thus share a common biological receptor site on a sensitive bacterium (1, 4), yet differ sufficiently from one another to permit the immunity mechanism to distinguish between the presence of a homologous or a heterologous protein attached to the receptor site of a cell carrying one of the Class E col factors. Colicins E₁, E₂, and E₃ also differ in the manner in which they bring about the death of a sensitive cell. Colicin E₃ has been reported to cause a cessation of all macromolecular synthesis.¹ In contrast, colicin E₂ causes destruction and solubilization of DNA with no immediate effects on the synthesis of RNA or protein, while colicin E₁ affects protein synthesis in the sensitive cell without inhibiting nucleic acid biosynthesis (6, 7).

The production of all three of the Class E colicins may be induced by agents which interfere with DNA synthesis, such as ultraviolet light irradiation or mitomycin C (8). The macromolecular events accompanying the induction of each of the Col factors of the E class appear to be similar (8). Finally, the inducibility of at least factors Col E₁ and Col E₃ is similarly affected in recombination-deficient mutants (9).

These various properties of the Class E colicins provide a useful system in which to investigate the relationship between protein structure and biological specificity at a variety of levels. In addition, a common evolutionary origin for the three Class E colicinogenic factors is suggested by their physiologic properties and by the existence of a common receptor site for their colicin products. Thus, the structural characterization of these proteins may yield information on the evolutionary relationships among these extrachromosomal genetic factors by an examination of their gene products, a technique not used to date for these genetic elements. The present paper describes the purification of colicins in 17 classes, designated alphabetically, on the basis of the existence of a specific receptor site for each class.

The potential to produce colicin depends on the acquisition of a colicinogenic factor (Col factor), a stable extrachromosomal genetic element. The presence of the Col factor also confers on the host bacterium immunity to its homologous colicin. This property permits more detailed classification of Col factors and their corresponding colicin products. On the basis of the immunity phenomenon, Fredericq (2) was able to subdivide the Class E colicins, which bind to a common receptor site, into colicins E₁, E₂, and E₃. Each of these three colicins is able to overcome the immunity directed by the other two Class E Col factors. (For a review of colicin classification see Reeves (3)).

¹ C. Levinthal and F. Levinthal, quoted by Luria (5).

Bacteriocins are high molecular weight extracellular antibiotics produced by various species of bacteria. These antibiotics are active only against closely related species of bacteria, in contrast to the antibiotics of relatively low molecular weight, which have a far wider activity spectrum. The best studied of the bacteriocins are the colicins, bacteriocines produced by Escherichia coli and by other closely related species.

Resistance to the action of a colicin, like resistance to a bacteriophage, is a stable genetic characteristic which may be acquired as the result of a mutation in a sensitive cell. Fredericq (1) initially used this phenomenon to group the various colicins
E. coli W3110(ColE2) was obtained from M. Nomura. The source of the ColE2 factor in this strain is Shigella sp. P9. Strain W3110(ColE2) was constructed by transferring both the ColI and the ColE2 factors from strain CA38(ColI, ColE2) (obtained from P. Frederiq) to W3110, and subsequently eliminating the ColI factor by the use of acridine orange as described by Kahn and Helinski (10). Strain YS40/V was used as the indicator strain for the assay of colicin activity. Casamino acids were used to supplement the M-9 medium used for the growth of colicinogenic cultures. This growth medium and the nutrient agar broth and plates used in the assay of colicin activity have been described previously (8).

Materials

Ampholyte preparations for isoelectric fractionation were obtained from the developmental laboratories of LKB Products, Stockholm. Mitomycin C was purchased from Kyowa Hakko Kogyo Company, Ltd., Tokyo. CM-Sephadex C-50 and DEAE-Sephadex A-50 were purchased from Pharmacia, Uppsala. Ammonium sulfate (special enzyme grade) was obtained from Mann. Acrylamide, \( N,N'\)-methylenebisacrylamide, and \( N,N,N',N'\)tetramethylethylenebisacrylamide for polyacrylamide gel disc electrophoresis were purchased from Eastman.

Colicin Assay and Protein Determination

The quantitative determination of colicin in bacterial cultures was performed by treating 1 ml of a colicinogenic culture with 0.1 ml of chloroform, serially diluting the chloroform-treated culture in nutrient broth, and spotting a drop of each dilution on a nutrient agar plate freshly seeded with \( 10^7 \) indicator bacteria. Cell-free preparations of colicin were assayed for activity by the same procedure, the chloroform treatment being eliminated. The number of colicin units per ml was defined as the highest dilution which gave a clear zone of inhibition of growth of the indicator bacteria.

Protein concentration was determined in the purification steps by the method of Lowry et al. (11), with bovine serum albumin as a standard. The extinction coefficients of colicins \( E_2 \) and \( E_8 \) were used to determine the concentration of purified preparations of these colicins for structural studies. Specific activity is defined as the number of colicin units per mg of protein.

Growth and Induction of W3110(ColE2) and W9110(ColE2) for Colicin Purification

Colicin \( E_2 \) Preliminary experiments showed that induced colicin \( E_2 \) production proceeds under anaerobic conditions as well as during aeration, while the increase in cell mass is sharply reduced by growth under anaerobic conditions. In order to take advantage of this disparity in the rates of colicin \( E_2 \) production and bacterial growth, the following procedure was used for growth and induction of W3110(ColE2).

Sterile M-9-Casamino acids medium (4 liters) was inoculated with W3110(ColE2) and grown with agitation at \( 37^\circ \) to late log phase. This culture was then used to inoculate 100 liters of freshly prepared, nonsterile M-9-Casamino acids medium previously equilibrated at \( 37^\circ \) in a New Brunswick model E-130 100-liter fermenter. Sufficient inoculum was added to adjust the initial cell concentration to \( 2 \times 10^8 \) cells per ml. For each liter of culture 0.1 ml of an antifoam (SAG 471, Union Carbide Company) was added. The culture was aerated vigorously, and was allowed to grow to approximately \( 5 \times 10^8 \) cells per ml with the fermenter agitation control set at 250 rpm. When this cell density was reached (approximately 3 hours after inoculation), mitomycin C was added at a concentration of 0.2 mg per liter. Aeration was discontinued, and the fermenter agitation control was reset at 40 to 50 rpm.

After a 2-hour induction period a culture sample was removed and treated with chloroform for assay. The culture exit port of the fermenter was then attached to a Sharples model AS-16 centrifuge equipped with a plastic bowl liner, and the induced cells were harvested by forcing the culture into the centrifuge under positive air pressure. Harvesting of a 100-liter culture took approximately 45 min. The induced culture had a titer of 25,000 units per ml.

Colicin \( E_8 \)—In contrast to the results described for colicin \( E_2 \) induction, a shift to anaerobic conditions after the addition of mitomycin C resulted in an inhibition of colicin \( E_8 \) production. In addition, induced cultures of W3110(ColE2) began to lyse approximately 1 hour after the addition of mitomycin C. For these reasons the procedure described above was modified as follows for the preparation of induced W3110(ColE2) bacteria.

A 2-liter overnight culture of W3110(ColE2) in M-9-Casamino acids medium was prepared and used to inoculate 50 liters of the same medium at \( 37^\circ \) in the fermenter. Antifoam was used as described previously. The cell density was adjusted to approximately \( 2 \times 10^9 \) cells per ml, aeration and agitation were initiated, and the culture was allowed to grow to approximately \( 5 \times 10^9 \) cells per ml. Mitomycin C was added to a final concentration of 0.2 \( \mu \)g per ml. Incubation was continued with aeration and agitation for an additional 50 min. The cells were then harvested in the Sharples centrifuge as described for the W3110(ColE2) culture. The activity of the induced culture was 50,000 units per ml.

Electrophoresis

Polyacrylamide disc gel electrophoresis, at pH 8.6, was carried out essentially according to the procedure of Ornstein (12). Disc gel electrophoresis at pH 4.0 was performed with the use of the buffer system and running gel described by Reisfeld, Lewis, and Williams (13). All gels were 6% acrylamide. "Ion focus" conditions (14) were used to run the protein into the gels at both pH values, rather than the corresponding stacking gels. The apparatus of Choudue and Zimm (15) was used for all acrylamide gel electrophoreses, but with platinum, rather than silver-silver chloride, electrodes.

Isoelectric fractionation with density gradient-stabilized pH gradients was performed as described by Vesterberg and Svensson (16). Protein samples were dissolved in 0.0 ml of 0.5% ampholylte of the appropriate pH range or dialyzed against a solution of 0.5% ampholyte. Electrophoresis was continued until a stable minimal current was achieved (48 to 72 hours). Fractions of 20 drops were collected and monitored for colicin activity, absorbance, and pH. Measurements of pH were made at 22°C with the use of a Radiometer type TTT1e pH meter equipped with a PHA6301a scale expander.
Ultracentrifugal Analysis

Sedimentation velocity and molecular weight analyses were performed in a Beckman-Spinco model E ultracentrifuge equipped with schlieren optics. Velocity sedimentation analysis was performed at 20°, with the use of 4° 12-mm Kel-F centerpieces in standard cell housings. To compare the two colicins, simultaneous sedimentation runs were carried out by using a 1° wedge window to displace the schlieren image of one cell. Molecular weight determinations were performed with the use of schlieren optics as described by Yphantis (17). Synthetic boundary runs to determine initial concentrations for molecular weight calculations were carried out in a capillary type, double-sector synthetic boundary centerpiece. In order to compare the rate of attainment of equilibrium more precisely, single runs were performed with the multichannel short column equilibrium centerpiece described by Yphantis (18). In this manner both proteins were analyzed simultaneously.

Amino Acid Analysis

Samples of colicin E2 and colicin E3 were dialyzed for 72 hours against three changes of distilled water and then lyophilized. The weight of the protein was determined after the lyophilized preparations were dried in an Abderhalden drying apparatus at 100° in a vacuum for 24 hours over phosphorus pentoxide. The protein samples were then dissolved in 10 ml of 6 N HCl, and 2-ml aliquots were distributed to hydrolysis tubes. The tubes were sealed under vacuum, hydrolyzed at 105-110° for 12, 24, or 48 hours, and analyzed for amino acid composition on a Beckman model 120 automatic amino acid analyzer. The tryptophan content was determined by amino acid analysis of 48-hour hydrolysates of the two colicins subjected to the performic acid oxidation procedure of Hirs (19). The tryptophan content was determined chemically by the use of the Ehrlich reagent procedure (20). Tryptophan and tyrosine contents were also determined spectrophotometrically from alkaline absorption spectra (21).

Immunochemistry

Colicin in Freund’s adjuvant (22) (7 mg of each colicin) was used for primary immunization of New Zealand white female rabbits. Each rabbit received an intravenous secondary boost of 3 mg of colicin 6 weeks after the initial toe pad injections, and 10 days after the secondary immunization 40 to 50 ml of blood were taken from each rabbit. The blood was allowed to clot in the cold for 24 hours, and the supernatant serum was then decanted after a low speed centrifugation to remove the remaining red cells. The antisera were stored at -20°. The antigen-antibody systems were analyzed for homogeneity and cross-reaction by the method of double diffusion in agar (23).

RESULTS

Purification of Colicin E2 and Colicin E3

All operations were carried out at 0-4°. The standard potassium phosphate buffer was 0.01 M, pH 7.0.

Extraction of Colicin Activity—Although colicins E2 and E3 are extracellular, over 90% of the activity of both colicins sediments with the cell pellet following induction (8). One of the most critical steps in the purification of these proteins is, therefore, to remove colicin activity from the surface of the cell without lysing the bacteria. Successive washes of the packed cells with 1.0 M NaCl in standard potassium phosphate buffer solubilized about 40% of cell-bound colicin E2 or colicin E3 activity, without lysis. The cell pellet from a 100-liter induced culture of W3110-(ColE3) was extracted for 30 min in a Waring Blendor at low speed with 333 ml of 1.0 M NaCl in standard potassium phosphate buffer. After extraction the cell suspension was centrifuged for 10 min at 5000 x g. The supernatant was decanted and saved. Extraction of the cell pellet was repeated twice, and the three extracts were pooled.

The cell pellet from a 50-liter induced culture of W3110-(ColE2) was extracted in a similar fashion in the Waring Blendor with four 250-ml portions of 1.0 M NaCl in standard potassium phosphate buffer. The colicin E2-containing supernatants were pooled for subsequent purification.

Ammonium Sulfate Fractionation of Colicin E2 and Colicin E3—The volumes of the pooled colicin extracts were adjusted to 1 liter with the sodium chloride extraction buffer. Solid ammonium sulfate, 114 g, was added slowly to each crude colicin extract, with constant stirring. The suspensions were further stirred for 30 min and then centrifuged at 16,000 x g for 10 min. The precipitates were discarded and the supernatants were returned to the cold bath for precipitation of colicin activity. The majority of colicin E2 and colicin E3 activity (60 to 90%) precipitates between 20% and 40% ammonium sulfate. An additional 129 g of ammonium sulfate were slowly added to each of the two colicin preparations and the suspensions were stirred for 30 min. The suspensions were then centrifuged again at 16,000 x g, the supernatants were discarded, and the ammonium sulfate pellets were resuspended in 50 to 70 ml of standard potassium phosphate buffer. Following resuspension, neither the colicin E2 nor the colicin E3 precipitate was entirely solubilized. The suspensions were clarified by centrifugation at 48,000 x g for 10 min. After clarification, each colicin solution was dialyzed for 4 to 5 hours against 4 liters of standard potassium phosphate buffer. During dialysis additional precipitation occurred, and the solutions were again clarified by centrifugation at 48,000 x g.

The supernatant of this centrifugation is referred to as the 20 to 40% ammonium sulfate cut.

DEAE-Sephadex Chromatography—The 20 to 40% ammonium sulfate preparations of colicin E2 and colicin E3 were applied to individual DEAE-Sephadex A-50 columns, 4 x 50 cm, prepared in standard potassium phosphate buffer, and washed into the columns with two 10-m1 rinses of the same buffer. The yellow material present in the ammonium sulfate fractions of both colicins, E2 and E3, was banded tightly at the tops of the columns. Protein was eluted from the columns with a 1400-ml linear gradient of 0.0 to 0.5 M NaCl in standard potassium phosphate buffer. Fractions of 10 ml were collected and monitored for absorbance and colicin activity. Colicin E3 was eluted from its DEAE-Sephadex column after the passage of approximately 200 ml of the gradient, while colicin E2 was eluted after approximately 600 ml. Fractions containing high specific activities were combined, dialyzed overnight against distilled water in a continuous flow dialyzer, and lyophilized.

CM-Sephadex Chromatography—The lyophilized preparations from the DEAE-Sephadex columns were transferred to polypropylene centrifuge tubes and resuspended in 15 to 20 ml of 0.05 M potassium phosphate buffer, pH 6.0. Polypropylene tubes were routinely used for resuspending lyophilized protein preparations, since resuspension in glass containers appeared to cause
significant amounts of surface denaturation. The resuspended preparations were applied to CM-Sephadex C-50 columns, 2.5 x 30 cm, equilibrated with the same buffer, and washed into the columns with two 5-ml rinses. Colicin was eluted with an increasing pH gradient formed by adding 700 ml of pH 6.0 equilibration buffer to the mixing chamber of the gradient maker and 700 ml of 0.05 M dibasic potassium phosphate to the reservoir chamber. Fractions of 10 ml were collected and monitored for absorbance, colicin activity, and pH. The elution profiles for CM-Sephadex chromatography of colicins Er and Es are shown in Figs. 1 and 2. Fractions were pooled as described in the figure legends, dialyzed in the continuous flow dialyzer, and lyophilized. The procedures and results for purification of colicins Er and Es are summarized in Table I.

**Disc Gel Electrophoresis**

Polyacrylamide gel electrophoresis at two different pH values was used as the initial criterion of homogeneity for the two colicin preparations. The stained acrylamide gels are shown in Fig. 3. At pH 4.0 both colicin Er and colicin Es migrate as single zones. At pH 8.6 colicin Es also appears as a single zone. Densitometer tracings, made with a Joyce-Loebl microdensitometer, of Gels B, C, and D, and of similarly run gels with twice the protein concentrations did not reveal any minor components. Colicin Er at pH 8.6 migrated either relatively diffusely, as shown in Fig. 3A, or as two zones with very similar mobilities. Separation of colicin Er preparations from CM-Sephadex chromatography into two distinct zones was observed at pH 8.6 on several different purified preparations. In order to rule out the possibility that the preparations had two zones as a result of non-isolectric conditions for the ColEr factor, colicin Es was purified from a culture grown from a single cell resolate of *E. coli* W3110 (ColEr). This colicin Es preparation also gave two zones under these electrophoretic conditions.

**Isoelectric Density Gradient Electrophoresis**

Two alternative explanations for the double zone phenomenon of colicin Er preparations at pH 8.6 are immediately apparent: there may be two active forms of this colicin, or the colicin Er preparation may contain a contaminating protein. To distinguish between these alternatives, a preparative method was used which could separate the two electrophoretic species in the colicin Er preparations and allow measurement of the colicin activity and physicochemical characteristics of each.

Lyophilized colicin Es purified from a culture grown from a single cell isolate, was run in two isoelectric equilibrium electrophoresis columns (25 mg in each column), with the use of a “pH 8 to 9” ampholyte preparation, which in fact gave its shallowest gradient between pH 7 and pH 8. Two columns were run in parallel, in order to accumulate a sufficient amount of each electrophoretic species, with the same ampholyte preparation. The columns were subjected to electrophoresis for 48 hours before fractionation. Both columns gave essentially identical results.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Colicin Er</th>
<th>Colicin Es</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume</td>
<td>Activity</td>
</tr>
<tr>
<td>Pooled NaCl extracts</td>
<td>10³</td>
<td>5.0 x 10⁶</td>
</tr>
<tr>
<td>20 to 40% ammonium sulfate fraction</td>
<td>52</td>
<td>4.0 x 10⁶</td>
</tr>
<tr>
<td>DEAE-Sephadex column (peak fractions, dialyzed, lyophilized, and redissolved)</td>
<td>16</td>
<td>2.2 x 10⁶</td>
</tr>
<tr>
<td>CM-Sephadex column (peak fractions, dialyzed, lyophilized, and redissolved)</td>
<td>1.3 x 10⁸</td>
<td>150</td>
</tr>
</tbody>
</table>
Fig. 4 shows the results for one of the two parallel columns. Two peaks of colicin activity were observed, which presumably corresponded to the two protein zones observed in acrylamide gel electrophoresis.

Two possible explanations for this phenomenon were considered. Either “isocolicins,” i.e. two colicin E2 proteins with different primary structures, are produced by the bacteria, in analogy with isoenzyme systems such as mammalian lactic dehydrogenase (24), or a single colicin E2 molecule of unique primary structure is able to exist in two forms. Each of two isocolicins from a system of this nature would not be expected to give rise to the same proportion of both colicin forms when rerun. However, if two interconvertible conformational forms of a single molecule are responsible for this phenomenon, rerunning either isolated form might be expected to give the same ratio of the two colicin forms as observed initially.

Isolated fractions were rerun in order to distinguish between these alternatives. Fraction I (isoelectric point, 7.63) from both columns was pooled, as was Fraction II (isoelectric point, 7.41), as shown in Fig. 4. The pools were dialyzed against 1 m sodium chloride for 6 hours to remove the ampholyte and allow equilibration of the two forms to occur. The two pools were then dialyzed overnight against distilled water, adjusted to 0.5% ampholyte (pH 8 to 9), and run in separate ampholyte columns for 48 hours. Fractions were collected and analyzed as before. The results of these two electrophoresis runs are shown in Fig. 5. It is clear that Fraction II (isoelectric point, 7.41) was partially converted to Fraction I. In addition, Fraction I (isoelectric point, 7.63) shifted significantly to Fraction II. In an identical experiment carried out at another time, approximately 50% of an isolated Fraction I pool was converted to Fraction II. Thus, either colicin El form appears to be able to give rise reversibly to both forms, consistent with the fact that the material placed on the original ampholyte columns was of a single primary structure.
**Ultracentrifugal Analysis**

The sedimentation coefficients of purified colicins E₈ and E₆ were determined at four concentrations and extrapolated to zero concentration to determine the $s_{20,w}$ of both proteins. Fig. 7 is a reproduction of the sedimentation run at a protein concentration of 8 mg per ml for both proteins. No contaminating peaks could be observed in the analytical ultracentrifuge when sedimentation runs were performed at 10 mg per ml at a lower bar angle. Both preparations appear to be homogeneous in this respect. Fig. 8 shows the relationship of sedimentation coefficient to protein concentration for these two proteins. The extrapolated $s_{20,w}$ values for colicins E₈ and E₆ are 4.0 ± 0.1 and 4.1 ± 0.1 S, respectively.

The molecular weights of colicins E₈ and E₆, as determined by the Yphantis short column technique, are 62,000 ± 3,000 and 60,000 ± 3,000, respectively, with the use of an assumed partial specific volume of 0.725 ml per g. Fig. 9 shows the rate of attainment of equilibrium of the two proteins during the same equilibrium centrifugation, with the use of a multichannel, short column equilibrium centerpiece (18). As can be seen in Fig. 9, the two proteins attained equilibrium at rates which are indistinguishable with the method used. No significant difference in size or shape between the two colicins could be demonstrated by the ultracentrifugal techniques described above.

**Extinction Coefficients and Amino Acid Analyses**

Approximately 25 mg each of lyophilized colicins E₈ and E₆ were dissolved in 8 ml of distilled water and dialyzed exhaustively against several changes of distilled water. To determine neutral absorption spectra, 0.5 ml of each colicin solution was removed and added to 0.5 ml of 0.02 M potassium phosphate buffer, pH 7.0. An additional 0.5 ml of each colicin was added to 0.5 ml of 0.2 M NaOH for alkaline absorption spectra. A third 0.5-ml aliquot was removed from each colicin solution for tryptophan determination by the method of Spies and Chambers (20).

Tryptophan and tyrosine contents were also calculated from the alkaline absorption spectra by the method of Goodwin and Morton (21). Tryptophan values (Table II) determined chemically and spectrophotometrically were within 10% of each other.

A 6.0-ml sample of each colicin solution was subjected to the
Amino acid compositions of each preparation were determined. The recovery of dry weight as amino acids of the colicins was 85% for colicin E2 and 84% for colicin E6, including tryptophan. The extinction coefficients at 280 nm for 1.0% solutions of colicins E2 and E6 in standard potassium phosphate buffer are 9.73 and 12.42, respectively, based on these data.

The lack of complete recovery of dry weight as amino acids suggested either the presence of a nonprotein contaminant or the existence of carbohydrate or lipid or both components of the colicin molecule. In order to examine the possibility that there was a high molecular weight nonprotein contaminant, purified preparations of colicin E2 and colicin E6 obtained from CM-Sephadex chromatography were subjected to differential centrifugation. The purified protein preparations were centrifuged at 150,000 x g in a Spinco model L2 centrifuge for 4 hours at 4°C. The supernatant from the centrifugation was removed, and the colicin in the supernatant was examined for weight recovery as amino acids. The antibiotic activity of the two colicins was unaffected by the centrifugation. The requirement of centrifugation for the complete recovery of colicin as amino acids indicates the presence of a nonprotein, high molecular weight contaminant. This conclusion is supported by the absence of any contaminating Amido schwartz-staining material in the acrylamide gel analyses. Total phosphorus (26) and carbohydrate (27) contents of the centrifuged preparations were less than 1% total by weight for both colicin E2 and colicin E6.

Table II summarizes the amino acid composition data for colicins E2 and E6. Preparations of colicins E2 and E6 used to elicit antibody response were not subjected to differential centrifugation prior to injection. Primary and secondary immunization, bleeds, sera processing, and immunodiffusion analysis are described under "Experimental Procedure." Fig. 10 is a photograph of a double diffusion experiment to characterize the two antigen-antibody systems. Both colicins give single zones against their homologous antisera, indicating the immunochemical homogeneity of the antigens. The material removed by differential centrifugation does not appear to be antigenic. As shown in Fig. 10, colicin E6 exhibits a zone of identity when tested against the colicin E1-anticolicin E1 system. The homologous system, however, contains a spur in addition to the zone of identity. Similar results are obtained when colicin E2 is tested against the colicin E2-anticolicin E2 system. These results indicate that the two proteins share common antigenic determinants but that each colicin in addition has determinants which it does not share with the other.

**Table II**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Colicin E2</th>
<th>Colicin E6</th>
<th>E. coli K-12</th>
<th>Total Protein[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>45.1 ± 0.7</td>
<td>38.7 ± 0.5</td>
<td>35.7</td>
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</tr>
<tr>
<td>Histidine</td>
<td>11.4 ± 0.4</td>
<td>11.5 ± 0.5</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>30.8 ± 0.6</td>
<td>23.8 ± 0.6</td>
<td>33.9</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>77.8 ± 0.8</td>
<td>82.5 ± 0.7</td>
<td>64.0</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>20.8 ± 0.3[^a]</td>
<td>22.7 ± 0.5[^a]</td>
<td>30.8</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>38.9 ± 0.4[^b]</td>
<td>45.4 ± 0.5[^b]</td>
<td>32.0</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>52.4 ± 0.4</td>
<td>48.8 ± 0.7</td>
<td>78.8</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>30.3 ± 0.9</td>
<td>32.6 ± 0.5</td>
<td>29.6</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>59.1 ± 0.5</td>
<td>68.8 ± 0.3</td>
<td>54.8</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>55.8 ± 0.3</td>
<td>50.0 ± 0.8</td>
<td>67.1</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>36.1 ± 0.3[^c]</td>
<td>34.7 ± 0.5[^c]</td>
<td>39.4</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>6.5 ± 0.2</td>
<td>7.6 ± 0.1</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>20.4 ± 0.3[^c]</td>
<td>18.0 ± 0.5[^c]</td>
<td>27.1</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>22.9 ± 0.2</td>
<td>29.0 ± 0.2</td>
<td>50.6</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.8 ± 0.3</td>
<td>11.5 ± 0.1</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>17.3 ± 0.3</td>
<td>17.5 ± 0.2</td>
<td>24.6</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>10.0 ± 0.6[^d]</td>
<td>10.7 ± 0.3[^d]</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.0 ± 0.1[^*]</td>
<td>1.1 ± 0.1[^*]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[^a]: Calculated from Goldstein, Goldstein, and Lowney (25).
[^b]: Extrapolated value.
[^c]: Values are for the 48-hour hydrolysis only.
[^d]: Average of chemical and spectrophotometric determinations.
[^*]: Determined as cysteic acid.

**Fig. 10.** Immunochemical analysis of colicins E2 and E6. Double diffusion analysis was performed on microscope slides. Colicin E2 and colicin E6 concentrations were 1.0 mg per ml. Antisera (Ab) to colicins E2 and E6 were undiluted. Diffusion proceeded for 5 hours at 37°C in a water-saturated desiccator, and was terminated by placing the slides in 0.9% NaCl solution to remove unprecipitated protein.

**DISCUSSION**

The few bacteriocins that have been purified to a high degree of homogeneity appear to fall predominantly into two classes. Colicin K (28), colicin V (29), and colicin A (30) activities have all been isolated in association with lipocarbohydrate-protein complexes which form a portion of the surface antigens of the noncolicinogenic bacterial cell. Purified preparations of colicin E2-317 (colicin F) also contain large amounts of carbohydrate.
In contrast to these lipoprotein-carbohydrate complexes, the bacteriocins from certain strains of *E. coli* (32), as well as *Pseudomonas aeruginosa* (33, 34) and *Listeria monocytogenes* (32), resemble elements of bacteriophage or whole phage in the electron microscope. Megacin C, a bacteriocin produced by *Bacillus megaterium*, is the only bacteriocin previously reported to be a simple protein of relatively low molecular weight (35).

The complete recovery of both colicin *E*$_2$ and colicin *E*$_3$ as amino acids and the lack of any detectable carbohydrate associated with either of these antibiotics, in contrast to the results described for colicins K, V, and A, indicate that not all colicins are structurally integrated into the surface antigens of the cell. The nature of the colicin produced by the various Col factors and the intimacy of the association of the colicins with the bacterial cell wall may be another reflection of the various physiological classes of colicinogenic factors described previously (8, 9).

Although both colicins were purified in a similar fashion, isoelectric density gradient electrophoresis showed that colicin *E*$_2$ exists in two electrophoretically distinguishable forms, while colicin *E*$_3$ exists in only one electroformic form. Multiple forms of the same enzymatic activity are most frequently the result of isozyme systems. The ability of each of the two electrophoretically distinct forms of colicin *E*$_2$ to give rise to a similar extent to the second form following isolation indicated, however, that the existence of two forms of colicin *E*$_2$ is not due to the presence of two molecules of distinguishable primary structure. Aggregation of various degrees is not responsible for the multiple forms of colicin *E*$_2$ since the two electrophoretically distinct forms are indistinguishable by sucrase gradient analysis. Recent studies on a variety of proteins, including chicken mitochondrial malate dehydrogenase (36), the penicillinase of *Bacillus cereus* (37), mushroom tyrosinase (38), and a mutant glutamate dehydrogenase from *Neurospora crassa* (39), have shown that under certain conditions some protein species of unique primary structure are capable of reversible alteration in configuration leading to two distinguishable physical conformations under a single set of conditions. The term "conformers" has been proposed (36) to describe the multiple forms a unique protein molecule may attain. Colicin *E*$_3$ appears to be capable of existing reversibly in two distinguishable forms with isoelectric points of 7.63 and 7.41. The change in isoelectric point of 0.22 pH unit is consistent with a reduction of 1 net positive charge in going from the higher to the lower pH isoelectric form (40). It is of interest that colicin *E*$_2$ exists in only one electroformic form at pH 4.0. The equilibrium between the two forms of colicin *E*$_2$ is evidently a function of pH, as it apparently is for penicillinase (37) and tyrosinase (38).

Colicins *E*$_2$ and *E*$_3$ are indistinguishable by the ultracentrifugal techniques used. They are easily distinguishable electrophoretically, however. The higher isoelectric point (or points) of colicin *E*$_2$ is reflected in its greater mole percentages of arginine and lysine residues. The amino acid compositions of the two colicins, while distinguishable from one another, bear striking similarities, and differ considerably from the over-all amino acid composition of their host. Both colicins show a significant reduction in the hydrophobic amino acids, isoleucine, leucine, tyrosine, and phenylalanine, accompanied by an increase in the charged or polar residues. In addition, both colicin *E*$_2$ and colicin *E*$_3$ contain only 1 cysteine residue per protein molecule. The relatively low proportions of hydrophobic amino acids and the highly restricted number of cysteine residues are common properties of a number of other extracellular proteins, including amylases (41), penicillins (42-44), and flagella (45) of various species. These common properties of primary structure may be a factor in the process by which the extracellular proteins pass through the cell membrane and cell wall.

The similarity in purification procedures, physical data from analytical ultracentrifugation, and amino acid analysis all indicate a striking degree of similarity between these two proteins. Similarly, the immunochemical analysis of colicins *E*$_2$ and *E*$_3$ indicates that the two proteins share common antigenic determinants. However, each colicin has antigenic units not present on the other, as demonstrated by the spurring shown in Fig. 10. More extensive immunochemical analyses by complement fixation are being carried out to determine the extent of immunological cross-reaction between these two proteins.

The data presented here suggest that there are both structural regions common to colicins *E*$_2$ and *E*$_3$ and structural elements unique to each colicin, in agreement with the prediction based on a shared specificity for a common receptor site but differing modes of killing action and differing responses to immunity. Presently, studies on the possible subunit nature of both colicins are in progress, with the hope of elucidating the chemical nature of the shared and unique biological specificities.

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