Characterization of Multiple Circular DNA Forms of Colicinogenic Factor E_1 from *Proteus mirabilis**

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ABSTRACT: Colicinogenic factor E_1 deoxyribonucleic acid was isolated from *Proteus mirabilis* by deoxyribonucleic acid extraction and methylated albumin kieselguhr column chromatography followed by further purification by means of nitrocellulose filtration after denaturation and neutralization, and by dye-buoyant density centrifugation. The purified colicinogenic factor E_1 deoxyribonucleic acid consisted of three supercoiled deoxyribonucleic acid forms with molecular weights of 4.2, 8.5, and 12.7 $\times$ 10^6 as calculated from contour lengths, and sedimentation coefficients of 23, 31, and 37 S, respectively. Each form spontaneously gives rise to open circular deoxyribonucleic acid molecules. The degradation of the two smaller forms by pancreatic deoxyribonuclease was analyzed on sucrose gradients; the 23S and 31S forms give rise to 17S and 23S molecules, respectively, with first-order kinetics. The sedimentation properties of all three supercoiled forms are unchanged by treatment with Pronase. Thus by all criteria applied, the colicinogenic factor E_1 deoxyribonucleic acid molecules are indistinguishable in structure from other supercoiled deoxyribonucleic acid. The relationship of the sedimentation coefficient of colicinogenic factor E_1 supercoiled deoxyribonucleic acid in 1.0 M NaCl to molecular weight is $s_{20,w} = 0.034M^{0.428}$. This relationship agrees with observations for several other supercoiled deoxyribonucleic acid molecules, and implies that there is a constant number of tertiary turns per unit of length.

Colicinogenic factors are extrachromosomal genetic elements, which determine in certain enteric bacteria the production of specific antibiotically active substances termed colicins. Bacterial cells containing a colicinogenic factor acquire an immunity to the antibiotic product of the factor which they harbor (Fredericq, 1948). Certain of these colicinogenic factors are also fertility factors, while others exhibit no fertility properties and are transferable to female cells only through the action of distinctly separate fertility factors. All of the colicinogenic factors examined may be transferred during bacterial conjugation unlinked to the transfer of chromosomal markers. Colicinogenic factor E_1, a bacterial plasmid, determines the production of the antibiotic protein (Schwartz and Helinski, 1968) colicin E_1 and has no sex factor properties (Nagel de Zwaig and Puig, 1964; Kahn and Helinski, 1964).

Colicinogenic factor E_1 in *Proteus mirabilis* has been demonstrated to consist of double-stranded DNA of a buoyant density of 1.710 g/cc in a cesium chloride gradient (DeWitt and Helinski, 1965). More recently, this colicinogenic factor E_1 DNA was shown by electron microscopy to consist of two size classes of circular molecules with contour lengths of 2.3 and 4.7 μ (Roth and Helinski, 1967). Both open and supercoiled circular molecules were observed for the two size classes.

In the present study the supercoiled nature, and presumed covalently intact property, of the colicinogenic factor E_1 DNA was utilized to purify sufficiently large amounts of the various size classes of this circular DNA for physical and chemical characterization. Two method were employed as final purification steps for the colicinogenic factor E_1 DNA. The first method consisted of reversible alkaline denaturation followed by adsorption of the denatured DNA to nitrocellulose (Jansz *et al.*, 1967). The second method was the dye–buoyant density procedure described by Radloff *et al.* (1967). Colicinogenic factor E_1 DNA separated by these techniques from *P. mirabilis* chromosomal DNA was shown to consist of three closed circular forms of DNA of characteristic colicinogenic factor E_1 buoyant density which are related by contour length as monomer, dimer, and trimer. Each form by several criteria is indistinguishable in structure from other well-characterized naturally occurring, closed circular double-stranded DNA molecules.

Experimental Section

Bacterial Strain. P. mirabilis (*Col* E_1) (colicinogenic factor E_1 from *Escherichia coli* K-30) was made thymine requiring by Dr. F. Hickson in this laboratory by the procedure of Stacey and Simpson (1965). For the experiments in this study this strain was stored in Difco antibiotic 3 broth and was frequently reisolated from a single colony to maintain the level of colicinogenic cells in a culture at a minimum of 90%.

Reagents. Most of the reagents used have been previously described (Roth and Helinski, 1967). Technical grade CsCl for preparative equilibrium centrifugation was purchased from the Penn Rare Metals division of

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Kawecki Chemical Co. Ethidium bromide was a gift of the Boots Pure Drug, Ltd., Nottingham, England. Deoxyribonuclease solutions (General Biochemicals) were a gift of Dr. Masaki Hayashi. Carrier-free ^{32}P was obtained as phosphoric acid from the New England Nuclear Corp.

Growth and Labeling Conditions. Tris-buffered minimal medium was used as previously described (DeWitt and Helinski, 1965) with the following changes: glycerol (0.4%) was used as the carbon source; 0.25% Difco vitamin-free Casamino Acids and 10 μg/ml of thymine were added; and Na_2HPO_4 and KH_2PO_4 were not added. Cells were grown at 37° with vigorous aeration to 10^9 cells/ml from a 1% inoculum pregrown on the same media. All labeling was done by the addition of 5 mCi of carrier-free $^{32}P_i$/l. for a minimum of three generations.

DNA extraction was performed by the phenol technique previously described (Roth and Helinski, 1967) except that TES[1] buffer was used for all steps subsequent to the lysozyme treatment and the phenol–chloroform mixture was not saturated with buffer.

DNA Fractionation. DNA was fractionated on a MAK column prepared as described by Sueoka and Cheng (1962). For the preparation of the DNA used for analytical sedimentation studies 150 g dry weight of kieselguhr and 300 mg of methylated albumin were used in a 7-cm diameter column. DNA (20 mg) was applied in 1 l. of 0.2 M NaCl buffered with 0.05 M sodium phosphate (pH 6.7), and eluted with 1.2 l. of a linear gradient of 0.6–0.9 M NaCl in the same buffer. Fractions of 17 ml were collected and a recovery of 10.5 mg of DNA was estimated by OD_{260} measurements. Proportionally smaller columns were used for less starting material, with yields and elution profiles similar to those previously obtained (DeWitt and Helinski, 1965).

Alkali Denaturation–Renaturation. Solutions of DNA in 0.7 M NaCl and 0.05 M sodium phosphate (pH 7.0) were chilled in ice and then adjusted to 0.1 N NaOH by the addition of 1.0 N NaOH. The pH of the DNA solution was approximately 12.2 After 5 min at 0° the DNA solution was adjusted to pH 8.4 by the addition of a mixture of 0.3 N HCl and 0.7 M Tris.

Purification on Cellulose Nitrate Filters. After denaturation and neutralization, the DNA at a concentration of 20 μg/ml, or less, was passed through a double layer of S&S Bactiflex B-6 membrane filters to remove the denatured DNA. The capacity of these filters under these conditions was similar to that described by Wohlhieter *et al.* (1966).

Density Gradient Equilibrium Centrifugation. Analytical equilibrium centrifugation in a cesium chloride gradient was performed by the method of Meselson *et al.* (1957) as previously described (DeWitt and Helinski, 1965). ^{15}N *Pseudomonas aeruginosa* DNA was employed as reference DNA. The density of the reference was taken to be 1.742 g/cc.

Preparative Dye–Buoyant Density Equilibrium Centrifugation. Density gradient centrifugation was performed on a Spinco Model L4 ultracentrifuge in a type Ti 60 fixed-angle rotor for 42 hr at 44,000 rpm, 20°. Each polyallomer tube contained 12.9 g of CsCl, 2 ml of an ethidium bromide solution (700 μg/ml in 0.1 M sodium phosphate buffer, pH 7.0), and 11.6 ml of sample. The remainder of the tube was filled with light mineral oil. At the end of the run 0.35-ml fractions were collected from the bottom of the tube through a hollow needle.

Sucrose Gradient Velocity Centrifugation. Sucrose gradient centrifugation was performed in a Spinco Model L2 or L4 ultracentrifuge in a SW 25.1 swinging-bucket head at 25,000 rpm, 20°. A linear 5–20% sucrose gradient (30 ml) in TES buffer was used. Fractions of 1 ml were collected by drop or time from the bottom of the tube.

Moving-Boundary Analytical Velocity Centrifugation. Centrifugations were performed in 30-mm filled-Epon cells in a Spinco Model E ultracentrifuge equipped with a monochromator light source and a two-cell mask. A temperature of 20° was achieved and maintained by the method of Studier (1965). The DNA concentration was 2 μg/ml in a solvent consisting of 1.0 M NaCl and 0.001 M EDTA (pH 7.5); 1.6 ml of this solution was used for the neutral runs. At the end of the neutral runs the DNA in each cell was redistributed by repeated inversion, then 0.16 ml was withdrawn and replaced by 0.16 ml of 1.0 M NaOH for the alkaline runs. Neutral runs were made at 20,410 rpm and the alkaline runs at 16,200 rpm. Absorption photographs were taken at 260 mμ for 25 sec at exposure intervals of 8, 16, and 32 min, as appropriate to the sedimentation coefficients of each form.

A Joyce-Lobel microdensitometer was used to produce tracings of the films. A major and clearly defined boundary was observed in all cases. The half-height point of this boundary was used for the calculation of the apparent sedimentation velocity. These values were corrected for solvent density and viscosity by the factors 1.144 and 1.160 (Studier, 1965) for the neutral and alkaline velocities, respectively. Minor boundaries were observed in several runs, but could not be quantitated because of the low initial DNA concentration.

Electron microscopy was performed by Dr. T. Roth. Sample preparation and determination of contour lengths were performed as previously described (Roth and Helinski, 1967).

Pronase Treatment. Pronase (5 mg/ml in TES) was autolyzed 10 min at 50° to minimize nuclease activity. The colicinogenic factor E_1 DNA was tested for Pronase sensitivity in a reaction mixture containing 0.2 μg of DNA and 50 μg of Pronase in 0.95 ml of TES. The reaction mixture was applied to a sucrose gradient at the end of a 30-min incubation period at room temperature.

Pancreatic Deoxyribonuclease Degradation. The degradation reaction was performed essentially as described by Roth and Hayashi (1966). The reaction mixture contained approximately 0.2 μg of DNA and DNase as specified in 2 ml of TM buffer (0.03 M Tris–0.01 M $MgCl_2$, pH 7.3). The enzyme was allowed to act

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: TES, 0.05 M Tris-Cl–0.005 M EDTA–0.05 M NaCl (pH 8.0); MAK, methylated albumin kieselguhr.

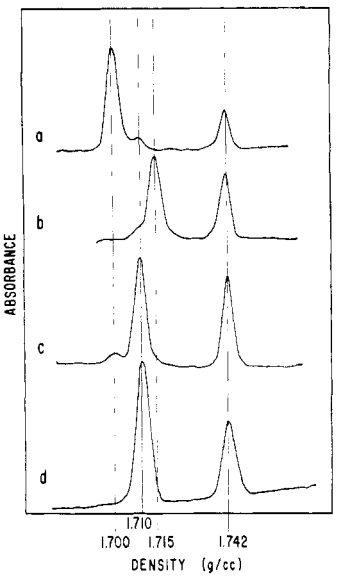

FIGURE 1: Analytical CsCl density equilibrum measurements at various stages in the purification of colicinogenic factor E_1 DNA. Each gradient contains approximately 2 μg of DNA. Native colicinogenic factor E_1 DNA has a buoyant density of 1.710 g/cc; the buoyant density of *Proteus* chromosomal DNA is 1.700 g/cc. *P. aeruginosa* ^{15}N DNA (1.742 g/cc) was added as a reference DNA. (a) Pooled MAK column fractions. (b) Pooled MAK column fractions, denatured and neutralized. (c) Denatured, neutralized, and filtered through nitrocellulose. (d) Filtrate concentrated, and again denatured, neutralized, and filtered.

at 24° and at intervals 0.65 ml was withdrawn and added to 0.1 ml of 0.1 M EDTA (pH 8.0) to stop the reaction. Immediately after the third aliquot was withdrawn, the reaction products were analyzed on sucrose gradients. The starting material was analyzed in a separate sucrose gradient run.

Counting of Radioisotope. DNA was applied to 1 × 1 in. squares of filter paper, or precipitated on nitrocellulose filters, and counted in a Beckman liquid scintillation counter as previously described (Hickson *et al.*, 1967).

Results

Purification of Colicinogenic Factor E_1 DNA and Physical Characterization. DNA was extracted from 16 l. of a *P. mirabilis* (*Col* E_1) culture and applied to a large MAK column as described in the Experimental Section. The initial four fractions from the column, containing 11% of the eluted DNA and enriched in colicinogenic factor, E_1 were pooled for further purification. The concentration of colicinogenic factor at this stage is approximately 9% as shown in Figure 1a. This material was denatured by treatment with alkali and neutralized. As shown in Figure 1b, essentially all of the colicinogenic factor E_1 DNA resisted permanent denaturation by this treatment (Pouwels *et al.*, 1968), while the *P. mirabilis* chromosomal DNA was completely denatured as indicated by an increase of 0.015 g/cc in its buoyant density. The treated DNA was then passed through nitrocellulose filters. The filtrate contained only a small residual amount of DNA of native chromosomal buoyant density, as shown in Figure 1c. The solution (100 ml) was concentrated to 1 ml by two steps of flash evaporation at room temperature, then once again denatured, neutralized, and filtered to remove the residual chromosomal contamination and any colicinogenic factor E_1 DNA molecules which may have sustained breaks in the course of concentration. The filtrate was then concentrated to 1 ml by brief evaporation at room temperature and dialyzed into TES buffer. A portion of the colicinogenic factor E_1 DNA was examined for purity by cesium chloride density equilibrium centrifugation. As shown in Figure 1d, there was no detectable contamination with chromosomal DNA. Over-all recovery of pure colicinogenic factor E_1 DNA was 30 μg which represents approximately 35% of the colicinogenic factor E_1 DNA that was eluted from the MAK column.

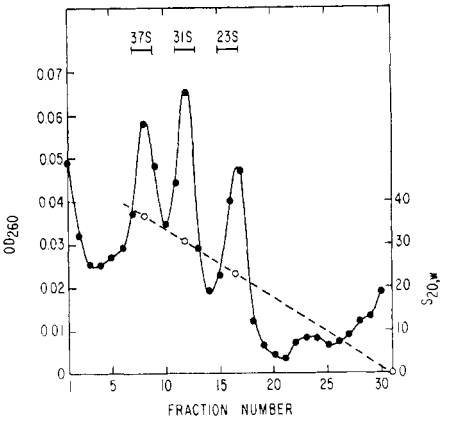

FIGURE 2: Sucrose gradient of purified colicinogenic factor E_1 DNA. The 1.0 ml of DNA solution was run for 8.75 hr under the conditions described in Experimental Section. (○–○) $s_{20,w}$; (●—●) OD_{260}; as determined by subsequent analytical centrifugation. The fractions pooled for further analysis are shown by the brackets above the peaks. Fractions are numbered in order as collected from the bottom of the centrifuge tube.

The pure colicinogenic factor E_1 was then subjected to sucrose gradient velocity centrifugation. The results of this centrifugation are shown in Figure 2. Three major peaks were observed with each containing approximately 6 μg of DNA. Three fractions from each of the peaks were pooled, as indicated in Figure 2. Each of these pools was dialyzed against two changes of TES buffer, then exhaustively dialyzed against a buffer consisting of 1.0 M NaCl and 10^{-3} M EDTA (pH 7.5). The latter buffer was used for analytical velocity centrifugation and storage of the DNA. Moving-boundary centrifugation, as described in the Experimental Section, was used to determine the actual sedimentation coefficients of the three isolated forms both in neutral and alkali molar salt. The neutral $s_{20,w}$ values of the three forms were 23, 31, and 37 S, and the alkaline $s_{20,w}$ values were 63, 77.5, and 102.3 S, respectively. The neutral sedimentation values are consistent with distances of migration of the three peaks in Figure 2 and are in good agreement with preliminary experiments in which the sedimentation velocity was estimated on sucrose gra-

TABLE I

Colicinogenic Factor E_1 DNA Form	Neutral $s_{20,w}$ (S)	Alkaline $s_{20,w}$ (S)	Single-Hit Product $s_{20,w}$ (S)	Contour Length, μ (%)	No. Measured	Mol Wt ($\times 10^6$)
Monomer	23.4	63.3	17	2.15 (±2)	11	4.2
Dimer	31.4	77.5	23	4.34 (±2)	8	8.5
Trimer	37.1	102.3		6.50 (±2)	7	12.7

dients relative to the replicating form of ΦX-174 DNA. The extremely large sedimentation coefficient of each form in alkali is consistent with the hypothesis that each form consists of a covalently closed circular molecule.

Electron photomicrographs taken of the resolved DNA preparations at the time of the analytical centrifugation showed that each of the pools contained only tightly twisted molecules. Measurements of contour lengths were performed, therefore, on electron photomicrographs of samples of the DNA which had been stored at 4° for 6 months. Of material from the 37S, 31S, and 23S classes, $^{48}/_{62}$, $^{64}/_{65}$, and $^{15}/_{65}$, respectively, of the molecules examined randomly at low magnification had been spontaneously converted to open circles during storage. The contour lengths of the major class of open circles present in the 37S, 31S, and 23S preparation were 6.50, 4.34, and 2.15 μ, with a sample standard deviation in each class of approximately 2%. The 37S preparation was the only one with a significant heterogeneity; $^{4}/_{18}$ of the molecules were of the 4.3-μ size class. These data are summarized in Table I, along with estimates of the molecular weights of the three size classes assuming a relationship of 1.96×10^6 daltons/μ (MacHattie *et al.*, 1965).

Purification by Dye–Buoyant Density and Kinetics of Nuclease Degradation. Recently Radloff *et al.* (1967) have described a method of purification of closed circular DNA which utilizes the buoyant density difference induced by preferential ethidium binding by linear and open circular DNA. Dialysis is sufficient to remove the ethidium bromide and return the DNA to native buoyant density (Hickson *et al.*, 1967). This technique was applied to the DNA of noncolicinogenic *Proteus* cells. No satellite DNA typical of closed circular DNA was observed in the DNA from a noncolicinogenic *Proteus* strain. However, partially purified colicinogenic factor E_1 DNA showed a band of higher density than the chromosomal DNA in a CsCl–ethidium chloride density equilibrium centrifugation in proportion to the amount of colicinogenic factor E_1 DNA present. In addition to confirming the closed circular structure of the colicinogenic factor E_1 DNA, this technique was utilized as a preparative procedure for purified colicinogenic factor E_1 DNA.

A *P. mirabilis* (*Col* E_1) culture (2 l.) was continuously labeled to a high specific activity with ^{32}P, as described in the Experimental Section. The DNA was extracted and partially purified by chromatography on a MAK column. The initial 21% of the eluted DNA was subjected to dye–buoyant density centrifugation with the resulting radioactivity profile shown in Figure 3. The dense satellite DNA was 4.3% of the recovered counts; thus, 0.89% of the counts eluted from the MAK column are attributable to colicinogenic factor E_1 DNA. The satellite DNA was dialyzed and resolved into its components by sucrose gradient centrifugation. The resulting sucrose gradient profile (Figure 4) indicated two major components which corresponded to the 23S

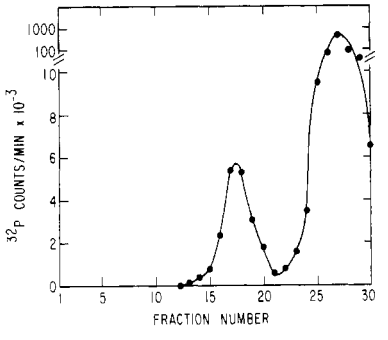

FIGURE 3: Dye–buoyant centrifugation of pooled MAK column fractions containing colicinogenic factor E_1 DNA. The amount of DNA used was 84 μg, with a specific activity of 5×10^4 cpm/μg of DNA. Fractions are numbered in order as collected from the bottom of the centrifuge tube. Collecting was discontinued after the chromosomal peak (visible as a red band) had been recovered, leaving approximately 5 ml in the centrifuge tube. Samples of 0.03 ml were used for determination of radioactivity. Fractions 17 and 18 and comparable fractions from an identical run were pooled for subsequent analyses.

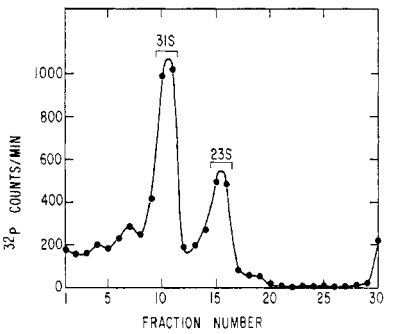

FIGURE 4: Sucrose gradient of dye–buoyant density satellite DNA. A 1.0-ml sample of satellite DNA obtained from the pooled preparation described in Figure 3 was run for 8.75 hr. Samples of 0.05 ml were used for determination of radioactivity. The two peak fractions of the major components were pooled as indicated by the brackets for further analysis.

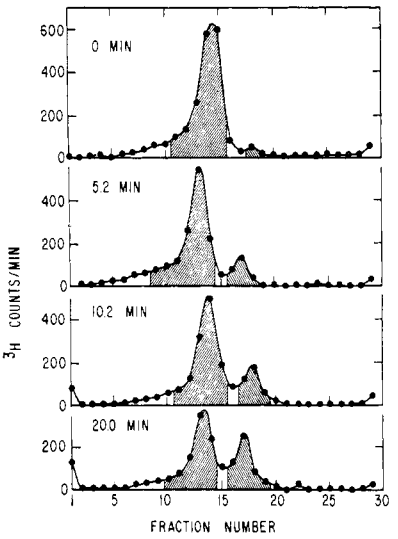

FIGURE 5: Sucrose gradient analysis of DNase degradation of 23S colicinogenic factor E_1 DNA. Samples of 0.65 ml were withdrawn from a reaction mixture containing 0.2 μg of DNA in 1.45 ml of Tris-$MgCl_2$ buffer and 5×10^{-10} g of DNase in 0.05 ml of Tris-$MgCl_2$ buffer after 5.2, 10.2, and 20.0 min and centrifuged for 9.25 hr in a sucrose gradient. The untreated material was analyzed by centrifuging a mixture containing 0.5 ml of DNA solution and 0.15 ml of Tris-$MgCl_2$ buffer for 8.75 hr. Each fraction from the sucrose gradient was counted on nitrocellulose filters after precipitation with trichloroacetic acid.

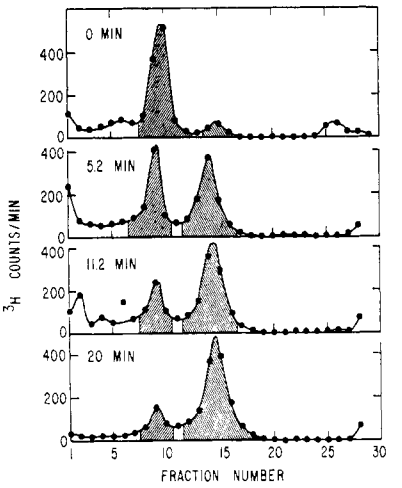

FIGURE 6: Sucrose gradient analysis of DNase degradation of 31S colicinogenic factor E_1 DNA. Samples of 0.65 ml were withdrawn from a reaction mixture containing 0.2 μg of DNA in 0.85 ml of Tris-$MgCl_2$ buffer, 1.15 ml of Tris-$MgCl_2$ buffer and 3×10^{-10} g of DNase in 0.03 ml of Tris-$MgCl_2$ buffer after 5.2, 11.2, and 20.0 min and centrifuged for 8.75 hr in a sucrose gradient. The untreated material was analyzed by centrifuging a mixture containing 0.25 ml of DNA solution and 0.40 ml of Tris-$MgCl_2$ buffer for 8.75 hr. Each fraction from the sucrose gradient was counted on nitrocellulose filters after precipitation with trichloroacetic acid.

monomer and 31S dimer molecules. Relatively small amounts of the 37S trimer were obtained in this preparation of colicinogenic factor E_1 DNA. The two major peaks were pooled separately for studies on the kinetics and products of pancreatic DNase degradation. The pooled fractions of the 23S and 31S forms were dialyzed against Tris-$MgCl_2$ buffer, and the enzyme reaction was performed on this material. Aliquots of the reaction mixture were removed at the times indicated in Figures 5 and 6 and the reaction was stopped by the addition of EDTA. The results of sucrose gradient analysis of these aliquots are shown in Figures 5 and 6 for the 23S and 31S forms, respectively. Assuming a linear ratio of distance migrated to sedimentation coefficient, the first degradation products of the 31S and 23S forms are 23 and 17 S, respectively. A plot of the remaining fraction of material of the initial S value against time (Figure 7) is consistent with first-order kinetics for this conversion.

Pronase Resistance. As a test of the possibility that the covalently closed structure of the colicinogenic factor is achieved by a peptide or protein linkage, ^{32}P-labeled colicinogenic factor E_1 DNA was analyzed by sucrose gradient velocity centrifugation before and after incubation with Pronase. It is apparent from the similarity of the two profiles (Figure 8) that the sedimentation properties of the three size classes of colicinogenic factor E_1 DNA were unaffected by Pronase under the conditions used.

Calculation of Twists. The data obtained in these experiments, summarized in Table I, can be used to ascertain the relationship between molecular weight and sedimentation coefficient for this series of twisted circular DNA molecules (Figure 9b). Several other well-characterized DNAs also fall on the same line, which implies some consistent relationship operating among these various DNA molecules. In light of the data consistent with the idea that supercoils arise in a natural DNA as a result of environment changes from the intracellular state (Wang *et al.*, 1967), the hypothesis that a constant number of twists per unit length are introduced is most appealing. Fukatsu and Kurata (1966) have presented a table of expected ratios, R, of the sedimentation coefficient of supercoils to linear molecules of the same molecular weight for various numbers, N, of rings on the basis of a theoretical consideration of a model in which the rings formed by the crossing of the DNA in the supercoiled forms are considered to be connected by universal joints. Figure 9d contains a plot of their calculations in the form $R = F(N)$, for $N = 1 +$ (mol wt/(2×10^6)). This condition generates an approximately linear plot on logarithmic coordinates. Applying the ratio thus obtained to the empirical relationship of molecular weight to $s_{20,w}$ derived by Studier (1965) (Figure 9c), a satisfactory fit to the data is obtained with the assumption of one ring for every 2×10^6 molecular weight (Figure 9a). It should be emphasized, however, that the expected sedimentation coefficient is not a very sensitive function of the number of rings per 10^6 molecular weight. The figure of one additional ring per 2×10^6 molecular weight is within the range predicted by Bloomfield (1966) from considerations of the ratio of sedimentation coefficients of the open circular and twisted circular forms of various other natural supercoiled DNAs. This analysis, however, gives a distinguishably different result from that of Vinograd *et al.* (1968) who find ap-

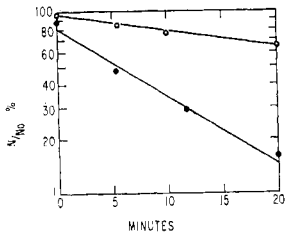

FIGURE 7: Kinetics of degradation of colicinogenic factor E_1 DNA by DNase. The fractions considered to contain starting material and product for the calculation of N/N_0 are designated by the hatched areas in Figures 5 and 6. (○—○) DNase treatment of 23S molecule; (●--●) DNase treatment of 31S molecule.

proximately 15 superhelical turns for polyoma DNA in buoyant CsCl.

Discussion

A previous study of colicinogenic factor E_1 isolated from *P. mirabilis* established that there are at least two twisted circular forms of this DNA. In this study it is established that there are three size classes of molecules of buoyant density characteristic of the colicinogenic E_1 factor. Furthermore, these three forms, related by size as monomer, dimer, and trimer, are indistinguishable in structure from other well-characterized twisted circular molecules by several criteria. Each form appears as a twisted circle on electron photomicrographs, and can give rise to an open circular form. In 0.1 M NaOH each form sediments as a very compact molecule and each form has after neutralization the characteristic density and sedimentation properties of the undenatured molecule. Each form is resistant to Pronase, and at least the 23S and 31S forms are degraded by DNase to a more slowly sedimenting form,

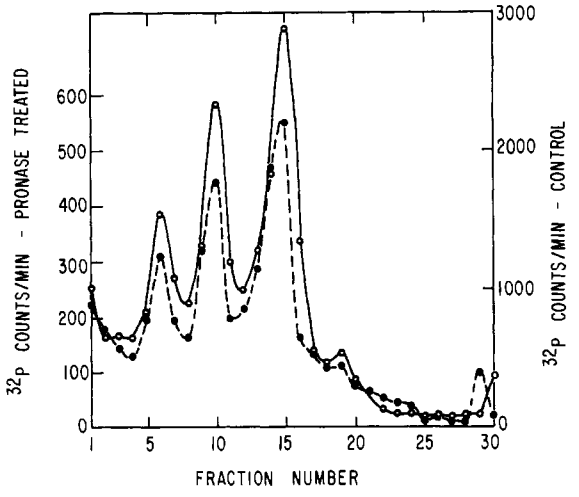

FIGURE 8: Sucrose gradient analysis of Pronase-treated colicinogenic factor E_1 DNA.(○—○) Control (1.10 μg of DNA in 1.0 ml of TES buffer); (●--●) Pronase-treated colicinogenic factor E_1 DNA (0.2 μg of DNA in 0.95 ml of reaction mixture). Both gradients were centrifuged for 8.75 hr in the same rotor and counted on nitrocellulose filters after precipitation of the whole fraction with trichloroacetic acid.

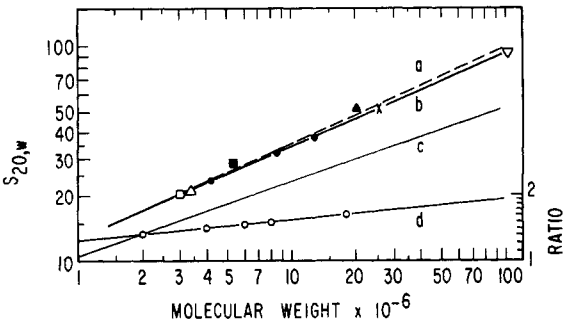

FIGURE 9: Sedimentation coefficient of supercoiled DNA as a function of molecular weight. (a) Expected plot generated as the product of lines c and d. (b) (—●—) Observed function, drawn through the points determined in this work, $s_{20,w} = 0.034M^{0.428}$. (c) Sedimentation coefficient of linear DNA as a function of molecular weight (Studier, 1965). (d) Expected ratio of the sedimentation coefficient of supercoiled DNA to the sedimentation coefficient of linear DNA as a function of the molecular weight from the calculations of Fukatsu and Kurata (1966). Points are plotted for several other supercoiled DNA molecules. (□) Polyoma (Weil and Vinograd, 1963); (△) ΦX-174 replicating form (Burton and Sinsheimer, 1965); (■) papilloma (Crawford, 1965); (▲) mitochondrial dimer (Clayton and Vinograd, 1967); (x) supercoiled $\lambda b_2 b_5 C$ (Kiger *et al.*, 1968); and (▽) *FColVCol-Btrycys* (F. Hickson and D. Helinski, unpublished observation; Hickson *et al.* 1967). All of the sedimentation coefficients referred to were determined in buffers in which the concentration of salts was at least 1.0 M.

presumably the open circle, with first-order kinetics. All three forms are found in a dense satellite band characteristic of twisted circular molecules upon dye–buoyant equilibrium centrifugation of a partially purified preparation. Finally, the relationship of molecular weight to sedimentation coefficient based on the experimental results for closed circular monomer, dimer, and trimer forms of colicinogenic factor E_1 DNA is in good agreement both with a theoretical relationship and with the experimental results for other supercoiled DNA molecules, implying that the colicinogenic factor E_1 forms have the hydrodynamic properties expected of supercoiled DNA. This closed circular structure of colicinogenic factor E_1 DNA permits the use of straightforward purification techniques based on the distinctive characteristics of these molecules.

In a carefully quantitated experiment, no DNA of denatured colicinogenic factor E_1 density was observed after denaturation and neutralization of partially purified MAK column fractions under conditions in which as little as 10% of the denatured DNA would be detectable. Of the material of colicinogenic factor density present in the MAK fractions, 71% was recovered after denaturation, neutralization, and filtration, implying that there is no exclusion of any quantitatively substantial form of the colicinogenic factor DNA. These results indicate that the colicinogenic factor E_1 DNA extractable by the phenol procedure is largely, if not entirely, in the covalently intact circular form. Attempts were made to minimize the bias among forms arising from the preferential elution of low molecular weight

DNA (Sueoka and Cheng, 1962) by pooling at least the initial 10% of the DNA eluted from the MAK column; however the possibility that the relative proportions of the forms of colicinogenic factor present in the final preparations differ considerably from those present *in vivo* cannot be excluded, and the same reservations must apply to the observed ratio of factor to chromosomal DNA.

The contour lengths of 2.15, 4.34, and 6.50 μ which were observed are clearly related as integer multiples. Contour lengths previously observed for the monomer and dimer forms were 2.3 and 4.7 μ. The differences are significant at the 95% level by a T test for the equivalence of means and can be attributed to systematic errors in details of sample preparation (Lang *et al.*, 1967) or in the determination of contour lengths.

At least 95% of the colicinogenic E_1 factor isolated from *E. coli* occurs as 23S molecules (Bazaral and Helinski, 1968). The 23S form must therefore contain all the information to determine colicinogenicity, and thus the 31S and 37S forms are most likely dimer and trimer molecules that are composed of repeats of the 23S monomer. Since all forms in *P. mirabilis* have the same bouyant density, distinct from chromosomal DNA, no significant amount of chromosomal DNA can be included in the dimer or trimer form. Although the distribution of monomer, dimer, and trimer among single cells of a population of *P. mirabilis* (*Col* E_1) is not known, all forms occur in populations isolated from a single colony. Thus either there is interconversion of the three forms within the approximately 40 generations required for a cell to give rise to a sufficient number of progeny for analysis, or all forms are present in each cell. The latter possibility is not very likely, since if the the segregating unit is a single chromosome of 2×10^9 molecular weight, and if the amount of colicinogenic factor E_1 DNA is approximately 1%, there could be only one copy of each form. It would be expected, then, that on a weight basis there would be three times as much trimer as monomer. This has never been observed. Since there are relatively few if any open circular molecules in the partially purified MAK column fractions, it can be assumed that the bias against trimers resulting from differential sensitivity to single-strand nicks is minimal. Therefore, if the phenol extraction and MAK chromatography do not bias against the trimer in some other fashion, it must be concluded that there is an interconversion among the forms. There is no evidence to date which would indicate whether the multiple length forms arise through a recombination process or through errors in replication.

Multiple length circular forms of DNA have also been observed in mammalian mitochondrial DNA (Clayton and Vinograd, 1967), and in the replicating form of ΦX-174 DNA (Rush *et al.*, 1967). Oligomers have also been observed to occur as catenated dimers in mitochondrial DNA from HeLa cells (Hudson and Vinograd, 1967), in leukaemic leucocytes (Clayton and Vinograd, 1967), and in sea urchin eggs (Piko *et al.*, 1968). The possibility that catenates occur at low frequency in colicinogenic factor E_1 DNA cannot be excluded on the basis of data presented in this paper.

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Histidine Decarboxylase of *Lactobacillus* 30a. IV. The Presence of Covalently Bound Pyruvate as the Prosthetic Group*

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ABSTRACT: Histidine decarboxylase from *Lactobacillus* 30a does not require pyridoxal phosphate for activity but is inhibited by reduction with sodium borohydride or by incubation with phenylhydrazine. In the latter reaction, 5 moles of [^{14}C]phenylhydrazine react/mole of enzyme to form a protein phenylhydrazone. This reaction is prevented by preliminary reduction with sodium borohydride, and also by the substrate, L-histidine, but not by D-histidine. These findings indicate that a carbonyl group forms part of the active center of the enzyme. When the enzyme is reduced with tritiated sodium borohydride, [α-^{3}H]lactate can be isolated from hydrolysates of the reduced protein, indicating that the unreduced enzyme contains bound pyruvate. Proteolytic digestion of enzyme [^{14}C]phenylhydrazone gives rise to a stable radioactive phenylhydrazone which, on purification and acid hydrolysis, yields phenylalanine as the only amino acid in amounts equimolar with the label. Following hydrogenation over platinum and hydrolysis, alanine is formed in nearly equimolar amounts. These results again demonstrate the presence of pyruvate in the native enzyme and indicate that it is combined with a phenylalanine residue of the protein by an amide linkage. *N*-Pyruvoylphenylalanine, synthesized by nonenzymatic transamination of alanylphenylalanine with pyridoxal, was converted into its phenylhydrazone and proved identical with the phenylhydrazone isolated from histidine decarboxylase in chromatographic, electrophoretic, and spectral properties and also in its behavior following reduction over platinum. Incomplete evidence indicates that the five pyruvoylphenylalanine residues in histidine decarboxylase occupy the N terminus of five of the ten peptide chains of this enzyme. A mechanism for the participation of pyruvate in the decarboxylation of histidine similar to that suggested for the participation of pyridoxal phosphate in the catalytic action of other amino acid decarboxylases is discussed.

Histidine decarboxylases (histidine carboxy-lyase, EC 4.1.1.22) catalyze the decarboxylation of histidine to yield histamine and CO_2. One such enzyme has been isolated in pure form from *Lactobacillus* 30a and has been partially characterized (Rosenthaler *et al.*, 1965; Chang and Snell, 1968a,b). Its structure is unusual in that it is apparently composed of ten subunits, giving a total molecular weight of 190,000 (Chang and Snell, 1968b). Unlike other amino acid decarboxylases studied thus far, this bacterial histidine decarboxylase does not require pyridoxal phosphate as a cofactor (Rosenthaler *et al.*, 1965; Chang and Snell, 1968a). However, it is inhibited by carbonyl reagents, implying that a carbonyl group is involved in the active site. The work to be described here shows that pyruvic acid is covalently bound to the enzyme through amide linkage with the amino group of a phenylalanyl residue, probably at the N terminus of a polypeptide chain. It is postulated that the pyruvoyl residue participates as a cofactor in the decarboxylation reaction in a manner analogous to the participation of pyridoxal phosphate in the enzymatic decarboxylation of other amino acids.

Methods

Histidine decarboxylase was purified as described by Chang and Snell (1968a) with the following slight modifications. Because of the relatively small preparative scale used, it was more convenient to employ the ratios and volumes described by Rosenthaler *et al.* (1965). After the heat step, the enzyme was collected by precipitation with ammonium sulfate (75% saturation) and the resulting concentrated fraction (*ca.* 10 ml) was clarified by centrifugation and then applied directly on the Sephadex G-200 column, omitting the acetone fractionation entirely. After recrystallization, the final material was indistinguishable from the preparation described by Chang and Snell (1968a) on the basis of ultracentrifugation, disc gel electrophoresis, and specific activity. Histidine decarboxylase activity was measured manometrically (Chang and Snell, 1968a). The concentration of protein in solutions of the pure enzyme was

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