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Multiple Forms of Colicin E_3 from *Escherichia coli* CA-38 (col E_3, col I)†

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ABSTRACT: Multiple forms of colicin E_3 have been isolated from the extracellular fluid of an induced culture of *Escherichia coli* CA-38 (col E_3, col I). After precipitation with ammonium sulfate the protein was collected by centrifugation and passed through a Sephadex G-50 column. Chromatography on a DEAE-cellulose column effected the separation of three forms of colicin E_3, designated E_3-I, E_3-II, and E_3-III. The specific activity of E_3-I was 2–5 $\times$ 10^6 units/mg while that of E_3-II was 1–3 $\times$ 10^6 units/mg and that of E_3-III was 6 $\times$ 10^5 units/mg. These three forms of colicin E_3 have identical molecular weights (60,000) as determined by equilibrium ultracentrifugation and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The amino acid compositions of E_3-I and E_3-II are very similar (E_3-III was not examined because of contaminating protein material). All three forms have glycine as the N-terminal amino acid. Rechromatography of E_3-I or E_3-II on DEAE-cellulose revealed that these forms are not stable; E_3-I can convert to E_3-II and E_3-III, while E_3-II can convert only to E_3-III. These conversions are irreversible. Studies using isoelectric focusing gradients have indicated that the three forms are converted to progressively more acidic molecules by steps of approximately one charge unit with a concomitant loss of biological activity. Furthermore, these studies have demonstrated that the conversion leads to at least two additional forms, E_3-IV and E_3-V.

Colicins are highly specific antibiotic proteins produced by certain strains of intestinal bacteria which kill other, usually closely related, strains of bacteria. The potential to produce colicin (colicinogeny) is conferred by the presence of extrachromosomal DNA called a col factor (DeWitt and Helinski, 1965; Clewell and Helinski, 1969). Colicin production can be induced by treatment of colicinogenic cells with ultraviolet radiation or mitomycin C. The colicin produced by induced cells is released into the medium where it kills sensitive bacteria by adsorbing to specific receptor sites on the cell surface rather than by penetrating and acting from within the cell (Fredericq, 1948; Maeda and Nomura, 1966). A colicinogenic cell is immune to the specific colicin it is capable of producing.

Colicins have been classified into groups according to their specificity for receptor sites (Fredericq, 1948); each group of colicins binds to a unique receptor. The three members of the E group of colicins, E_1, E_2, and E_3, all bind to a common receptor site. They were originally differentiated by the fact that a cell colicinogenic for one of the three was immune to its own colicin but sensitive to the other two. It was also shown that these three colicins have different biochemical targets; E_1 causes disruption of all macromolecular synthesis, presumably by interfering with oxidative phosphorylation (Luria, 1964), E_2 causes degradation of DNA (Jacob *et al.*, 1952), while E_3 causes cessation of protein synthesis (Nomura, 1963). Recently, the chemical lesion caused by colicin E_3 has been shown to be the specific cleavage of the 16S rRNA, resulting in inactive ribosomes (Senior and Holland, 1971; Bowman *et al.*, 1971).

Colicin E_3 has been purified and characterized by Herschman and Helinski (1967), who showed this colicin to be a

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protein with a molecular weight of about 60,000 which behaves as a single homogeneous species with an isoelectric point of 6.64. Their results suggested that colicin E_3 would be a good material for protein structural studies which might elucidate the interaction between the colicin and its specific receptor site. A new purification scheme has been devised which is relatively rapid and gentle, and which produces considerably greater yields of product than that of Herschman and Helinski (1967). In this procedure, colicin E_3 is isolated from the extracellular fluid of a culture of *Escherichia coli* CA-38 (col E_3, col I) which has been induced with mitomycin C. The isolated colicin E_3 differs in some properties from that of Herschman and Helinski (1967), which was isolated from cells rather than from the supernatant fluid. In particular, we have observed several separable forms of the protein which bear precursor–product relationships to one another, and which appear to differ significantly in specific biological activity.

Experimental Section

Materials

Mitomycin C was obtained from Sigma Chemical Co. Dansylamino acid standards and Special Enzyme grade ammonium sulfate were obtained from Schwarz–Mann. Randomly labeled L-[^{14}C]leucine and L-[4,5-^{3}H]leucine were obtained from New England Nuclear Corp. Glucostat reagent was a product of Worthington Biochemical Corp.

E. coli CA-38 (col E_3, col I) and *E. coli* A596 colicin I^S colicin E^r were obtained from M. Nomura, *E. coli* W 3110 (col E_3, col I) was obtained from D. Helinski, and *E. coli* row str^r colicin E^S was obtained from C. Shemin.

M-9 medium (pH 7.0) contains (per liter) 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 4 g of glucose, 10^{-4} M $MgSO_4$, and 10^{-6} M $FeCl_3$. M-9 cas medium is M-9 medium containing 5 g of Casamino Acids (Difco)/l., and M-9 low cas medium is M-9 medium containing 0.5 g of Casamino Acids/l. Nutrient agar was prepared with 8 g/l. of Nutrient Broth (Difco) supplemented with 1.5% agar. Soft nutrient agar contained 0.75% agar. Streptomycin plates are nutrient agar containing 400 μg/ml of streptomycin. TDE buffer is 0.03 M Tris-HCl (pH 8.0) containing 1 mM DTT[1] and 1 mM EDTA.

Methods

Colicin Assay. The solution to be assayed was diluted 100-fold with sterile saline. This solution was further diluted tenfold, followed by eleven twofold dilutions. Ten microliters of each dilution was spotted on an agar assay plate freshly overlaid with 10^8 indicator bacteria in 4 ml of soft agar. Streptomycin plates were used for assays using *E. coli* row str^r colicin E^S as an indicator strain while assays using *E. coli* A596 colicin I^S colicin E^r were carried out on nutrient agar plates without streptomycin. The plates were incubated at least 12 hr at 37°. The highest dilution which gave a clear zone of inhibition of growth in the lawn produced by the indicator bacteria after incubation is defined as the number of units per milliliter of colicin activity.

Protein Determination. Quantitative determinations of protein were carried out using the method of Lowry *et al.* (1954), with bovine serum albumin as the standard. Absorbance at 280 nm was used to estimate protein in fractions from the columns. One A_{280} unit of protein is equal to 1.0 mg of protein as determined by the method of Lowry *et al.*

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis at pH 9.5, 6.6, and 4.3 in 7.5% acrylamide gels was carried out essentially as outlined in the manual supplied by Canalco. However, samples were layered on the gels in 20% glycerol. Isoelectric focusing polyacrylamide gels were prepared according to the method of Wrigley (1968) using LKB 5–8 ampholyte mixture. These gels were run using 1% phosphoric acid as the anode solution and 1% ethanolamine as the cathode solution. Gels were removed from the tubes and fixed with 12.5% trichloroacetic acid for 30 min. Free ampholytes were partially removed by electrophoresis in a transverse destainer with 2.5% trichloroacetic acid. Finally, the gels were washed with water and stained with Coomassie Blue.

Isoelectric Focusing Gradients. Isoelectric focusing in 110-ml sucrose gradients was carried out according to the manual supplied by LKB. Samples were prepared by dialysis against 1% glycine overnight. The ampholyte range was pH 5–8, the applied potential was 700 V, and the length of a run was 48 hr at a temperature of 8°. Fractions of 1 ml were collected from the column and their pH was determined at 22° using a Model 700 Orion pH meter with an Ag–AgCl probe electrode.

Ultracentrifugation. Sedimentation experiments were carried out in a Beckman Model E ultracentrifuge equipped with uv optics. Sedimentation velocity experiments were carried out at 7° and 50,740 rpm. Sedimentation equilibrium experiments were carried out at 4° and 10,000 rpm for 48 hr.

Amino Acid Analysis. Protein samples were hydrolyzed with 5.7 N HCl for 6, 12, and 24 hr at 112° in sealed, evacuated tubes. After removal of the HCl under vacuum over NaOH pellets, the samples were analyzed on a Spinco Model 120 amino acid anaylzer using an accelerated system. Protein sulfhydryl groups were determined with DTNB (Ellman, 1959) in SDS solution according to the method of Damjanovich and Kleppe (1967).

Glucose Analysis. Protein samples were hydrolyzed by autoclaving with 0.125 N HCl for 45 min. Samples were neutralized, buffered with 0.5 M potassium phosphate (pH 7.0), and analyzed with Glucostat reagent.

Radiation Counting. Protein samples were precipitated with 5% trichloroacetic acid in the presence of 0.1 mg of bovine serum albumin. After filtration on Whatman GF/C-24 glass fiber filters, washing, and drying with acetone, the filters were placed in scintillation vials with 5 ml of phosphor solution containing 4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-[2-(5-phenyloxazolyl)]benzene per l. of toluene.

Results

Growth and Induction. E. coli CA-38 (col E_3, col I) was grown from a 10% innoculum of log-phase cells to mid-log phase (approximately 120 Klett units, 5×10^8 cells per ml) in 3-l. fernbach flasks containing 1 l. of M-9 cas medium. At this time 0.2 mg of mitomycin C/l. of culture was added. Approximately 40 min after the addition of mitomycin C the turbidity of the culture begins to decline sharply, continuing for an additional 80 min when it levels off. Colicin is released into the medium, reaching its maximum concentration in the supernatant about 120–180 min after the addition of mitomycin C, concomitant with the beginning of the plateau in turbidity. This is shown in Figure 1.

Isolation. At the time of estimated maximum colicin ac-

[1] Abbreviations used are: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

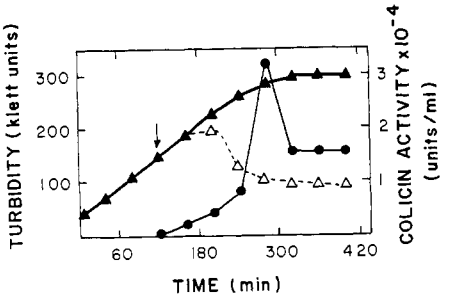

FIGURE 1: Growth and colicin induction of *E. coli* CA-38 (col E_3, col I). Mitomycin C (0.2 mg/l.) is added to induced culture at 120 min (arrow). Turbidity of uninduced culture (▲); turbidity after addition of mitomycin C (△); colicin activity in supernatant fluid (●).

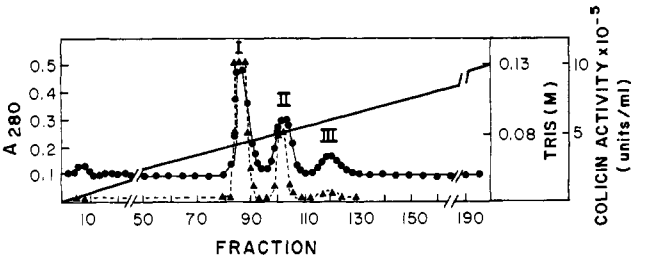

FIGURE 2: Chromatography of colicin E_3 on microgranular DEAE-cellulose. Conditions are described in the text. Straight line is the calculated concentration of Tris-HCl. Absorbance at 280 nm (●); colicin activity (▲). The peaks labeled I, II, and III are defined as colicins E_3-I, E_3-II, and E_3-III, respectively.

tivity the culture was cooled to 4° in an ice bath and the cells were removed by centrifugation. Solid ammonium sulfate was dissolved in the clear supernatant fluid (400 g/l. of fluid) and the resulting mixture was allowed to stand overnight at 4°. Precipitate from 6 l. of supernatant fluid was collected by centrifugation at 15,000*g* for 30 min and taken up in 15 ml of TDE buffer. This cloudy solution was centrifuged at 15,000*g* for 30 min and the supernatant was removed and stored in ice. The precipitate was resuspended in 10 ml of TDE buffer, and recentrifuged. The pooled supernatants were warmed to room temperature and passed through a 2.3 × 45.0 cm column of Sephadex G-50 equilibrated with TDE buffer. The protein eluting with the void volume, as monitored by absorbance at 280 nm, contained all the colicin activity.

The colicin solution was then applied to a 2.5 × 40 cm column of microgranular DEAE-cellulose (Whatman DE-52) equilibrated with TDE buffer and was eluted at room temperature with a 2-l. gradient of 0.03–0.13 M Tris-HCl (pH 8.0), containing 1 mM EDTA and 1 mM DTT, at a flow rate of 2 ml/min. Fractions of 10 ml were collected and monitored for absorbance at 280 nm and for colicin activity (Figure 2). Column chromatography separated three peaks of colicin activity, designated E_3-I, E_3-II, and E_3-III. In all experiments, E_3-I was present in the greatest amount, the quantity of E_3-II was approximately half that of E_3-I, and E_3-III occurred only in small amounts. In early experiments, gel filtration and column chromatography were carried out at 4°; there was clear evidence for at least two components, but resolution was poor at that temperature.

Yields and specific activities at each step of the purification are summarized in Table I. The large decrease in total activity seen after precipitation with ammonium sulfate appears to be due to the presence of the ammonium sulfate, as full activity is regained after removal of the salt. Total activity appears to increase in the purification procedure, but this is probably a reflection of the inaccuracy inherent in the assay.

The active fractions of each peak were pooled and concentrated by ultrafiltration using an Amicon PM-30 membrane. At a protein concentration of approximately 5 mg/ml, the colicin preparations appeared to be stable for up to 1 week at 4°. After 10 days about 90% of the activity of both E_3-I and E_3-II is lost. The least active fraction, E_3-III, loses all its killing activity in that time. Slow freezing and thawing of any of the colicin fractions causes nearly complete loss of activity. Protein samples for amino acid analysis and N-terminal analysis were desalted on a Sephadex G-50 column equilibrated with water, lyophilized, and stored at −20°. Activity loss could be reduced by 50% with this procedure.

Preparation of Radioactive Colicin E_3. Colicin labeled with isotopic leucine was prepared by growing the culture to mid-log phase in M-9 low cas medium, centrifuging the cells, and washing them twice with M-9 medium. The cells were resuspended in M-9 medium, warmed to 37°, and radioactive leucine and mitomycin C were added to the culture. Labeling with ^{14}C was effected by addition of 50 μCi of L-[U-^{14}C]leucine (specific activity 255 Ci/mole) per l. of culture, and ^{3}H labeling was accomplished with 250 μCi of L-[4,5-^{3}H]leucine (specific activity 38.5 Ci/mmole) per liter of culture. Production of colicin proceeded as described previously despite the absence of added Casamino Acids. The colicin was purified as usual and the same multiple forms were obtained.

Examination of E. coli W 3110 (col E_3, col I) for Multiple Forms of Colicin E_3. Herschman and Helinski (1967) reported the isolation and purification of a single, stable species of colicin E_3 from *E. coli* W 3110 (col E_3, col I). Under the conditions of growth used in the present work, the maximum level of colicin activity in the supernatant is reached at about the same time after induction in this strain as in *E. coli* CA-38 (col E_3, col I); however, the colicin activity does not decline in the following 2 hr. The same three forms of colicin E_3 were observed when the supernatant fluid of this culture was subjected to the isolation procedure outlined above.

TABLE I: Yields and Specific Activities at Each Step in the Purification of Colicin E_3 from the Supernatant of a Culture of *E. coli* CA-38 (col E_3, col I).

Stage of Purification	Sp Act.[a] (Units/mg)	Total Act. (Units)
Growth supernatant		1.8×10^8
$(NH_4)_2SO_4$ precipitate	2.5×10^3	1.2×10^6
G-50 eluate	5.0×10^5	2.0×10^8
DEAE-cellulose		
E_3-I	3.8×10^6	1.9×10^8
E_3-II	3.1×10^6	7.8×10^7
E_3-III	6.7×10^5	7.2×10^6
Total		2.7×10^8

[a] Protein concentration is taken from the absorbance at 280 nm. In various experiments the range of specific activities was: E_3-I, 2–5 × 10^6; E_3-II, 1–3 × 10^6; E_3-III, 6–7 × 10^5. The relative order of specific activities never varied.

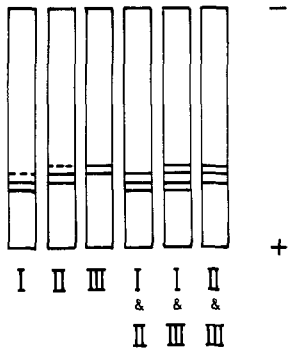

FIGURE 3: Diagramatic representation of isoelectric focusing polyacrylamide gels. Ampholyte range is pH 5–8. Samples (20 μg) of E_3-I, E_3-II, and E_3-III were run within 4 hr of their elution from DEAE-cellulose. Mixtures of 10 μg of E_3-I and E_3-II, E_3-I and E_3-III, and E_3-II and E_3-III were also run to confirm the overlap of species. Dashed lines represent minor bands.

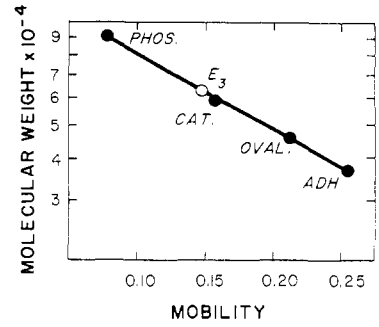

FIGURE 4: Mobility of colicin E_3-I and of markers of known molecular weights in polyacrylamide gels in the presence of SDS. Markers are phosphorylase *b* (92,000), catalase (60,000), ovalbumin (45,000), and yeast alcohol dehydrogenase (37,000). Identical results were obtained for colicins E_3-II and E_3-III. The apparent molecular weight of the colicins is 62,000.

Biological Properties of the Multiple Forms of Colicin E_3. Since multiple forms of colicin E_3 had not been reported previously, it was necessary to establish that the three peaks of colicin activity were in fact forms of colicin E_3 and not other colicins. All three components were tested for colicin I activity since the bacterial strain used as the source of the colicin preparation also carries the col I factor. However, none of the fractions showed any activity when tested against *E. coli* A596 colicin I^s colicin E^r as an indicator strain. Furthermore, if any of the isolated colicins are not colicin E_3, killing activity against a strain carrying col E_3 should be observed. The colicins were tested on *E. coli* W 3110 (col E_3, col I) and *E. coli* W 3110 (col E_2) as indicator strains. The isolated colicins were active only on the *E. coli* W 3110 which carried the col E_2 factor. The behavior of each of the peaks is consistent with known properties of colicin E_3.

Polyacrylamide Gel Electrophoresis. Samples of E_3-I, E_3-II, and E_3-III, when subjected to polyacrylamide gel electrophoresis in the presence of SDS (Weber and Osborn, 1969), gave single, sharp bands with either high or low concentrations of protein. E_3-I and E_3-II appear to be pure by this criterion while E_3-III has a minor low molecular weight component in some preparations. A mixture of the three forms also gave a single, sharp band. This result indicates that the three forms have the same monomer molecular weight.

The three forms of colicin E_3 were also subjected to electrophoresis in standard 7.5% polyacrylamide gels at pH 9.5, 6.6, and 4.3. In contrast to the results reported by Herschman and Helinski (1967) there were no distinct bands seen at either pH 9.5 or 4.3. The proteins appeared as very diffuse bands near the origin. This may be due to the presence of several charged forms. No migration occurred at pH 6.6 which is close to the isoelectric point of the proteins.

Isoelectric focusing in 7.5% polyacrylamide gels was also carried out on the three forms of colicin E_3. The resulting bands are shown diagrammatically in Figure 3. Each colicin, as isolated from the DEAE-cellulose column, is seen to be composed of several species with different isoelectric points. It appears from this experiment that the sets of species are different, although there clearly is overlap among the three colicins. Gels run as early as 1 hr after DEAE-cellulose chromatography show these profiles of multiple components.

Isoelectric Focusing Gradients. Colicins E_3-I, E_3-II, and E_3-III were subjected to isoelectric focusing in gradients of pH 5–8 stabilized by sucrose gradients. Each colicin gave a set of well-resolved peaks. Isoelectric points of the components are summarized in Table II. This tabulation shows that the p*I* 6.55 species of E_3-I is absent in E_3-II and E_3-III. Similarly, the p*I* 6.43 species of E_3-II does not appear in the profile of E_3-III. Designation of the species as I, II, III, IV, and V is intended to imply that I may be identical with E_3-I, II may be identical to E_3-II, etc. as these species elute from the DEAE-cellulose column.

Molecular Weight Determination. Molecular weights of the three forms were determined by polyacrylamide gel electrophoresis in the presence of SDS with markers of known molecular weights. These data are shown in Figure 4. The molecular weight of each form of colicin E_3 was calculated to be 62,000.

Ultracentrifugation of E_3-I and E_3-II immediately after elution from DEAE-cellulose gave $s_{20,w}$ values of 4.12 and 4.11 S, respectively. These are similar to the value of 4.1 S reported by Herschman and Helinski (1967). Equilibrium ultracentrifugation was carried out on all three forms of colicin E_3. From plots of ln *c vs.* r^2 molecular weights of 59,000 were calculated for each of the three forms. A partial specific volume of 0.720 was calculated from the amino acid composition. E_3-I and E_3-III gave linear plots, while E_3-II showed very slight deviation from linearity. This result agrees well with the molecular weight determined by Herschman and Helinski (1967) for their preparation, and the value of 60,000 will be used as the best estimate of the molecular weight.

Amino Acid Analysis. Amino acid analyses of E_3-I and E_3-II are shown in Table III. The amino acid compositions of the two forms are quite similar to one another and to that reported by Herschman and Helinski (1967) for colicin E_3. Amino acid determination was not carried out on E_3-III be-

TABLE II: Summary of p*I* Values From Isoelectric Focusing of Colicin Forms in Sucrose Gradients.

Colicin	p*I* I	II	III	IV	V
E_3-I	6.55	6.38	6.24		
E_3-II		6.43	6.30	6.16	
E_3-III			6.33	6.17	5.93
Average	6.55	6.40	6.29	6.16	5.93

TABLE III: Amino Acid Compositions of Colicins E_3-I and E_3-II.[a]

Amino Acid	Residues/60,000 Mol Wt		
	E_3-I	E_3-II	Herschman and Helinski (1967)
Lys	43.3	42.5	38.7
His	11.3	11.0	11.5
Arg	23.9	25.2	23.8
Asp	82.2	82.0	82.5
Thr[b]	24.4	23.7	22.7
Ser[b]	44.7	44.7	45.4
Glu	48.2	50.3	48.8
Pro	35.0	35.1	32.6
Gly	66.2	67.5	68.7
Ala	50.5	52.0	50.0
Val	37.5	38.4	34.7
Met	7.9	7.5	7.6
Ile	18.4	18.1	18.0
Leu	28.8	27.8	29.0
Tyr	10.8	11.6	11.5
Phe	17.0	17.9	17.5
Trp[c]	10.6	10.6	10.7
SH[d]	1.0	1.0	1.1

[a] Results of duplicate 24-hr hydrolysates. [b] Extrapolated to zero time from values obtained after 6-, 12-, and 24-hr hydrolysis. [c] Determined by Ehrlich method of Spies and Chambers (1948). [d] Determined by method of Damjanovich and Kleppe (1967) using DTNB and SDS.

cause of the contaminating protein material. Both E_3-I and E_3-II were found to have one sulfhydryl group per mole. This cysteine will react with DTNB only in the presence of SDS.

Amino-Terminal Residues. The discovery of an aminopeptidase in *E. coli* (Simmonds, 1970) suggested the possibility that the differences among the three forms might be caused by aminopeptidase degradation. The N-terminal amino acids of all three forms of colicin E_3 were found to be glycine[2] by the dansylation method of Gros and Labouesse (1969). These data indicate that the differences observed among the three forms are probably not due to degradation at the N terminus.

Sugar Content. Since colicin K contains the lipopolysaccharide of the somatic O antigen (Hinsdill and Goebel, 1966), colicins E_3-I and E_3-II were examined for sugar content. Enzymatic determination of glucose after mild acid hydrolysis showed less than 0.1 mole of glucose/mole of colicin in each of these proteins. The general sugar determination of Dubois *et al.* (1956) revealed no evidence for the presence of pentoses, hexoses, or deoxy sugars in E_3-I and E_3-II. Examination of the amino acid chromatograms of these proteins indicated the absence of glucosamine, galactosamine, and mannosamine.

Interconversion of Multiple Forms of Colicin E_3. To verify the relationship between E_3-I and E_3-II, two samples of colicin E_3 were prepared, one labeled with ^3H and the other with

[2] In similar experiments, the N-terminal amino acid of colicin E_2 was found to be serine.

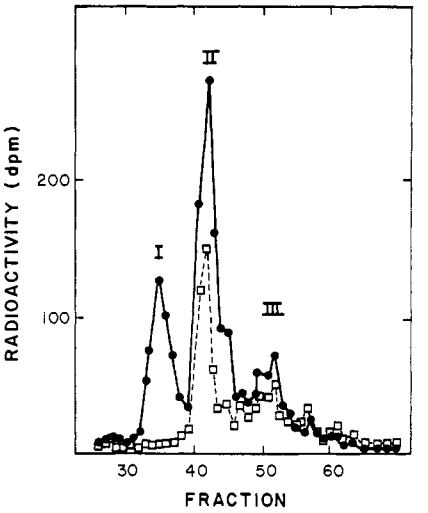

FIGURE 5: Rechromatography of a mixture of [^3H]E_3-I and [^{14}C]-E_3-II on DEAE-cellulose. Conditions are described in the text. Fraction volume is 5 ml. ^3H (●) and ^{14}C (□).

^{14}C. The peak tube of [^3H]E_3-I was mixed with the peak tube of [^{14}C]E_3-II and this solution was rechromatographed on a 0.9 × 45 cm DEAE-cellulose column using a gradient of 400 ml. The results are shown in Figure 5. It can be seen that E_3-I contains only ^3H whereas E_3-II contains both ^3H and ^{14}C. Similarly, the newly formed E_3-III contains both ^3H and ^{14}C and a hint of E_3-IV is seen in both isotopes. This confirms the results observed with isoelectric focusing and allows us to conclude that the conversion occurs in only one direction, from E_3-I to E_3-II to E_3-III and possibly to further forms of colicin E_3. Associated with the conversion is a loss of net positive charge resulting in more acidic molecules.

Discussion

A simple and efficient scheme for the isolation and purification of colicin E_3 has been described which involves few steps and allows very gentle handling of the colicin. The existence of multiple forms of colicin E_3 has not been reported previously although colicin E_2 has been shown to exist in two interconvertible forms in equilibrium (Herschman and Helinski, 1967). Colicins Ia and Ib have also been observed to exist in two chromatographically distinguishable forms, although the nature of the differences between these forms has not been explored (Konisky and Richards, 1970). Investigation of the properties of the three forms of colicin E_3 separated by DEAE-cellulose chromatography has shown that these forms have identical molecular weights, identical N-terminal amino acids, and that two of the forms, E_3-I and E_3-II, have closely similar amino acid compositions and sedimentation coefficients. Within the limits of the standard colicin assay, the specific activities of the three forms are different; E_3-I has the highest specific activity, E_3-II has a slightly lower specific activity, while E_3-III has relatively little killing activity.

The symmetry and spacing of the peaks of colicin E_3 activity obtained from DEAE-cellulose and isoelectric focusing suggest that the forms differ by single charge units. If we assume that the only groups in the protein that dissociate in the region of the isoelectric point, pH 6.5, are the eleven histidines and that these histidines are independent of one another and have a uniform pK_a of 6.5, we can easily calculate that the introduction of one negative charge, such as a car-

boxylate anion, will shift the p*I* down by 0.16 unit. If the pK_a of the histidines is 7.0 the shift will be 0.26 p*I* unit. The observed shift can reasonably be ascribed to the introduction of one negative charge or the removal of one positive charge.

One can propose, on the basis of these results, that DEAE-cellulose chromatography separates E_3-I, E_3-II, and E_3-III as single species with defined isoelectric points. After elution, unidirectional conversion takes place wherein these colicins are converted to more acidic molecules in steps of unit charge. E_3-I is converted to E_3-II, which in turn is converted to E_3-III. The isoelectric focusing experiments reveal that this conversion does not stop at E_3-III but goes on to at least two additional forms, E_3-IV and E_3-V. These last forms are probably not seen in the DEAE-cellulose chromatogram because they are present in small amounts.

In addition to charge, the only property which reflects any differences among the several forms of colicin E_3 is the specific killing activity. Conversion of the forms from E_3-I to at least E_3-III seems to be paralleled by a decrease in biological activity. This suggests that the transformation is a process of inactivation; however, the limitations of the colicin assay make such a conclusion tenuous.

Several mechanisms can be suggested to explain the observed conversion of the forms of colicin E_3. The possibility of contamination by an exoprotease or a deamidating enzyme has not been excluded conclusively. The similarity of amino acid compositions, N-terminal amino acids, and molecular weights indicates that the transformation is not caused by gross proteolysis. Specific deamidation of asparagine or glutamine residues would be difficult to detect. Hydrolysis of amide side chains to carboxylate groups is the basis of multiple forms of cytochrome *c*, which differ in p*I* by 0.22 unit (Flatmark, 1966). Although unsupported by evidence, the possibility exists that the colicin itself possesses enzymatic activity which brings about the conversion. Conversion could also be caused by discrete, stepwise conformational changes which result in a different charge distribution. The multiple forms of malate dehydrogenase (Kitto *et al.*, 1966) which, unlike those of colicin E_3, are stable, have been shown to differ only in conformation.

The biological significance of the multiple forms of colicin E_3 remains an intriguing question. A sensitive and accurate assay system which utilizes optimal conditions for determining colicin killing activity is required to confirm or disprove the differences in biological activity observed in this work.

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