Properties of a Supercoiled Deoxyribonucleic Acid–Protein Relaxation Complex and Strand Specificity of the Relaxation Event*

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ABSTRACT: Previously, colicinogenic factor E_1 (*Col*E_1) has been purified from *Escherichia coli* as a unique supercoiled circular DNA–protein complex that sediments slightly faster (at approximately 24 S) than protein-free or noncomplexed 23S supercoiled DNA. When treated with certain agents or conditions that denature or destroy protein structure, the complex undergoes a change in which the supercoiled DNA in the complex is converted into a relaxed, or open circular, state. For this reason, the complex has been designated a relaxation complex.

In this communication the following information is presented regarding the properties of the *Col*E_1 relaxation complex. (1) At low ionic strength the 24S relaxation complex initially is obtained as a larger complex that sediments through a sucrose density gradient with an approximate sedimentation coefficient of 34 S. Dilution and resedimentation of the material pooled from the 34S peak subsequently yield the 24S relaxation complex. If 0.5 M NaCl is present in the gradient during the initial sedimentation, the 34S form is not observed and the *Col*E_1 complex sediments only as the 24S form. (2) Kinetic studies on the susceptibility of the 24S relaxation complex to pancreatic DNase confirm the previous electron microscopy data indicating that the 24S complex contains supercoiled *Col*E_1 DNA. (3) When crude Sarkosyl lysates of *Escherichia coli* (*Col*E_1) cells were centrifuged to equilibrium in a dye-buoyant density gradient, only noncomplexed plasmid DNA was found in the dense satellite peak, while the complexed material, which relaxes under these centrifugation conditions, was found in a less dense shoulder of the large chromosome peak. On the assumption that the *Col*E_1 DNA is tightly bound only to protein, the extent of the buoyant density shift corresponds to an association of 240,000 daltons of protein with the relaxed *Col*E_1 DNA. (4) Heat treatment of cells (70° for 15 min) before lysis results in the recovery of virtually all of the *Col*E_1 DNA in the supercoiled, uncomplexed form. (5) The open circular form of *Col*E_1 DNA resulting from pronase treatment of the supercoiled *Col*E_1 DNA–protein complex was separated into equal amounts of circular and linear single strands by centrifugation in an alkaline sucrose gradient. Annealing studies of the separated circular and linear single strands indicate that the nick or gap in the relaxed molecule is strand specific. In addition to the above results various models for the nature of the protein and its association with the *Col*E_1 DNA are discussed.

Colicinogenic factor E_1 (*Col*E_1), a bacterial plasmid determining the production of the extracellular antibiotic protein colicin E_1, has been purified from *Escherichia coli* as a 23S supercoiled circular DNA molecule (Bazaral and Helinski, 1968) and a 24S supercoiled DNA–protein complex (Clewell and Helinski, 1969). The complexed form of *Col*E_1 DNA is characterized both by an unusually high affinity of the protein material to the small supercoiled DNA (molecular weight of 4.2×10^6) and a unique response to the action of protein-denaturing agents. In the presence of sodium dodecyl sulfate, Sarkosyl,[1] or the proteolytic enzymes pronase and trypsin, the supercoiled DNA within the complex relaxes to an open circular form. Noncomplexed *Col*E_1 DNA is not affected by these agents even in the presence of complexed *Col*E_1 DNA. The protein component in the complex is not readily dissociable from the supercoiled *Col*E_1 DNA. Even after buoyant density centrifugation a substantial amount of protein remains associated with the *Col*E_1 DNA.

In this report additional evidence is presented for the supercoiled nature of the *Col*E_1 DNA in the complex and an estimate is made of the amount of protein remaining associated with the *Col*E_1 DNA after centrifugation of the complex to equilibrium in a cesium chloride gradient. The differential recovery of supercoiled, noncomplexed *Col*E_1 DNA and relaxed, open circular, complexed *Col*E_1 DNA from various regions of a dye-buoyant density gradient of Sarkosyl lysates of heated and unheated colicinogenic cells is also described. Finally, alkaline sucrose gradient and hybridization studies are reported that indicate the presence of a single nick or gap within one specific strand of the relaxed *Col*E_1 DNA molecule produced by pronase treatment of the complex.

Experimental Section

Materials. Reagents and sources were as follows: Brij 58, from Atlas Chemical; sodium deoxycholate (DOC) from Mann Research Laboratories; sodium dodecyl sulfate from Fisher Scientific; Sarkosyl NL30 (sodium dodecyl sarcosinate

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[1] Abbreviations used are: TES, 0.05 M NaCl–0.005 M Na_2EDTA–0.03 M Tris, pH 8.0; TM, 0.01 M $MgCl_2$–0.03 M Tris, pH 7.4; SSC, 0.15 M NaCl–0.015 M sodium citrate, pH 7.3; Sarkosyl, sodium dodecyl sarcosinate; DOC, sodium deoxycholate.

from the Geigy Chemical Co.; pronase (B grade) and trypsin (pancreatic, crystalline, A grade) from Calbiochem (pronase solutions were allowed to autodigest for 30 min at 37° prior to use); egg white lysozyme and deoxyribonuclease I (RNase free) from Worthington Biochemical Corp.; CsCl (technical grade) from Penn Rare Metals division of Kawecki Chemical Co.; CsCl (optical grade) from Research Organic/Inorganic Chemical Co.; Ficoll (mol wt ~400,000) from Pharmacia; polyvinylpyrrolidone from Calbiochem; ethidium bromide was a gift of the Boots Pure Drug Co., Ltd., Nottingham, England; [^3H]methylthymine (18.2 Ci/mM), [2-^{14}C] thymine (55.2 mCi/mM), and carrier free [^{32}P]H_3PO_4 from New England Nuclear Corp.

Bacteria and Media. Escherichia coli K12 strains JC411-(*Col*E$_1$) (Clark and Margulies, 1965) and JC411 *thy*$^-$ (*Col*E$_1$) (a thymine-requiring strain, obtained from D. Kingsbury) were utilized in the present study. Each strain harbors the *Col*E$_1$ plasmid DNA which was transferred by conjugation from *Escherichia coli* K30. The JC411 (*Col*E$_1$) strain was grown in a Tris–casamino acids–glycerol medium containing deoxyadenosine that has been described elsewhere (Clewell and Helinski, 1969). During labeling with radioisotope, no additional cold thymine was added. The JC411 *thy*$^-$ (*Col*E$_1$) strain was grown in the same media as above except deoxyadenosine was omitted and thymine (2 μg/ml) and phosphate (0.001 M) were included.

Preparation of Cleared Lysate and Purified Complex. The lysing procedure was a modification of the method of Sinsheimer and coworkers (Godson and Sinsheimer, 1967; Komano and Sinsheimer, 1968). A typical preparation of complex involved harvesting cells in log phase (37°) from 30 ml of medium (containing 0.3 mCi of [^3H]thymine) and resuspending in 1 ml of cold 25% sucrose–0.05 M Tris, pH 8.0. Lysozyme (0.2 ml of 5 mg/ml solution in 0.25 M Tris, pH 8.0) was added, and after the suspension was maintained for 5 min at 0°, 0.4 ml of Na_2EDTA (0.25 M, pH 8.0) was added. The suspension was kept at 0° (swirling occasionally) for another 5 min after which lysis was brought about by the addition of 1.6 ml of a detergent mixture consisting of the following: 1% Brij 58, 0.4% sodium deoxycholate, 0.0625 M Na_2EDTA, and 0.05 M Tris, pH 8.0. After 5–10 min, the samples became relatively clear and viscous. The sample was then centrifuged at 2° for 25 min at 48,000*g*, a step which normally pellets about 95% of the ^{3}H-labeled chromosomal DNA while the complex is recovered in the supernatant. This supernatant is referred to as "cleared lysate." Sedimentation of cleared lysate on a 15–50% sucrose density gradient yields a well-defined peak of *Col*E$_1$ DNA (see Results section). A pool of the fractions in this peak is referred to as "purified complex," although as noted earlier (Clewell and Helinski, 1969) approximately 20–30% of the plasmid DNA in this fraction is not in the complexed state. Purified complex at this stage normally corresponds to a concentration of about 0.5 μg/ml of DNA. In the cases where material was prepared for the analytical equilibrium centrifugation study or for the hybridization study (see Results section), 1-l. cultures were harvested and resuspended in 10 ml of 25% sucrose (0.05 M Tris, pH 8.0). Lysozyme (2 ml of a 5 mg/ml solution) and 4 ml of Na_2EDTA (0.25 M pH 8.0) were added in the usual manner. After lysing by the addition of an equal volume (16 ml) of Brij–DOC detergent mixture, the sample was "cleared" by centrifuging for 15 min, at 48,000*g*.

Preparation of Sarkosyl Lysates. A 30-ml log culture labeled with [^3H]thymine was pelleted and resuspended in 3.4 ml of 25% sucrose (containing 0.05 M Tris, pH 8.0). A 0.4-ml portion of lysozyme (5 mg/ml) was added and, after 5 min at 25°, 0.8 ml of Na_2EDTA (0.25 M, pH 8.0) was added. After 5 min of further incubation, lysis was brought about by the addition of 2 ml of 2% Sarkosyl. The thick lysate was then drawn up and down a 5-ml pipet 10–15 times (this step is necessary to get good banding of the DNA on centrifugation). In the case where cells were heated prior to lysis, the 3.4-ml cell suspension was divided into two portions of 1.7 ml each. After heating of one of the portions, Sarkosyl lysates were prepared from each by the addition of lysozyme, Na_2EDTA, and Sarkosyl in the same manner as described above, except at one-half the designated volumes.

Preparation of Noncomplexed DNA. This material represents that minor amount of plasmid DNA which is not observed in the complexed state after lysis of the cells. The typical isolation procedure involves preparation of a cleared lysate from 60 ml of a log culture of JC411(*Col*E$_1$) grown in the Tris–CASA–glycerol medium containing 2 mCi of [^{32}P]H_3PO_4. The cleared lysate is then centrifuged to equilibrium in ethidium bromide–CsCl gradients. The dense satellite region representing covalently closed DNA is pooled and dialyzed and is referred to as 23S, noncomplexed, or marker *Col*E$_1$ DNA. Spontaneous breakdown of this DNA to the open circular form yields what is referred to as 17S noncomplexed open circular *Col*E$_1$ DNA.

Sucrose Density Gradients. Sucrose density gradient centrifugation was carried out as previously described (Clewell and Helinski, 1969). The 15–50% sucrose gradients containing 0.01 M Na_2EDTA, 0.06 M KCl, and 0.02 M Tris, pH 7.3, were centrifuged in a Beckman SW25.1 rotor. The 5–20% neutral sucrose gradients were in TES (0.05 M NaCl–0.005 M Na_2-EDTA–0.03 M Tris, pH 8.0). The 5–20% alkaline sucrose gradients contained 1.0 M NaCl–0.001 M Na_2EDTA–0.3 M NaOH. In the case of the 5–20% neutral and alkaline sucrose gradients, generally 0.2-ml samples were layered onto the gradient where centrifugations involved utilization of the Beckman SW50.1 or SW65 rotors. In the DNase experiments 0.3-ml samples were layered onto the gradients. Volumes of 1.0–1.5 ml of sample were layered onto gradients involving the SW25.1 rotor.

Preparative Buoyant Density–Equilibrium Centrifugation. Centrifugation was performed on a Spinco Model L4 ultracentrifuge in a type Ti-60 fixed angle rotor at 44,000 rpm and 15° for 60 hr. Each polyallomer centrifuge tube contained 15 g of CsCl and 12 ml of sample in TES. When dye was included, 15.5 ml of sample containing 3 mg of ethidium bromide was mixed with 15 g of CsCl. The remaining space in the centrifuge tube was filled with light mineral oil. At the end of the run, the bottom of the tube was punctured and 0.25- to 0.30-ml fractions were collected.

Analytical CsCl–Equilibrium Centrifugation. This procedure was performed by the method of Meselson *et al.* (1957), as previously described (DeWitt and Helinski, 1965). ^{15}N *Pseudomonas aeruginosa* DNA employed as reference DNA was previously calculated to have a density of 1.742 g/ml (Schildkraut *et al.*, 1962). Since the sample analyzed contained a high background of low molecular weight ultraviolet-absorbing material, an extended exposure period was used to detect the DNA bands.

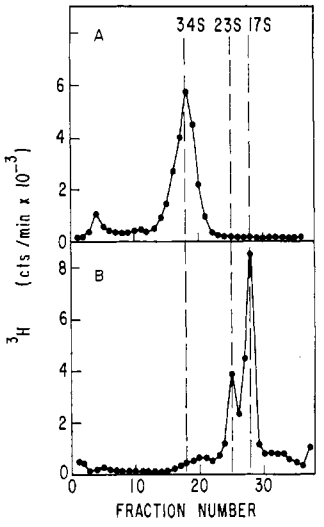

FIGURE 1: Sedimentation analysis of a cleared lysate incubated in the absence and presence of pronase. From a 30-ml culture (in log phase) of JC411(*Col*E_1) cells labeled with [^{3}*H*]thymine, a cleared lysate was prepared (see Experimental Section) and divided into two equal portions of 0.75 ml each. To one was added 0.25 ml of TES while 0.25 ml of a 5 mg/ml solution of pronase (in TES) was added to the other. After incubation at 25° for 15 min, 1.0 ml of each was analyzed. Sedimentation was through 15–50% neutral sucrose density gradients (from right to left) in the SW25.1 rotor (2°) at 25,000 rpm for 15 hr: (A) sample incubated in the absence of pronase; (B) sample incubated in the presence of pronase. Recovery of ^{3}H counts was close to 100% in each case.

Hybridization. The DNA–DNA hybridization was carried out according to a modification of the techniques of Warnaar and Cohen (1966) and Denhardt (1966). DNA samples used for immobilization on membranes and for hybridization inputs were pooled from alkaline sucrose density gradients. Immediately prior to fixation, the two ^{14}C-labeled DNA pools were adjusted to pH 8.5 and diluted 25-fold in a "fixing buffer" consisting of Tris-HCl (0.01 M, pH 7.3) and KCl (0.5 M). Portions of 0.5, 1.0, 2.0, and 4.0 ml were passed through membrane filters (S & S bactiflex B-6, presoaked in fixing buffer). Each passage was followed with a wash of 10 ml of fixing buffer. After drying in a 60° oven overnight the membranes were coated with bovine serum albumin by soaking for 24 hr at 67° in a solution of fixing buffer containing 400 μg/ml of bovine serum albumin and 0.01 M $MgCl_2$. The membranes were then dried with an infrared bulb and stored until ready for use. The ^{3}H-labeled DNA pools were sonicated and a 1-ml portion of each was adjusted to pH 8.5 and then brought to 5 ml with H_2O. The membranes with immobilized ^{14}C-labeled DNA were preincubated in 0.6 ml of a solution of 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin in 3 × SSC at 65°. After 5 hr, 0.15-ml samples of the above DNA were added to each mixture and the solutions incubated at 65° for 16 hr. The membranes were then washed with 10 ml on each side with a washing buffer consisting of 0.001 M Tris adjusted to pH 9.4. After drying, membranes were counted directly. The possibility of annealing of DNA strands in solution is not excluded by the procedure employed, but it is minimized by the storage of purified circles and linear strands in alkaline sucrose solution (pH 12.5) just prior to the fixation of the DNA on the membranes and the hybridization of the DNA to the DNA-containing membranes. In addition the concentration of ^{14}C- and ^{3}H-labeled DNA was less than 0.1 μg/ml during the immobilization step and the hybridization period.

Counting of Radioisotope. Samples were spotted or collected on 1-in. square filter papers immersed in cold 10% trichloroacetic acid, ethanol, and then ether, and counted in a Beckman liquid scintillation counter. Membranes were counted directly after drying without any treatment with trichloroacetic acid. Toluene containing 4.0 mg/ml of 2,5-diphenyloxazole served as the standard fluor throughout this work. In the case of some of the 5–20% sucrose gradients, the 10-drop fractions (on filter papers) were allowed to dry under an infrared bulb and counted directly.

Results

Sucrose Gradient Analysis of the ColE_1 DNA–Protein Complex. The 24S sedimentation property of the plasmid–protein complex was previously demonstrated by pooling the material in the *Col*E_1 DNA peak obtained by centrifugation of a cleared lysate of colicinogenic cells on a 15–50% sucrose gradient and recentrifuging the material on the more shallow 5–20% sucrose gradient in the presence of a differentially labeled 23S plasmid DNA marker (Clewell and Helinski, 1969). Further studies of the sedimentation properties of the *Col*E_1 DNA–protein complex in the 15–50% sucrose gradient have indicated that this complex initially sediments with a sedimentation coefficient greater than 24 S. As shown in Figure 1A, *Col*E_1 DNA present in a cleared lysate (see Experimental Section) of JC411(*Col*E_1) sediments with a relative sedimentation coefficient of approximately 34 S. The DNA material in this peak consists almost entirely of supercoiled *Col*E_1 DNA molecules (Clewell and Helinski, 1969). Pronase treatment of the cleared lysate prior to centrifugation in the sucrose gradient resulted in the conversion of the majority of the *Col*E_1 DNA in the 34S sedimenting form to the open circular form (17 S) while the remaining portion of the *Col*E_1 DNA was recovered as the 23S or noncomplexed supercoiled DNA form (Figure 1B).

Figure 2A represents the recentrifugation on 5–20% sucrose gradients of a mixture of ^{3}H-labeled *Col*E_1 DNA pooled from a peak similar to that shown in Figure 1A and ^{32}P-labeled noncomplexed *Col*E_1 DNA (23 S). The typical 24S plasmid–protein complex was observed with essentially complete recovery of the DNA. Pronase treatment of this mixture of ^{3}H-labeled *Col*E_1 DNA–protein complex plus ^{32}P-labeled noncomplexed *Col*E_1 DNA once again resulted in the relaxation of the complexed *Col*E_1 DNA with no significant change in the sedimentation properties of the noncomplexed DNA.

When ^{14}C- or ^{32}P-labeled noncomplexed *Col*E_1 DNA (23 S) is added to the cleared lysate of tritium-labeled cells, or added to the cell-suspension prior to lysis, the added *Col*E_1 DNA also sediments as a 34S complex. However, as reported previously (Clewell and Helinski, 1969) pronase treatment of such a mixture prior to sucrose gradient centrifugation results in conversion of the ^{3}H-labeled *Col*E_1 DNA complex into the 17S or open circular DNA form while the added noncomplexed *Col*E_1 DNA is recovered as the 23S, or supercoiled, form. Thus, in the cleared lysate both the complexed *Col*E_1 DNA that is present and the added noncomplexed *Col*E_1 DNA associate with macromolecular material which dissociates when the sample is diluted or dialyzed to remove the sucrose and

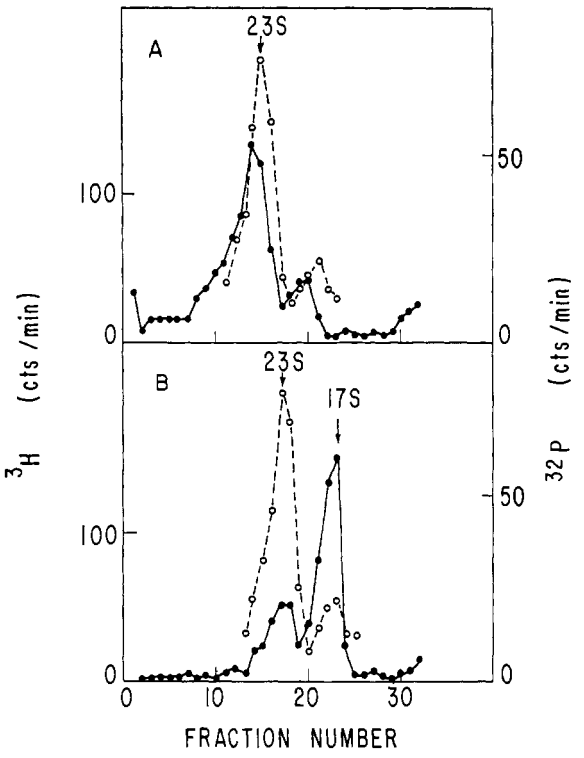

FIGURE 2: Sedimentation analysis of fractions pooled from the 34S peak of *Col*E$_1$ DNA. Pooled 34S peak material, as shown in Figure 1A, was diluted with TES and incubated with 1.25 mg/ml of pronase for 15 min at 25° (final dilution of ^{3}H was tenfold). An equivalent amount of material served as a control, incubating in the absence of pronase. In both cases, ^{32}P-labeled noncomplexed plasmid marker DNA was present during the incubation. Sedimentation was through 5–20% sucrose density gradients (from right to left) in the SW65 rotor (15°) at 50,000 rpm for 135 min: (A) control with no prior treatment with pronase; (B) treated with pronase; (-●-●-) ^{3}H-labeled plasmid DNA complex; (--O--O--) ^{32}P-labeled noncomplexed plasmid DNA.

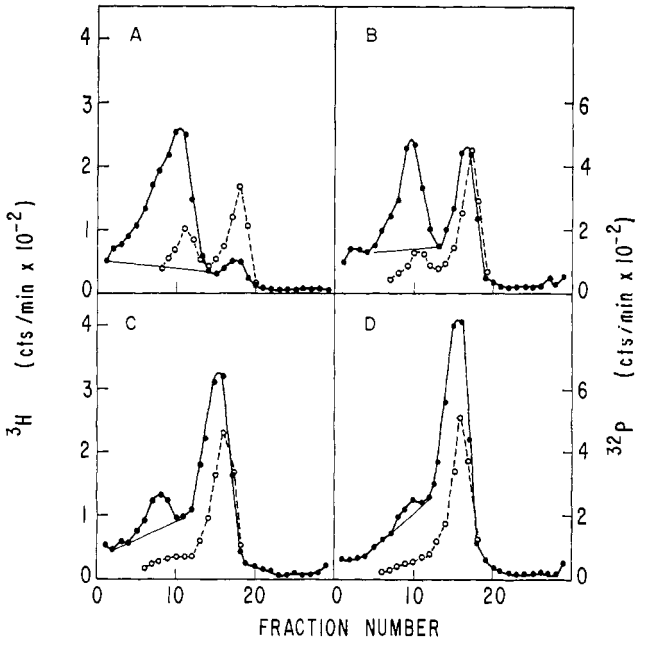

FIGURE 3: Sedimentation analysis of relaxation complex after treatment with a limiting amount of DNase I. Purified complex (see Experimental Section) was diluted fivefold with TM buffer (0.03 M Tris, pH 7.4, 0.01 M $MgCl_2$) and a 0.8-ml sample was mixed with 0.4 ml of a solution of ^{32}P-labeled noncomplexed DNA in both the supercoiled (23S) and open circular (17S) state. To this mixture was added 0.4 ml of a 10^{-2} μg/ml solution of DNase I in TM buffer. After 2, 5, and 10 min of incubation at 25°, 0.4-ml aliquots were removed and the reaction stopped by adding 0.1 ml of 0.25 M EDTA, pH 8.0, to each sample and cooling to 0°. A similarly prepared control solution was incubated for 20 min in the absence of DNase. Samples (0.3 ml of each) were analyzed immediately. Sedimentation was through 5–20% sucrose density gradients (from right to left) in the SW65 rotor (15°) at 50,000 rpm for 175 min. Recovery of ^{3}H counts in the gradients was similar for each time point, with an average recovery of 72%. A represents the control sample which was incubated in the absence of DNase I. B, C, and D represent samples incubated for 2, 5, and 10 min, respectively, in the presence of DNase I: (-●-●-) ^{3}H-labeled plasmid DNA complex; (--O--O--) ^{32}P-labeled noncomplexed plasmid DNA.

recentrifuged in the 5–20% sucrose gradient. When cleared lysates are centrifuged directly in a 5–20% sucrose gradient containing 0.5 M NaCl, the 34S form of complexed *Col*E$_1$ DNA is not observed and the complexed *Col*E$_1$ DNA sediments as the 24S form. If the centrifugation, however, is carried out under the same conditions except in the presence of 0.05 M NaCl once again the *Col*E$_1$ DNA exhibits a considerably higher sedimentation coefficient than 24 S.

*Pancreatic DNase Treatment of Col*E$_1$ *DNA–Protein Complex.* To further establish the covalently closed, circular nature of the double stranded *Col*E$_1$ DNA in the plasmid–protein complex the effect of limited pancreatic DNase on this material was examined. A sample of ^{3}H-labeled purified complex (see Experimental Section) was mixed with ^{32}P-labeled, noncomplexed *Col*E$_1$ DNA (present in both the supercoiled and open circular forms) and the mixture was digested with pancreatic DNase for various periods of time. The extent of conversion into the open circular form was followed by centrifugation of the samples on a 5–20% sucrose gradient. As shown in Figure 3, both the ^{3}H-labeled 24S complex as well as the ^{32}P-labeled, noncomplexed DNA (23S) were relaxed by the nicking activity of the pancreatic DNase. As a control, a sample of the same *Col*E$_1$ DNA complex was mixed with ^{32}P-labeled noncomplexed *Col*E$_1$ DNA, treated with sodium dodecyl sulfate, and centrifuged on a similar gradient. The result shown in Figure 4 demonstrates that the 24S complex typically was relaxed by the addition of sodium dodecyl sulfate while the 23S noncomplexed *Col*E$_1$ DNA was unaffected. The relaxation of the 24S *Col*E$_1$ DNA complex by pancreatic DNase followed first order kinetics (Figure 5), a characteristic of supercoiled circular DNA undergoing a single strand scission (Vinograd *et al.*, 1965; Vinograd and Lebowitz, 1966) and previously shown for noncomplexed *Col*E$_1$ DNA (Bazaral and Helinski, 1968).

The significance of the above DNase experiment is several fold. First, it primarily represents a confirmation of the supercoiled nature of the *Col*E$_1$ DNA in the 24S complex. Secondly, it argues against the possibility that the differential response of relaxation to the denaturing conditions shown by complexed and noncomplexed *Col*E$_1$ DNA is due to an intrinsic difference in susceptibility of the plasmid DNA to endonuclease when present in the complexed or noncomplexed state. It also is worth noting that after conversion of the *Col*E$_1$ DNA

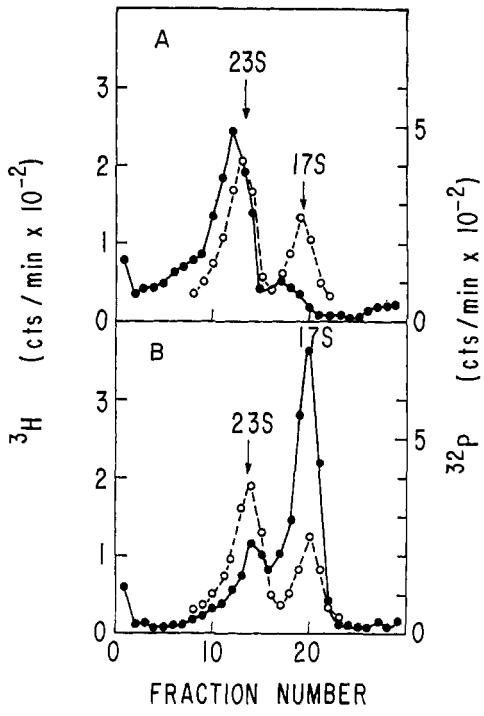

FIGURE 4: Sedimentation analysis of relaxation complex after treatment with sodium dodecyl sulfate. A 0.2-ml sample of purified complex, diluted in TM buffer, was mixed with 0.1 ml of ^{32}P-labeled noncomplexed plasmid DNA and 0.1 ml of TM buffer containing 0.1% sodium dodecyl sulfate. This mixture and a second sample of purified complex mixed only in TM buffer were incubated at 25° for 10 min and then 0.3 ml of each was removed for sucrose gradient analysis: (A) control, not treated with sodium dodecyl sulfate; (B) sample treated with sodium dodecyl sulfate. Sedimentation conditions were as described in the legend of Figure 3: (-●-●-) ^3H-labeled plasmid DNA complex; (--O--O--) ^{32}P-labeled noncomplexed plasmid DNA.

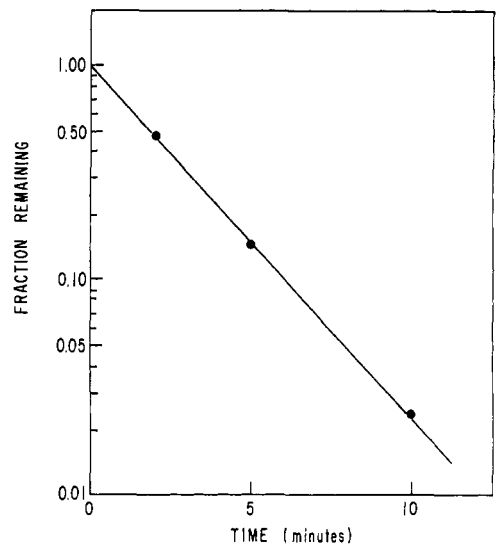

FIGURE 5: Kinetics of DNase-induced relaxation of complexed *Col*E_1 DNA. The fraction remaining was calculated using the control sample (see Figure 3A) to represent a zero time point. The corrected values used in each case were calculated from the area above the line drawn across the base of each 24S peak in Figure 3.

of the complex into the open circular state by pancreatic DNase, the DNA appears to sediment slightly ahead of the 17S noncomplexed open circular DNA (Figure 3). When the complex is relaxed by treatment with sodium dodecyl sulfate, the resulting open circular material always sediments coincident with 17S, or protein-free open circular marker material (Figure 4).

Recovery of Complexed ColE_1 DNA from Sarkosyl Lysates and Estimates of the Nunber of Copies of ColE_1 DNA per Chromosome. The purification of 24S *Col*E_1 DNA–protein complex involves a low speed centrifugation step (clearing spin) which normally pellets about 95% or more of the host DNA. Quantitation of the amount of plasmid DNA in the supernatant by sucrose gradient analysis gives estimates of the number of plasmid molecules per *E. coli* chromosome which are significantly higher than earlier published estimates. The previous estimate of a minimum of four copies per host chromosome was based on the relative amount of covalently closed circular DNA obtained when Sarkosyl lysates of *Col*E_1-containing cells were centrifuged in ethidium bromide–CsCl equilibrium buoyant density gradients (Bazaral and Helinski, 1968). It was shown earlier (Clewell and Helinski, 1969) that treatment of the purified *Col*E_1 DNA–protein complex with Sarkosyl or centrifugation of the complex to equilibrium in an ethidium bromide–CsCl gradient results in a conversion of the complexed supercoiled *Col*E_1 DNA to the open circular form. It was also shown that under these centrifugation conditions the supercoiled *Col*E_1 DNA not only relaxes but also bands at a less dense position due to a tight association with protein. It is clear, from these observations, that if the majority of supercoiled *Col*E_1 DNA is in the complexed state, then centrifugation of crude lysates in an ethidium bromide–CsCl gradient would result in a poor recovery of *Col*E_1 as supercoiled DNA.

These considerations lead to the prediction that when the total DNA in a crude Sarkosyl lysate of cells carrying the *Col*E_1 factor is subjected to dye-buoyant density centrifugation, the plasmid DNA in complex with protein should be found in the open circular form in a region coincident with or on the light side of the large chromosome peak. This prediction was tested by examining these regions of a dye-buoyant density gradient for the presence of a distinct 17S peak representative of open circular *Col*E_1 DNA. Six regions of a dye-buoyant density gradient centrifugation of a Sarkosyl lysate of JC411 (*Col*E_1) were pooled, dialyzed and analyzed by sucrose gradient centrifugation (see Figure 6). The results shown in Figure 7 and tabulated in Table I clearly demonstrate that open circular DNA (17 S) is present on the light side of the chromosome peak (predominately in fractions 3 and 4) to the extent of 2.7-fold more than the amount of the 23S supercoiled DNA in the more dense satellite peak in the gradient.

The experiment thus yields the result predicted above, and it is apparent that the percentage of *Col*E_1 DNA in the complexed state in a "cleared" lysate, as determined by the degree to which the DNA can be induced to relax, is reasonably representative of the percentage of the total plasmid DNA in the complexed state in a crude lysate.

Estimates of the proportion of total DNA recovered as *Col*E_1 DNA varied depending upon the quantitation procedure employed. Estimates of *Col*E_1 DNA (complexed plus non-

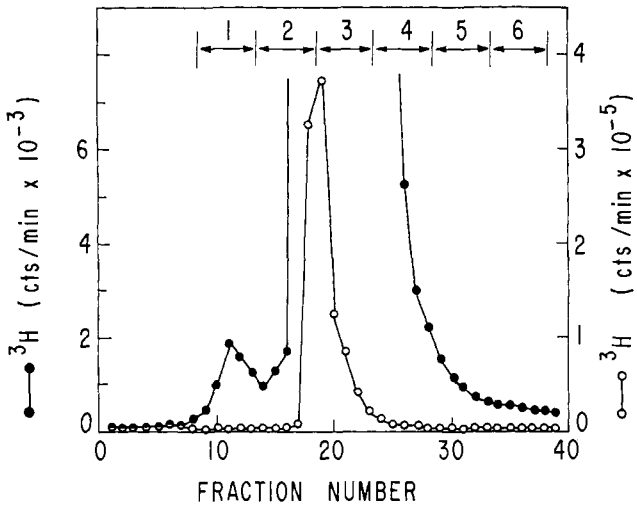

FIGURE 6: Dye-buoyant density gradient centrifugation of a Sarkosyl lysate of cells containing the complexed *Col*E$_1$ DNA. A Sarkosyl lysate was prepared from a 30-ml log phase culture of JC411(*Col*E$_1$) labeled with [^{3}H]thymine as described in Experimental Section. A 2.5-ml sample of the lysate was added to a mixture of 15 g of CsCl, 12 ml of TES, and 1 ml of a solution of ethidium bromide (3 mg/ml in TES). The solution was centrifuged in the Ti-60 rotor (15°) at 44,000 rpm for 60 hr. A 0.5-ml portion of each fraction was diluted fivefold and 0.05 ml of each dilution was used for counting. The DNA counts are plotted on two different scales. The numbers 1–6 designate fractions pooled from sucrose gradient analysis.

complexed) by the procedures of dye-buoyant density centrifugation (as described in Figures 6 and 7), sucrose gradient centrifugation analysis of a cleared lysate (as described in Figure 1A), and sucrose gradient centrifugation of crude lysates gave as an average of at least four different preparations for each procedure 1.32%, 1.57%, and 2.25%, respectively, for the proportion of total DNA recovered as *Col*E$_1$ DNA. Assuming a molecular weight of 2.8×10^9 for the *E. coli* chromosome (Cairns, 1963) these recoveries correspond

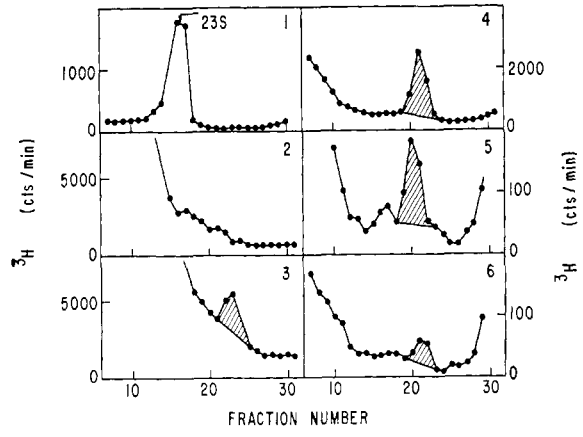

FIGURE 7: Sedimentation analysis of the fractions pooled after dye-bouyant density centrifugation of a Sarkosyl lysate shown in Figure 6. Sedimentation was through 5–20% neutral sucrose density gradients (from right to left) in the SW65 rotor (15°) at 50,000 rpm for 135 min. The shaded regions represent plasmid DNA in the "relaxed" state. Recovery of ^{3}H counts from the gradients ranged from 74 to 96%.

TABLE I: A Tabulation of the Relative Amounts of Supercoiled and Relaxed Plasmid DNA Recovered after Dye-Buoyant Density Centrifugation of a Sarkosyl Lysate.

Pool	Relative CPM in Pool[a]	Per Cent Plasmid[b]	Relative CPM Plasmid	
			Supercoiled	Relaxed
1	6,175	45.3	2797	
2	338,748			
3	646,971	0.66		4270
4	33,002	9.1		3303
5	4,860	4.0		194
6	2,597	2.2		57
Total	1,032,353	1.0	2797	7524

[a] Based on sum of "counted" aliquots of each fraction in the pool. [b] Based on sucrose density gradient analysis (*i.e.*, the amount of plasmid in each pool expressed as a percentage of the total counts recovered in each gradient).

to an average of 9 to 15 copies of *Col*E$_1$ DNA per chromosome per cell.

*Estimation of the Amount of Tightly Bound Protein in the Col*E$_1$ *DNA–Protein Complex.* Assuming that the major substance other than DNA in the plasmid–protein complex is of a protein nature, the extent of reduction of the buoyant density of *Col*E$_1$ DNA in a cesium chloride gradient should reflect the proportion of tightly bound protein in the complex. A liter of tritium-labeled JC411(*Col*E$_1$) cells was grown to log phase and harvested, and a cleared lysate was prepared after lysis of the cells by the Brij–DOC procedure (see Experimental Section). A sample of the cleared lysate was then centrifuged to equilibrium in a CsCl buoyant density gradient. As shown in Figure 8, a substantial amount of material is present on the "light" side of the main DNA band. The two regions indicated were pooled, dialyzed, and analyzed by sucrose gradient

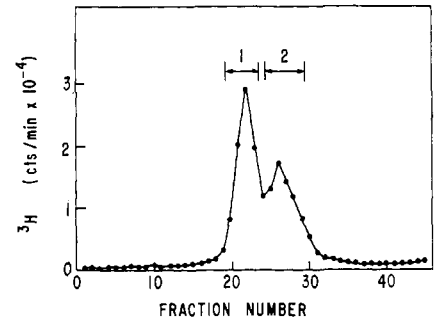

FIGURE 8: CsCl-buoyant density gradient centrifugation of a cleared lysate. A liter of ^{3}H-labeled (2.0 mCi of ^{3}H at 0.015 μg/ml of thymine) log phase JC411(*Col*E$_1$) cells was harvested and a cleared lysate prepared essentially according to the procedure described in the Experimental Section. A 10-ml sample of cleared lysate was mixed with 2 ml of TES and 15 g of CsCl and centrifuged in the Ti-60 rotor (15°) at 44,000 rpm for 60 hr. A 0.05-ml sample of each fraction was used for counting. Recovery of radioactivity was 68%. The numbers 1 and 2 designate regions where fractions were pooled for sedimentation analyses.

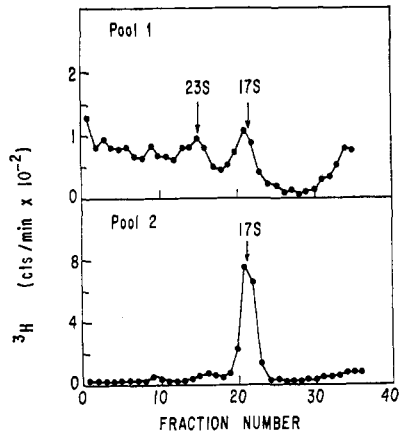

FIGURE 9: Sedimentation analysis of the pooled fractions shown in Figure 8. The pools were dialyzed and 0.1-ml samples were centrifuged through 5–20% sucrose density gradients (from right to left) in the SW65 rotor (15°) at 50,000 rpm for 135 min. Recovery of ^{3}H counts was 90% for pool 1 and 82% for pool 2.

centrifugation (Figure 9). The less dense region consisted predominantly of 17S or open circular *Col*E$_1$ DNA. When analyzed directly in the analytical ultracentrifuge in the presence of a density marker, material pooled from the less dense region (pool 2) of the preparative gradient exhibited the profile shown in Figure 10. The rather broad light band which presumably represents the complex exhibited a calculated buoyant density approximated at 1.685 g/ml. The additional band with a density of 1.707 g/ml apparently represents some host chromosomal DNA and possibly some noncomplexed *Col*E$_1$ DNA. If the original cleared lysate was treated with pronase, the less dense peak did not appear in the preparative CsCl gradient and the DNA taken from a region corresponding to the less dense region of the treated sample exhibited a density distribution coincident with the 1.707

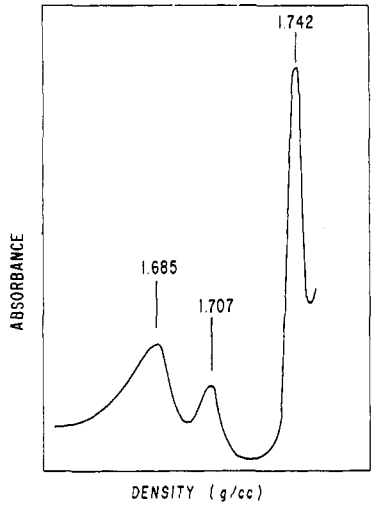

FIGURE 10: Analytical CsCl density equilibrium centrifugation of less dense *Col*E$_1$ DNA complex prepared from preparative CsCl centrifugation of cleared lysate as shown in Figure 8. Pool 2 as shown in Figure 8 was diluted twofold with a CsCl solution (ρ 1.700 g/ml), mixed with reference DNA and centrifuged as described in the Experimental Section.

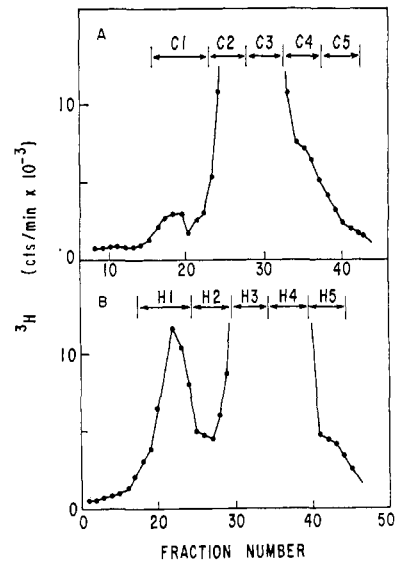

FIGURE 11: Dye-buoyant density gradient centrifugation of Sarkosyl lysates of untreated and heat treated cells. Sarkosyl lysates were prepared from JC411(*Col*E$_1$) cells before and after incubation of the cells at 70° for 15 min as described in the Experimental Section. Lysates were subjected to the centrifugation conditions described in the legend of Figure 6. A portion (0.05 ml) of each fraction was counted. Recovery of ^{3}H in each case was greater than 98%: (A) lysate of unheated cells; (B) lysate of heated cells. Fractions were pooled as shown for subsequent sedimentation analysis.

g/ml band. Thus, the *Col*E$_1$ DNA–protein complex recovered from the CsCl gradient exhibits a buoyant density in the CsCl gradient that is 0.022 g/ml less dense than noncomplexed *Col*E$_1$ DNA. Assuming that a simple protein has a buoyant density in CsCl of 1.300 g/ml, and that the density of a complex is the weight average of the densities of its components, it can be calculated that protein in the relaxed complex is present to the extent of approximately 5.4% of the total mass. Therefore, the amount of protein in the complex bound to the 4.2×10^6 daltons *Col*E$_1$ molecule is approximately 240,000 daltons. Some heterogeneity in the density of the relaxed complex, appearing as a skewing of the band toward the lighter end of the gradient (Figure 10), suggests the presence of a significant portion of *Col*E$_1$ DNA molecules with greater amounts of protein than the average molecule. While the heterogeneity of this material is not apparent to any significant degree in the 17S peak of the sucrose density gradient (Figure 9), some heterogeneity is often seen in the 24S complex upon sedimentation in sucrose (see Figures 2A, 3A, and 4A).

It is clear of course that the above calculation is based on the assumption that the material attached to plasmid DNA is entirely protein. Although the material responsible for the buoyant density difference is removed by treatment with pronase (Clewell and Helinski, 1969) the presence in the complex of lipoprotein, glycoprotein, or some other substance, however, has not been ruled out. It is also possible that during the preparative centrifugation procedure some protein material is removed from the plasmid–protein complex. This latter possibility is supported by the observation that the open circular *Col*E$_1$ DNA found in the less dense region of the gradient sediments practically coincident with 17S open circular noncomplexed *Col*E$_1$ DNA in contrast to the slightly

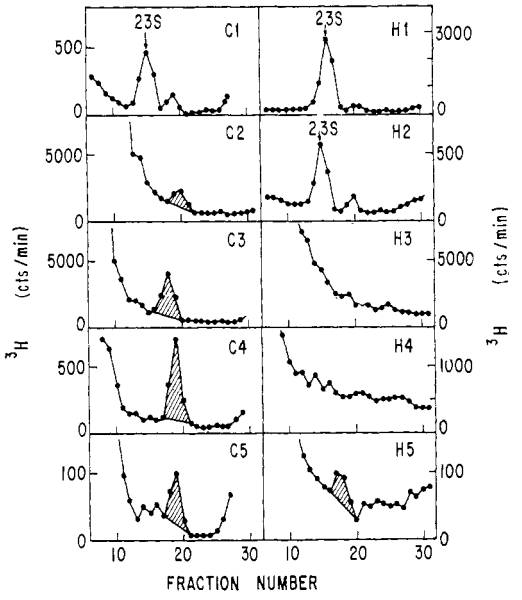

FIGURE 12: Sedimentation analysis of the pooled fractions shown in Figure 11. Sedimentation was through 5–20% sucrose density gradients (from right to left) in the SW50.1 rotor (15°) at 50,000 rpm for 100 min. The shaded regions correspond to the open circular form of *Col*E$_1$ DNA. Recovery of ^{3}H counts in the gradients ranged from 76 to 100%.

TABLE II: Effect of 70° Heat Treatment on the Relative Amounts of Supercoiled and Relaxed Plasmid DNA Recovered after Dye-Buoyant Density Centrifugation of Sarkosyl Lysates.

Pool	Relative CPM in Pool[a]	Per Cent Plasmid[a]	Relative CPM of Plasmid: Supercoiled[b]	Relative CPM of Plasmid: Relaxed
C1	18,340	18.1	3,320	
C2	1,015,360	0.28		2,843
C3	816,808	3.2		26,138
C4	37,268	8.7		3,242
C5	13,664	3.6		491
Total	1,901,440	1.9	3,320	32,714
H1	52,375	52.7	27,602	
H2	29,071	11.4	3,314	
H3	1,739,459			
H4	407,086			
H5	30,530	1.1		335
Total	2,258,521	1.4	30,916	335

[a] Calculated the same way as in Table I. [b] Since this pool represents satellite material, the small amount of relaxed DNA observed must have arisen from some random breakdown of supercoiled DNA.

more rapidly sedimenting nature of the *Col*E$_1$ DNA–protein complex obtained after pancreatic DNase treatment (see earlier section in Results).

*Effect of Heat Treatment of Cells on the Recovery of Col*E$_1$ *DNA–Protein Complex.* A 30-ml culture of JC411 *thy*$^-$ (*Col*E$_1$) cells, labeled with [^{3}*H*]thymine, was harvested in log phase and resuspended in 3.4 ml of 25% sucrose and 0.05 M Tris, pH 8.0. The suspension was then divided into two portions of 1.7 ml. One portion served as a control and was kept on ice while the other was heated at 70° for 15 min. Sarkosyl lysates were then prepared and centrifuged to equilibrium in dye-buoyant density gradients. As shown in Figure 11, the more dense satellite peak consisting of covalently closed circular *Col*E$_1$ DNA is present to a considerably greater extent in the heated sample when compared to the unheated control. Specific regions of the gradients were pooled, dialyzed, and analyzed on sucrose gradients. Figure 12 and Table II show that open circular *Col*E$_1$ DNA could not be found in the light side of the chromosome peak in the case of the DNA of heated cells, whereas the DNA of unheated cells exhibited a pattern similar to that demonstrated earlier (see earlier section in Results). At the same time, the increased DNA found in the more dense satellite peak of the DNA of the heated cells was approximately equivalent to the amount of plasmid DNA that is shifted to the light side of the chromosome in the case of the unheated control. Figure 13 demonstrates that the supercoiled DNA from the satellite region of the gradient of the DNA of heated cells is insensitive to pronase.

A reasonable interpretation of these observations is that heating cells prior to lysis with Sarkosyl results in the inactivation of the relaxation protein followed by either the detachment of the protein from the *Col*E$_1$ DNA or a failure of the protein to associate tightly with the supercoiled *Col*E$_1$ DNA during lysis of the cells. A comparison of the two profiles in Figure 11 indicates that the satellite DNA peak in both the heated and the unheated samples bands in approximately the same position relative to the chromosome peak. In a different, but identically performed experiment the refractive indices of the satellite peaks from both heated and unheated samples were within 0.0003 unit of each other. Careful comparison with noncomplexed *Col*E$_1$ DNA on sucrose gradients indi-

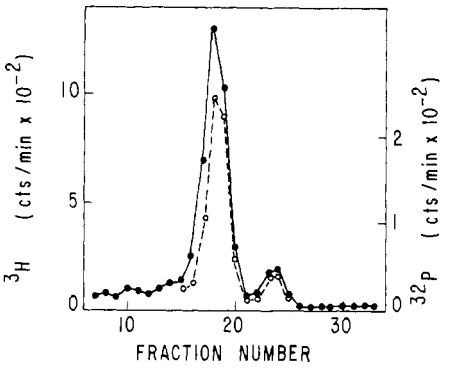

FIGURE 13: Sedimentation analysis of pool H1 after treatment with pronase. A sample (0.2 ml) of pool H1 (see Figure 11) was mixed with 0.1 ml of ^{32}P-labeled noncomplexed plasmid DNA and 0.1 ml of a 1 mg/ml solution of pronase (in TES). The mixture was incubated for 10 min at 25° before centrifugation. Centrifugation was through a 5–20% sucrose density gradient (from right to left) in the SW50.1 rotor (15°) at 50,000 rpm for 100 min. Recovery of ^{3}H counts in the gradient was 80%: (-●-●-)^{3}H-labeled DNA from pool H1; (--○--○--) ^{32}P-labeled noncomplexed plasmid DNA.

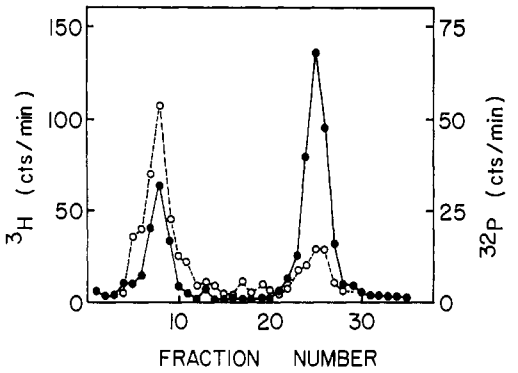

FIGURE 14: Sedimentation analysis of relaxation complex on an alkaline sucrose density gradient. This sample is identical with that used in the experiment of Figure 2. Figure 2A represents a neutral gradient control for this experiment. A 0.2 ml sample of ^{3}H-labeled complexed *Col*E$_1$ (diluted eightfold) was mixed with 0.1 ml of ^{32}P-labeled noncomplexed *Col*E$_1$ DNA and 0.1 ml of TES prior to layering (0.2 ml) on the gradient. Sedimentation was through a 5–20% alkaline sucrose gradient (from right to left) in the SW65 rotor (15°). Centrifugation was for 100 min: (-●-●-) ^{3}H-labeled complex; (--O--O--) ^{32}P-labeled noncomplexed *Col*E$_1$ DNA. Recovery of ^{3}H counts was 74%.

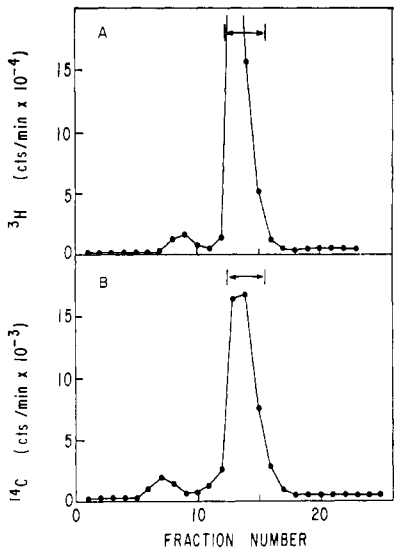

FIGURE 15: Dye-buoyant density gradient centrifugation of cleared lysate in the preparation of differentially labeled *Col*E$_1$ DNA. Log cultures (1 l.) of JC411(*Col*E$_1$) were labeled in one case with [^{3}*H*]-thymine (4.0 mCi at 0.03 μg/ml) and in the other case with [^{14}*C*]thymine (0.1 mCi at 0.228 μg/ml). The cultures were harvested and resuspended in 10 ml of 25% sucrose and cleared lysates were prepared as described in the Experimental Section. A 24-ml sample of the cleared lysate was mixed with 6 ml of a 5 mg/ml solution of pronase and incubated for 20 min at 37°. In each case, duplicate 12-ml samples were then mixed with 15 g of CsCl, 0.5 ml of TES, and 3 ml of a solution of ethidium bromide (1 mg/ml). The solutions were centrifuged in the Ti-60 rotor (15°) at 44,000 rpm for 60 hr. This procedure was performed in duplicate; however the result of only one sample is shown for each case. A and B represent the ^{3}H- and ^{14}C-labeled samples, respectively. Recovery of radioactivity was 64% in the case of A and 61% in the case of B.

cates that the supercoiled DNA from the heated sample sediments as a 23S molecule rather than the 24S form that is typical of the plasmid–protein complex. In addition, this DNA behaves as a covalently closed circular molecule when centrifuged through an alkaline sucrose gradient.

The 70° heat treatment renders the cells resistant to lysis by the Brij–DOC procedure; however, in cases where some lysis was obtained, the purified plasmid obtained by sucrose gradient centrifugation was resistant to induced relaxation by sodium dodecyl sulfate.

The differences between Table I and Table II regarding total recovery of plasmid DNA may be due to differences in the amount of binding of the complexed DNA to the wall of the centrifuge tube during centrifugation. Purified complexed DNA binds readily to the centrifuge tube, but this can be prevented by centrifuging in the presence of bovine serum albumin (5 mg/ml) (Clewell and Helinski, 1969). In crude lysates, where the bulk chromosomal DNA as well as cellular protein is present, this problem is not as serious, although some binding may occur. Noncomplexed supercoiled DNA does not appear to bind to the tube wall to any significant extent.

*Alkaline Sucrose Gradient Centrifugation of the Col*E$_1$ *DNA–Protein Complex.* Centrifugation of the purified *Col*E$_1$–protein complex in an alkaline sucrose gradient resulted in a relaxation of the supercoiled *Col*E$_1$ DNA as indicated by the presence of a relatively small amount (20–30%) of rapidly sedimenting, or covalently closed, *Col*E$_1$ DNA in the alkaline gradient (Figure 14). Supercoiled noncomplexed DNA sediments as a rapidly sedimenting molecule. It has been consistently observed that the percentage of relaxation that can be produced by sodium dodecyl sulfate, as demonstrated by neutral gradient centrifugation, is the same percentage of relaxation that is observed upon sedimentation on alkaline gradients. If the purified plasmid–protein complex is centrifuged for a longer period of time in the alkaline sucrose gradient, the single stranded DNA obtained from the relaxed or open circular form of *Col*E$_1$ can be separated into two peaks one of which sediments 1.13 times faster than the other (see next section of Results and Figure 16). These are interpreted as representing the circular and linear single strands of the open circular *Col*E$_1$ DNA (Vinograd and Lebowitz, 1966). Approximately equal amounts of the circular and linear strands are recovered and there is no indication of gross heterogeneity in the length of the linear strand. These results are consistent with the presence of a single nick or gap in only one of the two strands of the relaxed form of the *Col*E$_1$ DNA in the plasmid–protein complex.

*Strand Specificity of the Relaxation of Supercoiled Col*E$_1$ *DNA.* To determine whether the relaxation phenomenon is strand specific, the relative degrees of hybridization between the circular and linear single-stranded DNA obtained from the relaxed *Col*E$_1$ DNA was examined. Both ^{3}H- and ^{14}C-labeled *Col*E$_1$ DNA were prepared by growing 1-l. cultures of JC411(*Col*E$_1$) separately with [^{14}*C*]thymine (0.1 mCi) and [^{3}*H*]thymine (4.0 mCi) for radioisotope labeling of the DNA. The cells of each culture were harvested and Brij–DOC lysis was carried out as described in an earlier section (Experimental Section). Cleared lysates were prepared and then incubated with pronase at 37° for 30 min. The pronase treatment induces a relaxation of the complexed *Col*E$_1$ DNA and at the same time removes protein tightly bound to the DNA. The samples were then centrifuged to equilibrium in dye-buoyant density gradients. The resulting profiles are seen in

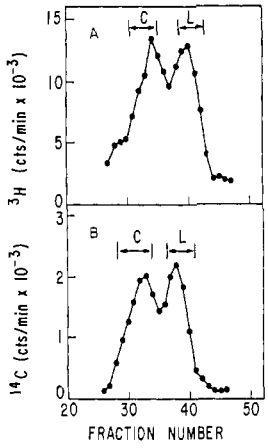

FIGURE 16: Alkaline sucrose gradient analysis of relaxed *Col*E$_1$ DNA purified by cesium chloride gradient centrifugation. The pooled fractions shown in Figure 15 (duplicate pools were combined) were dialyzed and centrifuged through 5–20% alkaline sucrose density gradients (from right to left) in the SW25.1 rotor (20°) at 22,500 rpm for 24 hr. Fractions (25 drop) were collected and a 0.05-ml portion of each fraction was counted. The ^{3}H- and ^{14}C-labeled DNA are seen in A and B, respectively. The pools designated C and L represent single-strand circular and linear DNA, respectively. The total gradient corresponds to 80 fractions, and C sediments 1.13 times faster than L. Recoveries of radioactivity in the gradients were close to 100%.

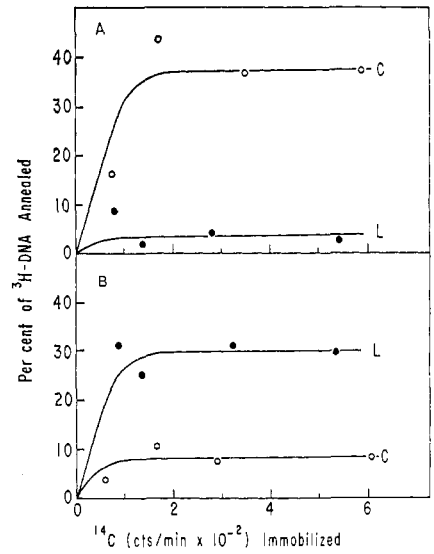

FIGURE 17: Hybridization analysis of circular and linear single stranded DNA purified from relaxed *Col*E$_1$ DNA. In each case varying amounts of [^{14}*C*]DNA were immobilized on membranes. ^{14}C-labeled L and C strands were fixed in A and B, respectively. The amount of ^{3}H-labeled DNA tested for hybridizability to the fixed [^{14}*C*]DNA was constant in each case and represented 4407 cpm for the C DNA and 5518 cpm for the L DNA. The specific activities of the DNA's were 173,000 and 15,000 cpm/μg for ^{3}H and ^{14}C, respectively. The open circles (○) represent the degree of hybridization of ^{3}H-labeled C DNA while the solid circles (●) represent the degree of hybridization of ^{3}H-labeled L DNA. Each point represents the average of triplicate hybridization mixtures.

Figure 15. The material in the major peak (containing relaxed *Col*E$_1$ DNA circles) in each case was pooled, dialyzed, and centrifuged on alkaline sucrose density gradients. The resulting separation of the single strands corresponding to C (circular) and L (linear) DNA is seen in Figure 16. Fractions were pooled as indicated and used for hybridization as described in Experimental Section. Varying amounts of ^{14}C-labeled C and L strands were immobilized on membrane filters, while a constant amount of ^{3}H-labeled C and L strands was tested for the degree of hybridization with the ^{14}C-labeled material. Figure 17 indicates the amount of ^{3}H-labeled DNA that annealed to the varying amounts of ^{14}C-labeled DNA fixed to the membranes. It is quite evident that there is a considerable degree of preferential hybridization between the L and C strands indicating that the relaxation phenomenon is strand specific. The low degree of apparent self-annealing is undoubtedly at least partly due to some cross contamination in the original circular and linear strand pools.

Effectiveness of Various Agents and Conditions in the Induction of Relaxation. Agents and conditions which have been tested for ability to promote relaxation of the *Col*E$_1$ DNA–protein complex are summarized in Table III. Sodium dodecyl sulfate was tested at several concentrations and was found to induce relaxation consistently at concentrations as low as 0.025%. Concentrations of sodium dodecyl sulfate as low as 0.0025% have induced relaxation, but not consistently. The level of EDTA in the routinely used buffer, TES, is 0.005 M. At EDTA levels as high as 0.06 M, the effect of 0.25% sodium dodecyl sulfate was not inhibited. Similarly, the presence of 0.01 M $MgCl_2$ had no observable effect on sodium dodecyl sulfate (0.25%) induced relaxation.

Sodium dodecyl sulfate and Sarkosyl (both active agents) are ionic detergents, while Brij 58 (an inactive agent) is a neutral detergent. DOC, an ionic detergent, is not an effective inducing agent as indicated by its failure to produce relaxation in the Brij–DOC lysis mixture (DOC is at a level of 0.2% at this point). However, if purified complex is exposed to 0.25% DOC, some degree of relaxation occurs. While this amount of DOC does not affect the complex in the cleared lysate stage, exposure of the latter to 1% DOC results in about 50% relaxation of the complex. If DOC is omitted from the lysis procedure, a typical relaxation complex is still recovered if the remainder of the procedure is unchanged.

The following agents also failed to promote relaxation: ATP, DPN, DPNH, cyclic AMP, dATP, dCTP, dTTP, dGTP.

TABLE III: Summary of Agents and Conditions Tested for Promotion of Relaxation of the Complex.

Relaxation	No Relaxation
Pronase, 1.25 mg/ml, 2.5 μg/ml	Bovine serum albumin, 250 μg/ml
Trypsin, 250 μg/ml	DOC, 0.2%
Sarkosyl, 0.25%, 0.025%	Brij 58, 1%, 0.5%
Sodium dodecyl sulfate, 1.0%, 0.25%, 0.025%	Brij 58–DOC, 0.5%–0.2%
Alkali, pH 12.5	NaCl, 1 M
Phenolization	Ethanol, 24%
Heat, 60° for 20 min	$MgCl_2$, 0.04 M, 0.01 M
	$CaCl_2$, 0.04 M
	2-Mercaptoethanol, 0.022 M

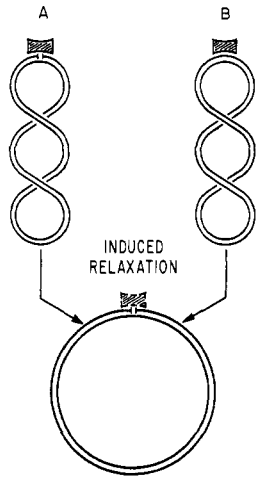

FIGURE 18: Two possible models for the relationship of the protein to supercoiled *Col*E$_1$ DNA. (A) *Col*E$_1$ DNA within the complex possesses a nick or gap at a specific site on one of the two helical strands. Protein material with binding properties is associated at the open site and constrains the open strand so that the circular duplex exhibits the property of a supercoiled, or covalently closed, circular molecule. (B) *Col*E$_1$ DNA within the complex is covalently closed, and an inhibited nickase is associated at a specific site on the supercoiled *Col*E$_1$ DNA. Treatment of the complex with agents or conditions which affect protein structure results in an alteration, or loss of the binding protein in the case of A, or an activation of the strand specific nickase in the case of B. Depending on the treatment, some or all of the protein may be dissociated from the DNA.

All of the latter agents were tested at a concentration of 0.25 mg/ml and in the presence of 0.01 M $MgCl_2$.

Discussion

Gentle lysis of colicinogenic *E. coli* cells permits the recovery of a substantial proportion of the supercoiled *Col*E$_1$ DNA in the form of a complex with cellular material. At low ionic strength the *Col*E$_1$ DNA initially sediments as a 34S complex which contains loosely bound cellular material. The latter readily falls apart upon dilution or dialysis, and gives rise to supercoiled DNA appearing mainly (*i.e.*, to the extent of 70–80%) as the 24S relaxation complex. It is not clear whether or not the loosely bound cellular material is plasmid specific. It does not appear to be related to the relaxation phenomenon since 23S supercoiled (noncomplexed) DNA when added to the lysate of colicinogenic cells, or to intact cells prior to lysis, does not form a relaxable complex (Clewell and Helinski, 1969); however, the added supercoiled DNA does sediment initially at approximately 34 S.

The unusually high affinity of the *Col*E$_1$ DNA for the protein material has permitted an estimation of the amount of protein in the complex by cesium chloride buoyant density centrifugation. The estimate of 240,000 daltons of protein complexed with 4.2×10^6 daltons of open circular DNA is of course based on the assumption that lipid, polysaccharide, or RNA is not present in the complex. Furthermore, this estimate applies only to the relaxed form of the complex and does not necessarily give any indication of the amount of protein present when the DNA is in the supercoiled configuration. An estimate of the amount of protein present in the unrelaxed complex cannot be made by this procedure since the conditions of buoyant density centrifugation promote relaxation. As shown earlier the buoyant density of noncomplexed *Col*E$_1$ DNA is unaltered by treatment with pronase (Clewell and Helinski, 1969).

The fact that the *Col*E$_1$ DNA in the 24S complex is relaxed by the conditions of equilibrium centrifugation in a cesium chloride gradient prevents an accurate quantitation of the molecules of *Col*E$_1$ DNA within an *E. coli* cell by an estimation of only DNA in the dense satellite peak observed in the dye-buoyant procedure. The amount of purified *Col*E$_1$ DNA that can be induced to relax by treatment with sodium dodecyl sulfate or pronase indicated the association of approximately 70–80% of the supercoiled molecules in the form of a relaxation complex under the growth conditions employed. This estimate corresponds well with the finding that greater than 70% of *Col*E$_1$ DNA is recovered as an open circular molecule after dye-buoyant density centrifugation of a Sarkosyl lysate. When the relaxed *Col*E$_1$ DNA found in the less dense shoulder of the chromosomal DNA peak is taken into account, the number of copies of *Col*E$_1$ DNA determined by this procedure is consistent with the 10–15 copies of *Col*E$_1$ DNA per chromosome that is observed by the procedure of sucrose gradient analysis of cells lysed by the Brij–deoxycholate procedure. Heat treatment of cells prior to lysis with Sarkosyl allowed practically complete recovery of *Col*E$_1$ DNA as covalently closed circular DNA in the dense satellite peak after dye-buoyant density centrifugation, while relaxed DNA could no longer be recovered from the less dense shoulder of the chromosome peak. The additional material now appearing in the dense peak resembled noncomplexed DNA and represented supercoiled *Col*E$_1$ DNA that was resistant to pronase, detergents, or alkali.

On the basis of the properties exhibited by the relaxation complex, two models have been considered for the nature of the association of protein material with the *Col*E$_1$ DNA. As shown in Figure 18A, one possibility is that the protein possesses binding properties and is associated at the specific site of a nick or gap in the circular DNA molecule in such a way as to sufficiently constrain the *Col*E$_1$ DNA giving it the sedimentation properties and electron microscopy characteristics of a supercoiled circular duplex. Treatment of the complex with agents, or conditions, that destroy or denature protein would result in a release, or inactivation, of the bound protein followed by a relaxation of the supercoiled DNA.

Alternatively, the complex may consist of a covalently intact circular duplex associated with an inhibited nickase (Figure 18B). Treatment of the complex with a protease or protein denaturing agent would then result in a release of the inhibition and "trigger" the catalysis of a nick specifically in one of the two strands in the duplex. Conceivably the protein could consist of more than one subunit where one of the subunits possesses a nicking capability while another subunit(s) is present as an inhibitor(s) of the nicking activity. There have been several reports of the modification of the allosteric properties of an enzyme by treatment with detergents and proteases (Lee and Wang, 1968; McClintock and Markus, 1968; Taketa and Pogell, 1965).

The data presented thus far do not argue strongly for one model in favor of the other. It could be reasoned that if conditions were obtained that inactivated the complex (*i.e.*, rendered the complex resistant to proteases, detergents, and alkali), this would be evidence against the binding or "linker"

model (model A). While the results of heat treatment of whole cells prior to lysis could be interpreted as an inactivation of the relaxation complex, the possibility of a heat activated repair of a preexisting nick (*e.g.*, involving ligase) during this procedure cannot be ruled out. Heat treatment of the purified *Col*E_1 relaxation complex results in relaxation (Clewell and Helinski, 1969). If model B were correct the fact that only supercoiled noncomplexed plasmid DNA is recovered from heat-treated cells could be interpreted as resulting from a heat-induced dissociation of the *Col*E_1 DNA from the nicking protein within the intact cell. It is possible that *in situ* the *Col*E_1 DNA complex is part of, or associated with, a region of the cellular membrane. Heat may dissociate the *Col*E_1 DNA from protein in this environment whereas exposure of this hydrophobic membrane region to a hydrophilic environment by lysis (without prior heat treatment) may be responsible for the high affinity of the relaxation protein to the *Col*E_1 DNA.

It has been possible to demonstrate a relaxation complex in the *Col*E_2 system of *E. coli* which is very similar to the *Col*E_1 system with the exception that the *Col*E_2 complex can be inactivated *in vitro* by a 60° heat treatment (Clewell *et al.*, 1970a; D. G. Blair, D. J. Sherratt, D. B. Clewell, and D. R. Helinski, to be published). The fact that the *in vitro* heat treatment renders the *Col*E_2 complex insensitive to relaxation by pronase, detergent, or alkali argues more strongly against model A, but once again the possibility of a heat-activated sealing action cannot be completely ruled out in this case.

A possible structure for the relaxation complex that has certain features of both the binding "linker" model (model A) and the latent nickase model (model B) is one where the relaxation complex consists of a stable enzyme–substrate intermediate in which specifically one of the duplex strands is activated for either nicking or closure. In this case certain treatments (*e.g.*, protease, detergents, or alkali) would favor the reaction in the nicking direction while other treatments (*e.g.*, heat *in vivo* for *Col*E_1, or *in vitro* for *Col*E_2) favor the direction of complete closure.

The hybridization data which demonstrate strand specificity in the relaxation event is without doubt the most important finding thus far regarding physiological significance of the complex. An endonuclease-catalyzed nick in a covalently closed circular duplex is generally considered to be one of the early biochemical steps in the semiconservative duplication of a covalently closed circular duplex. It is possible that the *Col*E_1 DNA relaxation complex represents this biologically significant enzyme and its substrate. Assuming that strand elongation of *Col*E_1 DNA occurs at the same rate as that in chromosomal DNA, about 4–7 sec are required for the duplication of the plasmid. Thus individual plasmid molecules must spend the majority of the time during the cell cycle in a nonreplicating state. If "nicking" activity in the *Col*E_1 DNA relaxation complex is a representation of the initial nick in the *Col*E_1 duplication process, it would suggest that the early events in *Col*E_1 DNA duplication involves a "signal" within the cell which activates the inhibited strand specific nickase.

Several laboratories have reported evidence for the association of DNA with protein or membranous material during the replication process (Smith and Hanawalt, 1967; Ganesan, 1968; Botstein, 1968; Earhart *et al.*, 1968; Knippers and Sinsheimer, 1968) and the role of proteins in the initiation of DNA duplication (Maaløe and Hanawalt, 1961; Lark *et al.*, 1963). Salivar and Sinsheimer (1969) have shown an association of λ and φX-174 RF DNA with cell material having properties similar to that of cell membrane components. The λ and φX-174 RF DNA–cell component complexes contain considerably more cellular material than is indicated by the sedimentation properties of the *Col*E_1 DNA–relaxation complex and would appear to represent a considerably larger portion of the attachment site for circular extrachromosomal DNA than is possibly present in the 24S *Col*E_1 DNA complex.

Work is currently in progress examining the properties and physiological significance of the relaxation complex and the mechanism of the strand specific relaxation event. Recent results have shown that *Col*E_1 DNA synthesis continues linearly in the presence of chloramphenicol and the amount of *Col*E_1 DNA isolated as the relaxation complex remains at the level found prior to the addition of chloramphenicol (D. B. Clewell and D. R. Helinski, manuscript in preparation). It has also been found that the percentage of *Col*E_1 DNA molecules that can be isolated in the complexed state varies depending on the particular medium employed for cell growth (D. B. Clewell and D. R. Helinski, manuscript in preparation). Finally, regarding the general existence of relaxation complexes, it is noteworthy that complexes similar to that of the *Col*E_1 and *Col*E_2 relaxation complexes have been found for the extrachromosomal elements *Col*E_3-CA38, *Col*I_b-P9 (Clewell *et al.*, 1970b), and F_1 (Kline and Helinski, 1970) systems of *E. coli*.

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Limited Heterogeneity of the Major Nonhistone Chromosomal Proteins*

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ABSTRACT: There has been much discussion but little detailed work on the chemistry and biology of nonhistone chromosomal protein. The principal hindrances in their study have been the tendency of the proteins to aggregate and the difficulty in dissociating them from histone and DNA. For this study purified chromatin was used as starting material. The histones were extracted with 0.4 N H_2SO_4, and the remaining nonhistone chromosomal proteins then solubilized by 1% sodium dodecyl sulfate (SDS) in 0.05 M Tris (pH 8).

DNA was next removed by ultracentrifugation. The nonhistone chromosomal proteins were then examined by SDS gel electrophoresis (molecular weight sieving). Our preparations of rat liver nonhistone chromosomal protein include 13 major polypeptide bands of molecular weight *ca.* 5000 to *ca.* 100,000. Homologous peptides are found in chicken liver nonhistone chromosomal protein, while an additional high molecular weight band is found in preparations from chicken erythrocyte. Rat kidney nonhistone chromosomal protein lacks two and possesses one additional band relative to the rat liver protein fractions. Pea bud nonhistone chromosomal proteins include half of these same bands. The striking similarity of the nonhistone chromosomal proteins of different organs and creatures suggests that some of them are common enzymes, such as those of nucleic acid metabolism, and/or common structural proteins (analogous to histones). Some of the apparent differences may be organ and species specific.

Isolated interphase chromatin is composed of DNA, RNA, histones, and nonhistone chromosomal proteins (NHC proteins).[1] Little is known about the latter; their isolation and fractionation have been severely hampered by the tendency of NHC proteins to aggregate with DNA, histones, and one another. Chromatin possesses several enzymatic activities which may be associated with NHC proteins. These include RNA polymerase (Weiss, 1960; Huang *et al.*, 1960) and a neutral protease that preferentially degrades deoxyribonucleohistone (Furlan and Jericijo, 1967; Furlan *et al.*, 1968). Several general findings suggest that the NHC proteins play some role in the regulation of template activity. Studies of chromatin of different pea tissues (Bonner *et al.*, 1968b), of different stages of the sea urchin embryo (Marushige and Ozaki, 1967), and of different stages of spermatogenesis in trout testis (Marushige and Dixon, 1969) indicate that the more template-active chromatins of a given organism contain more NHC protein than do less template-active chromatins. Teng and Hamilton (1969) have reported that one of the major events in the early action of estrogen in the uterus of the ovariectomized rat is an increased rate of synthesis and accumulation of NHC protein in the uterine chromatin. Interestingly, Sadgopal and Bonner (1970) have found a striking increase in the NHC proteins of HeLa metaphase chromosomes as compared to HeLa interphase chromatin. Histones turn over at a low rate and are conserved in cell division (Byvoet, 1966; Hancock, 1969); in contrast it appears that at least some of the NHC proteins turn over very rapidly (Holoubek and Crocker, 1968). The NHC protein fraction may also include nuclear membrane components. In eukaryotes DNA synthesis appears to be initiated at the nuclear membrane (Comings and Kakefuda, 1968); isolated crude

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[1] Abbreviations used are: NHC proteins, nonhistone chromosomal proteins; SDS, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; DOC, sodium deoxycholate.