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# Purification and Characterization of Colicin D

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Colicin D-CA23, obtained by sonic treatment of mitomycin C-induced cells of *Escherichia coli* K-12 W1485 (*colD*), was purified by ammonium sulfate precipitation, gel filtration on Sephadex G200, ion-exchange chromatography on diethylaminoethyl cellulose, and isoelectrofocusing. Polyacrylamide-gel electrophoresis, sedimentation velocity analysis, and antigenic analysis indicated that the preparation was homogeneous. Colicin D is composed entirely of amino acids and hence is a simple protein uncomplexed with lipid or lipopolysaccharide. It contains six residues of cysteine per molecule. The molecular weight of colicin D is approximately 92,000, as determined by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis and gel filtration on Sephadex G200. Its sedimentation coefficient is 4.41*S*. The behavior of colicin D in solutions of sodium dodecyl sulfate and 2-mercaptoethanol indicates that it does not consist of subunits and exists as a single polypeptide chain. Its high molecular weight and presence of six cysteine residues per molecule distinguish colicin D from all colicins previously described. Although colicins D and E3 have similar modes of action, their gross molecular properties are entirely different.

Colicins are macromolecular bactericidal substances synthesized by some strains of *Escherichia coli* and are active against some other closely related strains of bacteria.

Although colicins are classified into more than twenty groups, representatives from only three, groups E, I, and K (10, 15, 16; Schwartz and Helinski, Bacteriol, Proc., p. 53, 1968), have been subjected to rigorous characterization. The results from these studies showed certain colicins to be simple proteins with molecular weights between 55,000 and 80,000 daltons, but they were at variance with earlier reports which stated colicins to be lipoglycoprotein complexes (e.g., reference 11). To generalize about the molecular properties of colicins, it is necessary to examine representatives of other colicin groups. This report describes the purification and characterization of colicin D-CA23, a colicin from a group not previously described in detail.

The receptor and immunity specificities of individual colicins may be reflected in their gross molecular properties (10, 15). It is possible that the third type of specificity inherent in colicin molecules, the mode of action, may also be reflected in their molecular properties. At present, essentially only three types of killing action exerted by colicins on sensitive cells have been described. These are: the inhibition of a wide range of energy-requiring cellular processes (e.g., reference 18), presumably due to an interference with oxidative phosphorylation; the inhibition of protein synthesis (E3; 14); and the degradation of deoxyribonucleic acid (E2; 22). Almost all colicins which have been examined for their killing activity appear to inhibit oxidative phosphorylation; only one representative of each of the other types of killing action has until now been reported. However, it has recently been found that colicin D specifically inhibits protein synthesis (K. Timmis and A. J. Hedges, *submitted for publication*) and hence has a mode of action like that of colicin E3. The question arose, therefore, as to whether its properties are similar to those of E3.

## MATERIALS AND METHODS

**Bacterial strains.** The following strains from the Bristol stock collection were used (stock numbers in parenthesis): *E. coli* K-12 W1485 $F^-$ $str^s met$ $\lambda^-$ $\lambda^r$ (UB34); *E. coli* K12 J5-3 (R1-19) *pro met* (UB268), obtained from N. Datta; *E. coli* C6 (UB67) colicin indicator strain; and *E. coli* CA23 (*colD*-CA23) (UB95). [Abbreviations: *pro*, proline requiring; *met*, methionine requiring; $str^s$, streptomycin sensitive. R1-19 is a derepressed (repressor minus) antibiotic resistance factor determining resistance to chloram-

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phenicol (CM), kanamycin, ampicillin, streptomycin, and sulfonamide.]

**Media.** The basic minimal medium (MM) contained: 0.5% $NH_4Cl$, 0.1% $NH_4NO_3$, 0.2% $Na_2SO_4$, 0.3% $K_2HPO_4$, 0.1% $KH_2PO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.2% glucose. Amino acids and antibiotics were added as required to a final concentration of 20 μg/ml. Minimal agar (MA) was MM solidified with 2% Difco agar, and yeast extract-peptone medium (YEP) consisted of MM fortified with 0.5% Difco yeast extract and 3% Difco peptone. Nutrient broth (NB) contained 1.6% Difco nutrient broth and 0.5% NaCl, nutrient agar (NA) consisted of 1.5% Oxoid blood agar base no. 2, and soft NA was 0.7% Oxoid blood agar base no. 2. The standard buffer used (PB) was 10 mM potassium phosphate buffer, *p*H 7.4.

**Materials.** Whatman diethylaminoethyl celluloses DE11 and DE52 were purchased from H. Reeve Angel Ltd., London; Sephadex G100 and G200 were obtained from Pharmacia Ltd., London; Ampholine ampholytes were obtained from LKB Instruments Ltd., Croydon; mitomycin C, ovalbumin (crystallized), α-chymotrypsinogen A (crystallized, type II), and bovine serum albumin (crystallized) were all purchased from Sigma, London; hemoglobin (crystallized), gamma globulin (Cohn fraction II), and myoglobin (crystallized) were obtained from Koch-Light Laboratories, Colnbrook; and Hibitane (chlorhexidine acetate) was purchased from ICI, Macclesfield.

**Plasmid transfers.** Equal volumes of cultures of donor and recipient strains in the early logarithmic phase of growth in NB were mixed and incubated at 37 C without shaking. After 2 hr, the conjugation mixture was blended for 30 sec, and dilutions of this mixture were plated on selective medium. Where appropriate, $col^+$ recipients were detected by the double-layer technique of Frederico (9).

**Assay of colicin.** Soft NA (5 ml) seeded with *E. coli* C6 ($10^6$ bacteria/ml) from an overnight culture in NB was poured onto a 5-ml base layer of NA. The plate was dried at 37 C for 15 min, and 0.01-ml quantities of dilutions of colicin (made immediately before in sterile PB) were applied to marked segments of the plate with a standard loop. After overnight incubation, assay plates were examined, and the end point of the titration was estimated to be the greatest dilution which would produce a zone of complete inhibition in the indicator lawn. The reciprocal of this dilution was expressed as arbitrary units per ml (AU/ml). By using strong oblique illumination when viewing assay plates, it was possible to make preliminary estimates of colicin titers after incubation of assay plates for only 2.5 hr.

**Protein determinations.** Protein concentrations were determined by the method of Lowry et al. (19), or occasionally by the biuret method (17), with bovine serum albumin used as standard.

**Molecular-weight determinations with Sephadex G200.** The molecular weight of colicin D was determined by Sephadex G200 chromatography by the method of Andrews (1), except that the eluant buffer used was PB containing 0.0002% Hibitane as a bacteriostat. To maintain a constant hydrostatic pressure on the column of Sephadex during sample application, samples in 30% sucrose were layered under the eluant buffer. The column was calibrated with the standard proteins given in the legend to Fig. 6.

**Molecular-weight determination by SDS-polyacrylamide-gel electrophoresis.** The method for determining molecular weight by sodium dodecyl sulfate (SDS)-polyacrylamide-gel electrophoresis was essentially that described by Shapiro et al. (24), except that the gel buffer contained 8 M urea in addition to 0.1% SDS. After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid and stained in 12.5% trichloroacetic acid containing 0.25% Coomassie Blue.

**Polyacrylamide-gel electrophoresis.** Polyacrylamide-gel electrophoresis was performed by the method of Davis (6).

**Isoelectric focusing.** Electrofocusing of colicin D was performed in an LKB analytical electrofocusing column by the method described by Vesterberg and Svensson (28).

**Ultracentrifugal analysis.** Colicin D was analyzed by sedimentation velocity centrifugation using a 4° aluminum centerpiece in a Spinco model E analytical ultracentrifuge equipped with a schlieren optical system.

**Antigenic analysis.** Colicin in sterile PB was introduced into rabbits via the ear vein route according to the following regime: day 1, 1 mg of colicin D in 0.5 ml of PB; day 4, 2 mg of colicin D in 0.5 ml of PB; day 7, 4 mg of colicin D in 0.5 ml of PB. On day 27, the rabbits were bled, and the serum obtained was pooled and stored at −20 C. Antiserum and antigen were subsequently analyzed by the double-diffusion precipitin reaction in agar (5). In addition, neutralization reactions were performed by incubating antiserum with dilutions of antigen at 37 C for 30 min and subsequently applying the mixtures to wells in NA seeded with *E. coli* C6 ($10^7$ cells/ml). After overnight incubation, the seeded plates were examined for inhibition zones, and the amount of colicin neutralized by the serum was calculated.

**Amino acid analysis.** Exhaustively dried samples of purified colicin D were hydrolyzed with 6 N hydrochloric acid for 22 hr at 105 C and subsequently analyzed for amino acids. Cysteine and methionine were determined by analysis of hydrolysates of samples which had been subjected to performic acid oxidation (12). Tryptophan was estimated from the alkaline adsorption spectrum of colicin D (8).

## RESULTS

**Production of a strain singly colicinogenic for colicin D.** Starch-gel electrophoresis of crude preparations of colicin from the original colicin D-producing strain *E. coli* CA23 (*colD*-CA23) revealed that this organism produces two colicins: colicin D and another as yet unidentified colicin (Timmis, Ph.D. thesis, Univ. of Bristol, Bristol, England, 1970). It is

well known that segregation of multiple plasmids may occur during transfer from one bacterial strain to another and, therefore, in order to obtain a strain producing only colicin D, an attempt was made to transfer *colD* from *E. coli* CA23 to *E. coli* K-12 W1485 by conjugation. No *col*$^+$ recipients were isolated either from low-frequency or high-frequency transfer crosses (27) and therefore a "helper" plasmid, the derepressed R-factor (R1-19), was introduced into *E. coli* CA23 by mixed culture with *E. coli* K-12 J5-3 (R1-19). R$^+$ recipients were selected on NA containing 20 $\mu$g of CM and 5 AU of colicin D per ml; 67% of the recipients received R1-19. Transfer of *colD* by conjugation from *E. coli* CA23 (*colD*) (R1-19) to *E. coli* K-12 W1485 then took place at a detectable rate. Selection for recipients which were either antibiotic resistant (on MA containing methionine and CM) or colicin D immune (on MA containing methionine and 5 AU of colicin D per ml) demonstrated that R1-19 was transfered to 0.1% of the recipients and *colD* was transfered to 0.01%. Since the rate of transfer of R1-19 in this cross was typical of a repressed plasmid, it is probable that one of the *col* factors in *E. coli* CA23 is fi$^+$ (21).

Thus the use of a derepressed R factor not only provided fertility functions which increased the rate of transfer of *colD* but also permitted indirect selection of *colD*$^+$ recipients, since 10% of all the R$^+$ recipients were *colD*$^+$.

The *colD*$^+$ recipients obtained were tested by the macrocolony test (9) for sensitivity to the colicins produced by *E. coli* CA23 and, although 42% were totally resistant, 58% were found to be slightly sensitive to these colicins. That is, 58% of the *colD*$^+$ recipients were not completely immune to the two colicins produced by *E. coli* CA23 and, hence, were likely to be singly colicinogenic. This was confirmed by starch-gel electrophoresis. The partially immune recipients produced only one electrophoretic species of colicin (colicin D), whereas those completely immune produced, in addition, a minor species identical with the minor species produced by *E. coli* CA23.

In addition to the separation of the two *col* factors, transfer of *colD* to *E. coli* K-12 W1485 resulted in a 20-fold increase in yield per cell over that obtained from *E. coli* CA23. A recipient singly colicinogenic for colicin D, *E. coli* K-12 W1485 (*colD*) (R1-19) (UB1082), was chosen as the producer strain for the large-scale production of colicin D.

**Preparation of colicin D.** Preliminary experiments showed that the synthesis of colicin D is highly inducible and is a lethal event for the producer cell.

An aerated overnight culture of UB1082 in YEP (3.2 liters) was used to inoculate 32 liters of the same medium which was then incubated at 36 C with vigorous agitation. Antifoam was added as required. After incubation of the culture for 195 min (approximately $10^9$ cells/ml), mitomycin C was added to a concentration of 0.4 $\mu$g/ml and, after a further 210 min (about $10^6$ viable cells/ml), the cells were harvested by passing the culture through a Sharples continuous-action centrifuge.

The cell paste (125 g) was washed with PB, suspended in 400 ml of 100 mM PB, stored overnight at 4 C, and then sonically treated with a Dawe Soniprobe, type 1130, using a flow-through cell cooled by a water-alcohol mixture maintained at 0 C. The sonic extract was centrifuged at 25,000 $\times$ *g* for 60 min, and the supernatant fluid was retained. All subsequent procedures were carried out at 2 C.

**Purification of colicin D.** Solid ammonium sulfate was added slowly to the stirred crude colicin solution to the level of 30% saturation. After 15 min, the solution was centrifuged and the precipitate was discarded. Salt was further added to the supernatant fluid to produce a final saturation of 50% and, after stirring for 15 min, the precipitate was collected by centrifugation. This precipitate, which contained 93% of the total activity present in the sonic extract, was dissolved in a minimum volume of PB.

To perform high-resolution chromatography, the crude material was subjected to a preliminary cleaning and concentration procedure. This consisted of low-resolution gel filtration on Sephadex G200, which removed considerable very-high- and low-molecular-weight compounds, adsorption to and stepwise elution from diethylaminoethyl cellulose (DE11, Fig. 1a), and lyophilization. The colicin thus obtained was dissolved in 6 ml of PB and divided into two portions. Each portion was chromatographed on a column of Sephadex G200, 25 $\times$ 300 mm (Fig. 1b). Active fractions eluted from the Sephadex column were applied directly to a column of microgranular diethylaminoethyl cellulose (DE52), 10 $\times$ 200 mm, and washed thoroughly into the column with PB. Colicin D was subsequently eluted as a single peak by application of a 200-ml linear 10 to 200 mM PB gradient (Fig. 1c). Fractions containing the highest specific activity were pooled, dialyzed against distilled water, and lyophilized. This colicin will subsequently be referred to as DE52 colicin D.

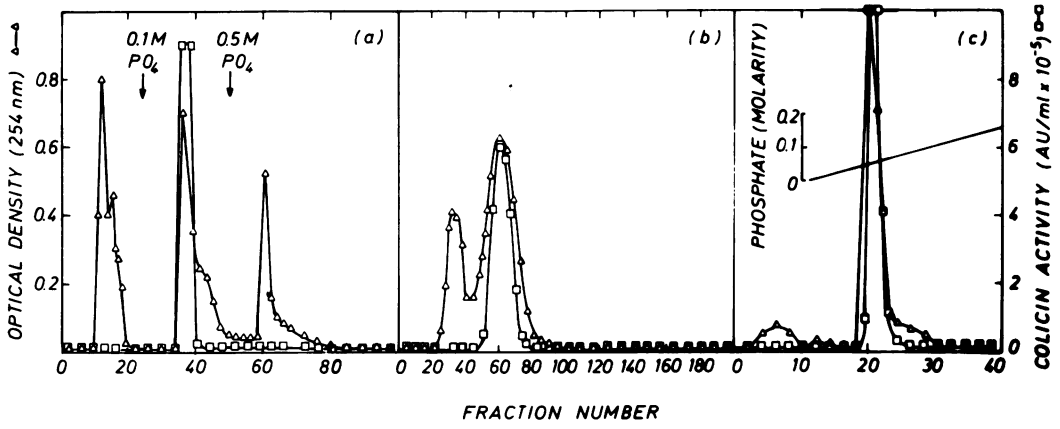


FIG. 1. *Chromatography of colicin D. To concentrate the colicin D solution after low-resolution Sephadex G200 chromatography, it was applied directly to a diethylaminoethyl (DEAE) cellulose (DE11) column, 25 × 150 mm. After washing the column with $10^{-2}$ M PB, colicin D was eluted by $10^{-1}$ M PB (a). Peak fractions of colicin activity were bulked, dialyzed against 3 × $10^{-3}$ M PB, and lyophilized. This material was dissolved in 6 ml of $10^{-2}$ M PB and divided into two portions; each was separately chromatographed on Sephadex G200 (b). Fractions with high specific activities eluted from the Sephadex were applied directly to a high-resolution DEAE cellulose (DE52) column and, after extensive washing of the column with PB, colicin D was eluted by the application of a linear $10^{-2}$ to 2 × $10^{-1}$ M phosphate buffer gradient (c). Peak fractions of colicin activity were bulked, dialyzed against distilled water, and lyophilized (= DE52 colicin D).*

Since a minor contaminant was still present in this material (as revealed by antigenic analysis, see Fig. 5), a portion was further purified by isoelectric focusing. As may be seen from Fig. 2, colicin D activity banded tightly in a single peak, which suggests the absence of conformers (10). However, it precipitated at its isoelectric point (*p*H 4.70), and only 3 mg of active material out of the 10 mg applied to the column were recovered. Nevertheless, isoelectrofocusing did remove the minor contaminant detected in DE52 colicin D by antigenic analysis, and probably precipitation during isoelectrofocusing could be avoided by the use of solubilizing agents such as urea.

From 125 g (wet weight) of cells, we obtained 368 mg of DE52 colicin D. The specific activity was increased from 1.54 × $10^5$ AU/mg of protein to 2.0 × $10^5$ AU/mg of protein by the isoelectrofocusing stage (Table 1).

**Estimates of purity.** Figures 3, 4, and 5 demonstrate that, by three criteria, polyacrylamide-gel-disc electrophoresis, velocity centrifugation, and antigenic analysis, the colicin D preparation was pure: only one molecular species could be detected when high concentrations of protein were used. When colicin D was electrophoresed in a starch gel which was subsequently sliced, one slice treated with a protein stain and the other overlayered with NA seeded with *E. coli* C6, the single zone of colicin D activity detected corresponded exactly with the single zone of protein.

DE52 colicin D contained a minor contaminant species which, at high concentrations, produced a faint second precipitin line in a double-diffusion antigen-antibody reaction in agar (Fig. 5a). This contaminant was not revealed by other analytical techniques and was removed by isoelectrofocusing (Fig. 5b).

**Characterization of colicin D.** Colicin D was characterized by determining its chemical nature, sedimentation coefficient, molecular weight, amino acid content, and immunochem-

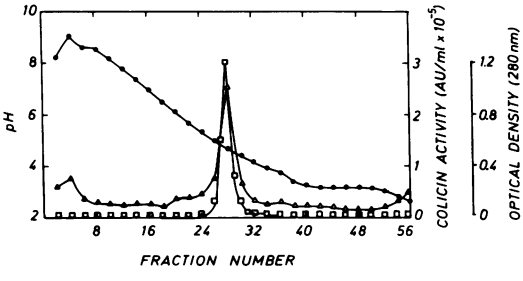


FIG. 2. *Isoelectric focusing of colicin D. A 10-mg amount of DE52 colicin D was electrophoresed in an analytical isoelectrofocusing column (LKB) containing broad-spectrum pH 3 to 10 Ampholines at an initial voltage of 250 v and current of 4 ma. After electrophoresis for 27 hr at 18 C, the current had stabilized at a minimum value, and electrophoresis was terminated. The column was emptied (30-drop fractions) by using an Ultrorac fraction collector (LKB), and the pH (●), absorbance at 280 nm (Δ), and colicin activity (□) of each fraction were determined.*

TABLE 1. *Purification of colicin D*

| Step | Activity (AU)[a] | Protein (mg) | Specific activity (AU/mg of protein) | Yield (%) |
|---|---|---|---|---|
| Sonic treatment | $2.22 \times 10^8$ | 15,170 | $1.46 \times 10^4$ | 100 |
| 30–50% Ammonium sulfate fraction | $2.06 \times 10^8$ | 5,665 | $3.64 \times 10^4$ | 93 |
| Crude G200 | $1.14 \times 10^8$ | 2,139 | $5.33 \times 10^4$ | 51 |
| DEAE[b] cellulose | $1.20 \times 10^8$ | 1,201 | $1.00 \times 10^5$ | 54 |
| G200 | $7.60 \times 10^7$ | 511 | $1.49 \times 10^5$ | 34 |
| DE52 cellulose | $5.67 \times 10^7$ | 368 | $1.54 \times 10^5$ | 26 |

[a] AU, Arbitrary unit.
[b] DEAE, Diethylaminoethyl.

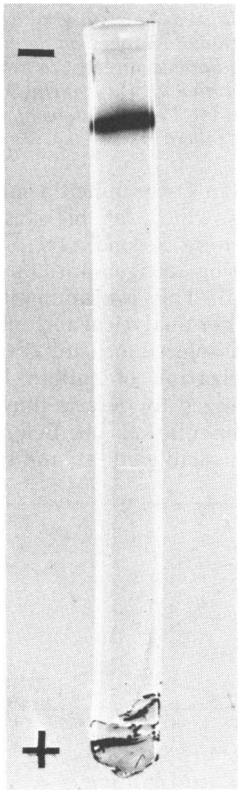


FIG. 3. *Polyacrylamide-gel electrophoresis of colicin D. Colicin D (DE52 colicin D, 40 μg) in 20 μliters of reservoir buffer containing 10% sucrose was layered onto the surface of 10% acrylamide gels buffered at pH 9.5 under the reservoir buffer, and electrophoresis was carried out at room temperature for 60 min at 70 v (constant voltage) in a Shandon disc electrophoresis apparatus. Gels were subsequently stained in a 1% solution of acetic acid saturated with amido black, and unbound stain was removed by successive washes in 1% acetic acid.*

ical properties.

**Chemical nature.** Although most colicins appear to be simple proteins, it has been stated that a few colicins and other bacteriocins are lipoglycoprotein or glycoprotein complexes (2, 13, 26). Colicin D is extremely sensitive to heat (50% inactivated in 8 min at 80 C at a concentration of 20 μg/ml in PB) and trypsin (50% inactivated in 5 min at the same concentration with trypsin at 200 μg/ml). No hexose could be detected in 2 mg of colicin D by using the anthrone reagent (7). Direct estimates of the amino acid content by amino acid analysis, and the peptide bond content using the biuret reagent, of a thoroughly dried sample of colicin D indicated that greater than 99% of the material consisted of amino acids. Thus, colicin D is a simple protein, uncomplexed with other molecular species. Its neutral adsorption spectrum was typical of nonchromogenic proteins with a maximum absorption at 279 nm and a specific absorbancy of 0.726 liter per g per cm at 280 nm.

**Ultracentrifugal analysis.** Sedimentation analysis of colicin D was performed at three concentrations, 5, 10, and 15 mg/ml (in 10 mM PB, pH 7.0, containing 100 mM NaCl), and the $S$ values obtained were extrapolated to zero concentration. This value was then adjusted to the standard sedimentation coefficient, $S_{20,w}$, which was 4.41$S$.

**Molecular-weight determination.** The molecular weight of colicin D was determined to be 96,000 by analytical Sephadex G200 chromatography and 89,000 by SDS-polyacrylamide-gel electrophoresis (Fig. 6a and 6b).

In addition to migrating as a polypeptide with a molecular weight of 89,000 in SDS-polyacrylamide gels, colicin D maintained full biological activity during the preelectro-

phoresis reduction-denaturation procedure (incubation at 37 C in the presence of 1% SDS and 1% 2-mercaptoethanol). Even when this treatment was prolonged for 7 hr, no loss of activity, as determined by rapid dilution and immediate assay, could be detected. It therefore seems probable that colicin D is not composed of subunits. In addition, colicin D which had not been preincubated with 2-mercaptoethanol had a mobility during SDS electrophoresis identical to that which had been subjected to the reduction procedure. This suggests that the molecule consists of a single polypeptide chain.

**Amino acid analysis.** After isoelectrofocusing, colicin D was exhaustively dialyzed against distilled water, chromatographed on a small column of Sephadex G100 equilibrated with distilled water, and lyophilized. One portion of this material was subjected to performic acid oxidation, and then both portions were analyzed for amino acids (Table 2). From the amino acid analysis, the partial specific volume of colicin D was calculated to be 0.74 ml/g (4).

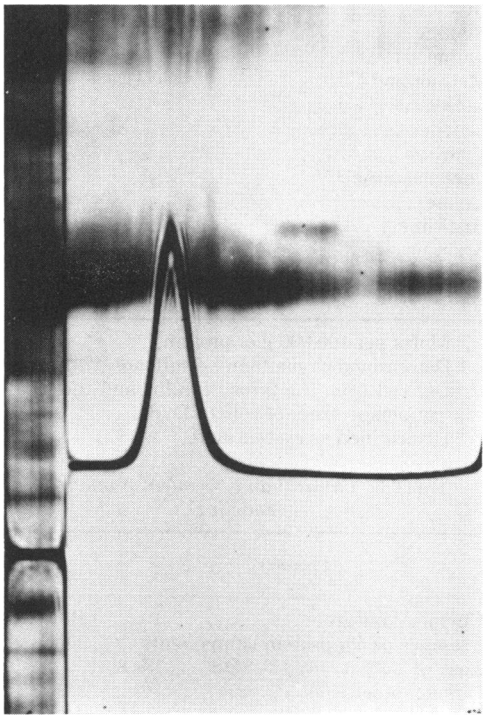

FIG. 4. *Velocity sedimentation of colicin D. Colicin D (DE52 colicin D) at a concentration of 15 mg/ml in $10^{-2}$ M PB (pH 7.0) containing $10^{-1}$ M NaCl was centrifuged at 59,780 rev/min in a Beckman model E analytical ultracentrifuge. Photographs were taken at 8-min intervals with a bar*

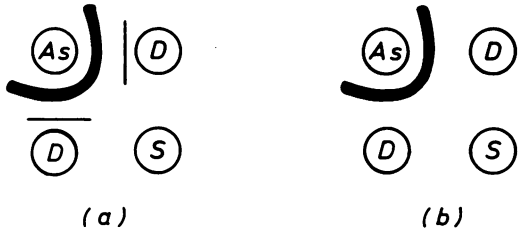


FIG. 5. *Antigenic analysis of colicin D. Double-diffusion analysis in agar was performed on microscope slides. Diffusion was allowed to proceed for 24 hr at 37 C in water-saturated sealed petri dishes, and slides were then examined for precipitin lines by using strong oblique illumination. As, Undiluted antiserum raised against DE52 colicin D; S, undiluted preimmunization serum; D, colicin D at a concentration of 3 mg/ml in standard PB. In (a), D is DE52 colicin D; in (b), D is colicin D further purified by isoelectrofocusing.*

**Antigenic analysis.** Purified colicin D formed a single precipitin line with specific antiserum in a double-diffusion analysis (Fig. 5b). In addition, the biological activity of colicin D was completely neutralized by this serum: 1 ml of serum inactivated about 5 μg of colicin D. This antiserum did not neutralize colicin E3 at all. Although colicin E3 antiserum with which to perform the reciprocal neutralization was not available, serum prepared against purified colicin E2 (which neutralized colicins E2 and E3 with equal efficiency) was shown to have no effect on colicin D. Thus no antigenic homology between colicins D and E3 could be demonstrated.

## DISCUSSION

Table 3 summarizes the properties of colicin D-CA23. Like other colicins which have recently been characterized, it is a protein uncomplexed with polysaccharide or lipopolysaccharide. It seems probable that the earlier lipoglycoprotein colicins isolated without induction and after considerable cell autolysis were colicin-receptor complexes. The fact that, during SDS electrophoresis, a technique which permits determination of the molecular weight of protein subunits, colicin D migrated as a protein with a molecular weight similar to that obtained from gel filtration studies, and the fact that this colicin maintained full biological activity for extensive periods in reducing conditions which favor dissociation of protein

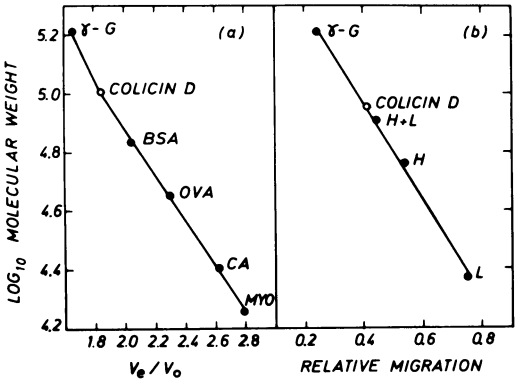


FIG. 6. *Molecular-weight determination of colicin D by gel filtration on Sephadex G200 (a) and by sodium dodecyl sulfate (SDS)-polyacrylamide-gel electrophoresis (b). (a) Protein samples in eluant buffer containing 30% sucrose were layered under the reservoir buffer onto the surface of the column of Sephadex (90-cm long, 1-cm diameter), and the column effluent was continually monitored for adsorption at 254 nm with a Uvicord I (LKB) ultraviolet analyzer coupled to an LKB single-channel direct-current recorder. Up to three standard proteins, at concentrations of 8 to 15 mg/ml, or colicin D were used in each run; all samples contained 0.2% Blue Dextran. Abbreviations: γ-G, gamma globulin; BSA, bovine serum albumin; OVA, ovalbumin; CA, α-chymotrypsinogen A; MYO, myoglobin; Ve, elution volume; Vo, void volume of the column = elution volume of Blue Dextran. (b) Quantities (100 μg) of reduced or unreduced protein [i.e., incubated for 3 hr in 10 mM PB (pH 7.1) containing 1% SDS either in the presence or absence of 1% 2-mercaptoethanol, respectively] were used for each gel. Included in each sample were 25 μg of reduced hemoglobin as internal reference. All migration values are expressed relative to the migration of this standard. Abbreviations: γ-G, unreduced gamma globulin; H + L, dimer consisting of one heavy and one light chain of reduced gamma globulin; H, heavy chain, and L, light chain, of reduced gamma globulin.*

weight of approximately 92,000.

The high molecular weight plus the presence of six cysteine residues per molecule distinguish colicin D from all other colicins which have been examined. Unlike colicin E2, but like E3, it does not exist as conformers (10).

It is generally accepted that colicin receptors are situated close to the cell membrane, and presumably large protein molecules do not have ready access to these inner regions of the cell envelope. The large size of colicin D might imply some difficulty in its adsorption to sensitive cells and, indeed, this has proved very difficult to detect by direct measurement (although this may be due equally to a scarcity of receptors). In addition, the lethal adsorption of colicin D, as measured by the loss of viability of sensitive cells treated with colicin, was demonstrated to be extremely sensitive to environmental conditions, and agents known to increase cell wall permeability, such as ethylenediaminetetraacetic acid, produced a dramatic increase in this lethal adsorption (Timmis, K. and A. J. Hedges, J. Gen. Microbiol. **66:** ii–iii, 1971).

Several workers refer to bacteriocins as extracellular antibiotics, because considerable quantities are often released from the cells into the culture medium after induction, apparently without cellular lysis, and they consider the presence of less than two residues of cys-

TABLE 2. *Amino acid composition of colicin D*

| Amino acid | Amt[a] |
|---|---|
| Aspartic acid | 97.8 |
| Threonine | 55.5 |
| Serine | 53.0 |
| Glutamic acid | 92.6 |
| Proline | 66.8 |
| Glycine | 54.3 |
| Alanine | 51.7 |
| Valine | 85.9 |
| Methionine[b] | 20.1 |
| Isoleucine | 53.7 |
| Leucine | 77.0 |
| Tyrosine | 22.3 |
| Phenylalanine | 27.5 |
| Lysine | 50.9 |
| Histidine | 21.7 |
| Arginine | 49.3 |
| Tryptophan[c] | 10.5 |
| Half-cystine[d] | 6.8 |

[a] Moles per 100,000 g of protein.
[b] Determined as methionine sulfone.
[c] Derived from the tyrosine value and the alkaline adsorption spectrum of colicin D (8).
[d] Determined as cysteic acid.

TABLE 3. *Physical and chemical properties of colicin D*

| Property | Determination |
|---|---|
| Chemical nature | Protein |
| Presence of nonprotein components | – |
| Loss of activity in 1% SDS[a] + 1% 2-mercaptoethanol | – |
| May exist as conformers | – |
| pI[b] | *p*H 4.70 |
| $E_{1\,cm,\,1\%}$ | 7.26 |
| $S_{20,w}$ | 4.4*S* |
| Molecular weight | ca. 92,000 |
| Partial specific volume (ml/g) | 0.74[c] |

[a] SDS, Sodium dodecyl sulfate.
[b] Isoelectric point.
[c] Calculated from the amino acid analysis (4).

teine in several colicins as consistent with this hypothesis. However, Pollock (23) suggested that for proteins to be considered extracellular their secretion should not be accompanied by irreversible cell damage, and this is certainly not the case for inducible colicins. Furthermore, although considerable colicin activity may be found in culture fluids after induction of $col^+$ cultures, even with $col^+$ strains which lack the homologous receptor, up to 90% of the total activity is usually cell bound (Timmis, Ph.D. thesis, University of Bristol, 1970). Although lysis of $col^+$ strains apparently does not occur after induction, permeability changes accompanying cell death may account for some activity being present in culture supernatant fluids soon after induction. Thus, at present, there is insufficient evidence to warrant use of the term "extracellular" to describe bacteriocins, and certainly the high molecular weight and lack of subunits of colicin D, and its cysteine content are suggestive of a cellular location for this protein. The presence of six cysteine residues in colicin D does not necessarily imply the presence of disulfide bridges, and the flexibility associated with the lack of disulfide bridges commonly found for colicins may reflect a function of the colicin molecule concerned in the lethal process, perhaps in the primary initiation of the lethal action after adsorption to a colicin receptor, rather than its extracellular nature.

It is well known that several colicins with different receptor specificities have similar modes of action, but it is not certain that stimulation of the lethal process in sensitive bacteria is identical with the adsorption process, nor that the region of the molecule concerned with adsorption is the same as that which initiates the lethal process. Indeed, there is some evidence that colicins specifically adsorb to lipopolysaccharide in the cell wall (20) but that initiation of the lethal process takes place directly on the membrane (3, 25). Therefore it is possible that colicins with the same mode of action but different receptor specificities have certain molecular properties in common, reflecting similar "lethal sites." The physical and chemical properties of colicin D are entirely different from those of E3 which has a similar mode of action. In particular, they differ in immunity specificity (both $col^+$ strains are sensitive to the heterologous colicin), molecular weight (E3: 60,000), isoelectric point (E3: *p*H 6.64), antigenic structure, and amino acid content (see reference 10). Those colicins which appear to inhibit oxidative phosphorylation also show little similarity in their molecular properties (15, 16; Schwartz and Helinski, Bacteriol. Proc., p. 53, 1968). Thus it has been shown for colicins representing two classes of killing action that gross molecular structure is in no way correlated with mode of action.

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