Purification and Characterization of Covalently Closed Replicative Intermediates of ColEl DNA from *Escherichia coli*†

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ABSTRACT: Pulse-labeled ColEl DNA molecules, undergoing replication in *Escherichia coli* cells either in the absence or presence of chloramphenicol, were extracted and purified by neutral sucrose density gradient sedimentation and equilibrium centrifugation in an ethidium bromide–cesium chloride gradient. In the dye–buoyant density gradient, the replicating molecules were found in regions between the supercoiled and open-circular nonreplicating plasmid DNA, as well as in the open-circular region. In a neutral sucrose gradient, peaks of pulse label were found in the region of 26 to 38 S as well as at the 23 and 17 S positions corresponding to the positions of supercoiled and open-circular ColEl DNA. In alkaline sucrose gradients, nascent ColEl DNA was found to sediment as discrete peaks corresponding to 5–6, 7–9, and 14–16 S, indicating that at least one growing strand of the replicating molecule is produced discontinuously. In the electron microscope, many of the molecules appeared as partially supercoiled structures containing two open-circular branches of equal length, of less than 20% to more than 90% replicated. Branched open-circular molecules were not observed to any significant extent without prior treatment to induce single-strand scissions. The parental strands of the replicating molecules were determined to be covalently closed, but the superhelical density of the DNA was shown to be progressively decreased as replication proceeded.

The colicinogenic El (ColEl) plasmid, a covalently closed, circular DNA element of a molecular weight of 4.2×10^6 (Bazaral and Helinski, 1968) has been isolated from *Escherichia coli* in the form of supercoiled DNA and as a relaxation complex consisting of supercoiled DNA and protein (Clewell and Helinski, 1969). In an exponentially growing culture there are 20 to 30 copies of the plasmid per cell (Clewell and Helinski, 1972). In the presence of chloramphenicol, replication of the plasmid continues and more than 3000 copies of ColEl DNA per cell have been found after 16 h of incubation in the presence of the drug (Clewell and Helinski, 1972; Clewell, 1972).

In recent years, much attention has been devoted to the understanding of the mechanism of replication of circular DNA elements. In two well-studied cases, replicating intermediates of SV40 and polyoma viruses have been shown by electron microscopy to have the appearance of partially supercoiled–partially open circular structures (Sebring et al., 1971; Jaenisch et al., 1971; Bourgaux and Bourgaux-Ramoisy, 1972; Roman et al., 1974). In both cases, unwinding of the superhelical turns in the molecule has been shown to take place gradually and continuously throughout the replication cycle through a proposed transient nick and repair process.

ColEl molecules purified from *E. coli* mini cells and having the appearance of replicating molecules upon electron microscopy analysis have been observed as circular molecules with two forks (Cairns intermediate) and with a linear double-strand tail (rolling circle intermediate), as well as partially supercoiled and partially open-circular structures that were analogous in appearance to the covalently closed replicative DNA of the papovaviruses (Fuke and Inselburg, 1972). Since this report, evidence has been obtained both from whole cells of *E. coli* (Helinski et al., 1974; Lovett et al., 1974a,b) and mini cells (Oka and Inselburg, 1975) in support of a covalently closed circular structure as the principal replicative intermediate in ColEl DNA replication.

The study of replicating ColEl DNA molecules in whole cells of *E. coli* is difficult, largely due to the fact that this plasmid is present in the amount of less than 2.0% of the total cellular DNA and that a very small proportion (less than 5%) of the plasmid molecules in an exponentially growing culture or chloramphenicol-treated culture are undergoing replication at any particular time (Clewell and Helinski, 1972; Katz and Helinski, 1974). Recent observations, however, have shown that after a brief period of thymine starvation in the presence of cyclic adenosine 3′,5′-monophosphate a stimulation of at least fourfold in the amount of plasmid DNA that can undergo simultaneous replication during a subsequent thymidine pulse takes place (Katz and Helinski, 1974). This stimulation is also seen in cells previously treated with chloramphenicol and occurs in the presence of the drug. Employing the above methods, it is possible to extract and purify from whole *E. coli* cells replicating ColEl DNA molecules that are pulse-labeled from the site of the origin of replication. The characterization of the structure of the replicating forms of this plasmid is the subject of this report.

Experimental Procedure

Bacteria. The *E. coli* K12 strain, JC411 (ColEl) *thy*⁻, carrying the ColEl plasmid has been described previously (Clewell and Helinski, 1972).

Medium. M9 salts (Roberts et al., 1963) were supplemented with 0.2% glucose, 0.5% casamino acids (Difco), and 1.5–2.5 μg/mL thymine.

Growth and Labeling Conditions. Preparative amounts of

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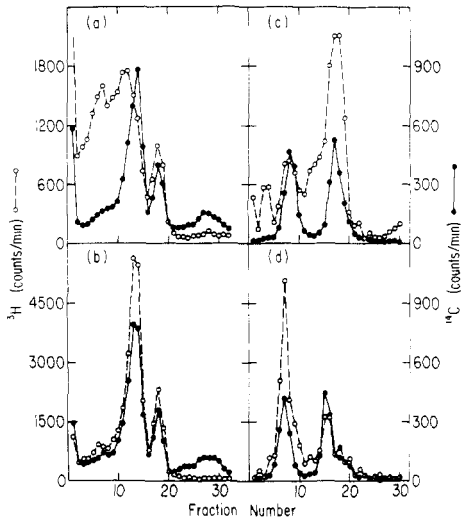

FIGURE 1: Identificatin of ColEl DNA from pulse-labeled cells. A 10-mL culture was prelabeled with [^{14}C]thymine to a density of 5×10^8 cells/mL, thymine starved in the presence of cAMP for 30 min. and then pulsed with [^{3}H]thymidine. A sample was taken after 15 s (pulsed sample) and unlabeled thymine and thymidine were added to the balance of the culture for 1 min (pulse and chased sample.) Spheroplasts of each sample were produced and lysed by the addition of Triton X-100. Neutral sucrose gradient sedimentation was carried out on the pulsed sample (a) and pulsed and chased sample (b). The gradients contained 5 mL of 5–20% sucrose, 0.05 M Tris-HCl (pH 7.5), 0.005 M EDTA, and 0.5 M NaCl. After centrifugation for 2 h at 50 000 rpm in a Beckman SW 50.1 rotor, fractions were collected dropwise from the bottom of the tubes. Dye-buoyant density gradient centrifugation was carried out on the pulsed sample (c) and pulse and chased sample (d). Each sample was mixed with 3.6 g of CsCl, 300 μg/ethidium bromide, and TES buffer (0.05 M Tris, pH 8.0, 0.005 M EDTA, 0.05 M NaCl) in a final volume of 5 mL and centrifuged at 38 000 rpm for 44–60 h in a Beckman Ti50 rotor. Fractions were collected from the bottom of the tubes.

replicating forms of ColEl DNA were obtained by growth of cells at 37 °C in 250–500-mL batch cultures containing [^{14}C]thymine (0.5 μCi/mL) to a density of 5×10^8 cells/mL, harvesting by centrifugation at 25 °C, and resuspending with an equal volume of medium lacking thymine and containing 4.5×10^{-3} M cAMP[1] and incubated at 37 °C for an additional 30 min. The culture then was aerated at 25 °C for 10 min and pulsed for 30 s with [^{3}H]thymidine (10 μCi/mL; 0.3 μg/mL). In cases where chase experiments were performed, a sample was removed from the culture and unlabeled thymidine (500 μg/mL) and thymine (20 μg/mL) were added to the balance of the culture. Incorporation was stopped by addition of sodium azide (5×10^{-2} M, final concentration) and the cells were frozen quickly in a dry ice–ethanol bath.

In the cases where CM was used, 200 μg/mL was added to a culture with a cell density of 5×10^8/mL. After 3.5 h of additional incubation, the culture was then subjected to the thymine starvation conditions. The drug was also employed during the subsequent thymine starvation treatment and pulse.

Extraction of ColEl DNA. The cells were thawed, collected by centrifugation, and resuspended to a density of $5\text{–}8 \times 10^{10}$ cells/mL into a solution containing 10% sucrose and 0.05 M Tris-HCl, pH 7.5. Lysozyme (200 μg/mL) and EDTA (0.02 M, final concentration) were added and the mixture was allowed to incubate at 0 °C for 45 min to permit the formation of spheroplasts. The spheroplasts were lysed by employing either of the following procedures. (1) The method of Schekman et al. (1972) employed addition of Triton X-100 (0.05%, final concentration). After lysis occurred, the mixture was centrifuged at 46 000*g* for 30 min and the supernatant fluid (cleared lysate) was saved. (2) The method of Hirt (1967) employed the addition of sodium dodecyl sulfate to the spheroplasts in the cold to a final concentration of 1%. Upon lysis, NaCl was added with occasional stirring to a final concentration of 1 M. The mixture was incubated at 0 °C for a minimum of 4 h, at which time a white precipitate was clearly visible. The mixture was centrifuged at 37 000*g* for 20 min and the supernatant fluid (Hirt supernatant) was saved. Both the cleared lysates and Hirt supernatants could be subjected directly to sucrose density gradient sedimentation or equilibrium centrifugation for fractionation of the ColEl DNA.

Centrifugation Procedures. Dye-buoyant density gradient equilibrium centrifugation in cesium chloride–ethidium bromide (Radloff et al., 1967) and neutral and alkaline sucrose density sedimentation procedures have been previously described (Bazaral and Helinski, 1968; Clewell and Helinski, 1970). All gradients were collected dropwise from the bottom.

Treatment of DNA with DNase I. Highly purified DNase I was kindly provided by J. Streiffel and P. Price. The enzyme was diluted to the appropriate concentration to introduce an average of six nicks per ColEl DNA molecule in 10 min at 25 °C. Reaction mixtures (50 μL) contained ColEl DNA (0.1–0.5 μg), 2.5×10^{-5} M $CaCl_2$, 4×10^{-3} M $MgCl_2$, 10^{-3} M EDTA, and 10^{-2} M Tris, pH 8.5. The reaction was terminated by the addition of EDTA to a final concentration of 5×10^{-3} M. The entire reaction mixture could be used directly for electron microscopy.

Electron Microscopy. DNA was prepared for electron microscopy by the aqueous and formamide techniques described by Davis et al. (1971). The DNA was mounted on parlodion-coated 400-mesh copper grids, rotary shadowed with 80% platinum–20% palladium in a Denton evaporator, and examined in a Phillips 200 electron microscope.

Results

Identification of Replicating ColEl DNA. To determine whether or not ColEl DNA molecules in the process of replication could be isolated from intact *E. coli* cells grown in the presence of cAMP, cells were prelabeled at 37 °C with [^{14}C]thymine followed by starvation for thymine in the presence of cAMP for 30 min. After incubation at 26 °C for 5 min, [^{3}H]thymidine was added and after 15 s, a sample was taken, and the balance of the culture chased for 1 min. The cleared lysates, obtained by the Triton X-100 procedure, were analyzed by sedimentation through neutral sucrose gradients and by dye-buoyant density gradient centrifugation. In the pulsed sample it can be seen (Figure 1a) that the majority of the ^{3}H pulse-labeled counts incorporated sediments at the 28–38S and the 17S positions. The material at the bottom of the gradient (>50 S) most likely represents fragmented chromosomal DNA. The prelabeled ColEl DNA (^{14}C-labeled material) in the sample sediments at the 23–24S position (corresponding to noncomplexed and complexed supercoiled ColEl DNA (Clewell and Helinski, 1969)) and at the 17S position (open-circular DNA). After a 1-min chase period, it can be seen that the ^{3}H pulse-labeled DNA cosediments with the ColEl [^{14}C]DNA at the 23S and 17S positions (Figure 1b).

In the ethidium bromide–cesium chloride density gradients of both samples, the ^{14}C prelabeled DNA is distributed into

[1] Abbreviations used are: CM, chloramphenicol; cAMP, cyclic adenosine 3′,5′-monophosphate; ET buffer, 0.01 M Tris, pH 8.5, 0.001 M EDTA; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

TABLE I: Recovery of ColEl DNA in Cleared Lysate Using Sodium Dodecyl Sulfate Extraction Method.[a]

Preparation	cpm		
	^3H	^{14}C	$^3H/^{14}C$
Total lysate	26 698 380	11 813 535	2.25
Supernatant	1 829 900	293 350	6.24
17S–38S position in sucrose gradient[b]	1 575 500	279 020	5.74

[a] The cells were prelabeled with [^{14}C]thymine and pulse-labeled with [^{3}H]thymidine as described under Experimental Procedure. [b] See Figure 2a.

2 peaks, the more dense containing supercoiled ColEl DNA and the less dense peak containing open-circular ColEl DNA and linear chromosomal DNA (Figure 1c,d). In the pulsed sample, the ^{3}H pulse-labeled material appears in three regions: <10% of the radioactivity in a region more dense than the supercoiled ColEl position, <20% in the position of the supercoiled DNA, and 55–60% as a broad band less dense than the supercoiled DNA (Figure 1c). In the sample taken after the chase, >80% of the ^{3}H pulse-labeled DNA bands coincidently with the supercoiled ColEl molecules. The chase of the ^{3}H pulse-labeled material into mature ColEl DNA forms suggests that the material sedimenting ahead of the supercoiled plasmid molecules in neutral sucrose and banding less dense than the supercoiled DNA in ethidium bromide–cesium chloride density gradients represents the replicating forms of the ColEl DNA.

Isolation of Replicating ColEl DNA. Lysis of spheroplasts obtained from large cultures of cells by the Triton X-100 procedure often yielded considerable amounts of contaminating chromosomal DNA in the cleared lysate. The sodium dodecyl sulfate lysis method of Hirt (1967), therefore, was employed to extract plasmid DNA from cells of large (250 mL) cultures pulse-labeled as described in the Experimental Procedure. Using this procedure, approximately 2.5% of the total prelabel (^{14}C radioactivity) and 6.9% of the total pulse-labeled (^{3}H radioactivity) were recovered in the cleared lysate (Table I).

The profile of the preparative neutral sucrose density gradient sedimentation of the sodium dodecyl sulfate supernatant is shown in Figure 2a. Two peaks of the ^{14}C prelabel were observed: sedimenting at the 23S (supercoiled) and 17S (open circular) ColEl DNA positions. The appearance of the 17S material probably is the result of the sodium dodecyl sulfate induced relaxation of plasmid DNA that existed in the form of a supercoiled DNA–protein relaxation complex in cells grown under the above conditions (Katz et al., 1973). A slight skewing also was observed on the leading edge of the 23S peak. The profile of the pulse-labeled DNA in this gradient was found to be quite similar to that observed for the Triton-X100-treated smaller culture described in Figure 1a with a large peak at 34 S, smaller peaks in the 23 to 28S region and a peak at 17 S (Figure 2a).

Figure 2b shows the sucrose gradient profile of a sodium dodecyl sulfate supernatant containing DNA from cells grown and pulsed in the presence of CM. A single 23S peak of ^{14}C-labeled DNA, with a small shoulder at approximately 36 S, was found. The absence of 17S material probably is due to the fact that cells grown in the presence of CM contain very little of ColEl DNA in the form of relaxation complex (Clewell and

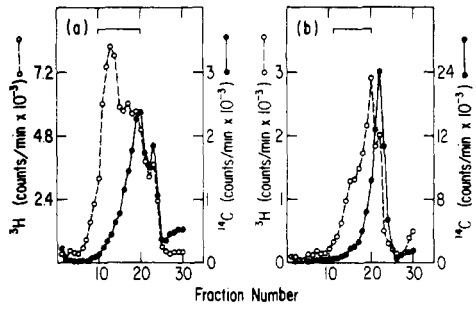

FIGURE 2: Purification of replicating forms of ColEl DNA by neutral sucrose density gradients: (a) A culture (500 mL) of cells was prelabeled with [^{14}C]thymidine and, at a cell concentration of 5 × 10^8 cells/mL, thymine was starved in the presence of cAMP for 30 min and pulsed at 25 °C with [^{3}H]thymidine for 30 s. Lysis was carried out by the sodium dodecyl sulfate procedure. (b) A 300-mL culture was prelabeled with [^{14}C]thymine and in a and then incubated in the presence of CM for 3.5 h. At the end of this time, the culture was pulsed for 30 s with [^{3}H]thymidine as in a. The gradients contained 36 mL of 15–50% sucrose, 0.05 M Tris-HCl (pH 7.5), 0.005 M EDTA, and 0.5 M NaCl. After 16 h of centrifugation at 25 000 rpm in a Beckman SW27 rotor, 1-mL fractions were collected from the bottom of the tubes and small portions were used for the determination of radioactivity. Fractions in each gradient as shown were pooled, precipitated with 0.3 M sodium acetate, pH 6.0, and 2 volumes of EtOH and resuspended into small volumes of ET buffer.

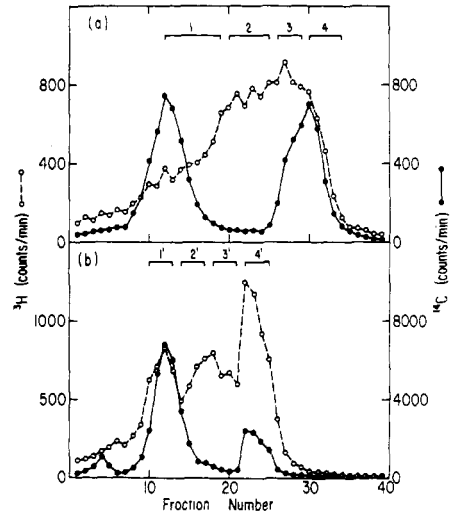

FIGURE 3: Dye–buoyant density gradient fractionation of replicating ColEl DNA. The pooled material shown in Figures 2a,b was centrifuged in ethidium bromide–CsCl gradients as described in Figure 1. The gradients were collected preparatively and small portions were used for the determination of radioactivity. (a) Pooled fractions from the gradient described in Figure 2a. (b) Pooled fractions from the gradient described in Figure 2b.

Helinski, 1972). The ^{3}H pulse-labeled DNA appeared as a major peak at 31 S with a smaller peak at 23 S and a shoulder at approximately 36 S.

The material that sedimented between the 23 and 38S positions in the sucrose gradients of ColEl DNA pulse-labeled in the presence and absence of CM was independently pooled as indicated in Figure 2a,b, alcohol precipitated, resuspended, and banded in an ethidium bromide–cesium chloride density gradient. In both cases, the majority of the ^{3}H-labeled DNA appeared in the regions of the gradient of intermediate and light density (Figure 3a,b). ^{3}H-labeled DNA was also observed in the region corresponding to the position of supercoiled ColEl DNA. Fractions of each gradient were pooled as shown in Figure 3, and the ethidium bromide was removed by 2-propanol extraction.

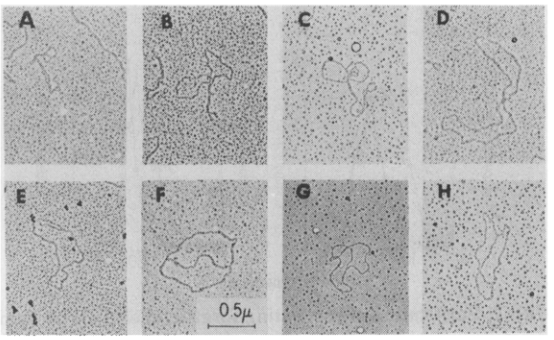

FIGURE 4: Replicative forms of ColEl DNA. Electron micrographs of selected molecules in pools 3 and 4 of Figure 3a mounted by the aqueous (A, B, C, E, and F) or formamide (C, G, and H) techniques (see Experimental Procedure) after no prior treatment (A, B, C, and D), DNase I treatment (E and F) or ethidium bromide and light treatment (G and H).

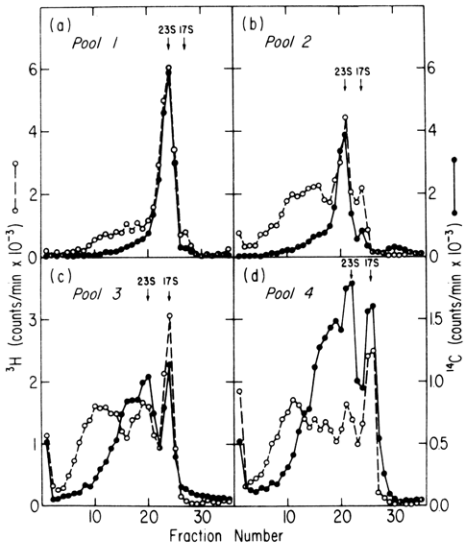

FIGURE 5: Neutral sucrose density gradient sedimentation of purified ColEl DNA replicative forms from cells growing in the absence of CM. Material pooled as shown in Figure 3a was precipitated and resuspended as described in Figure 2 and each sample was sedimented in a 5-mL neutral sucrose density gradient as described in Figure 1. The 23S and 17S positions in each panel were determined from the sedimentation positions of reference ^{32}P-labeled supercoiled and open-circular ColEl DNA in each gradient. (a) pool 1; (b) pool 2; (c) pool 3; (d) pool 4.

Electron-Microscopic Examination of Replicating ColEl DNA. Portions of pools 1–4 of Figure 3a were spread by the aqueous or formamide techniques and examined under the electron microscope. All the material in pool 1 was found to be supercoiled ColEl DNA. Plasmid DNA was not observed in pool 2 probably due to losses or an insufficient amount of DNA in this pool. In pools 3 and 4 a considerable amount of the material observed was open-circular ColEl DNA and linear fragments of chromosomal DNA. The balance of the material appeared as molecules containing a supercoiled region and two open-circular regions of equal size; four such molecules are shown in Figure 4A–D. ColEl DNA molecules of this form have been extracted previously from minicell preparations (Inselburg and Fuke, 1971; Fuke and Inselburg, 1972). To provide additional evidence that these latter structures were replicating ColEl DNA molecules, samples of pool 3 and 4 were treated with either DNase I or with ethidium bromide (20 μg/mL) and light to cleave a single strand of the DNA and

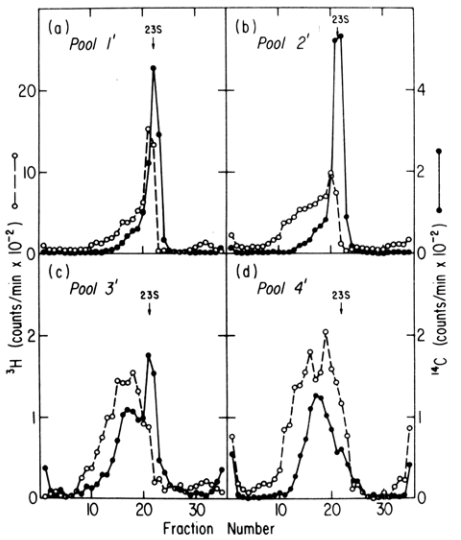

FIGURE 6: Neutral sucrose density gradient sedimentation of purified ColEl DNA replicative forms from cells incubated in the presence of CM. The precipitated and resuspended DNA in pools 1′–4′ of Figure 3b was sedimented through neutral sucrose as described in Figure 4. (a) pool 1′; (b) pool 2′; (c) pool 3′; (d) pool 4′.

subsequently reexamined under the microscope. After this treatment, open-circular ColEl DNA molecules containing an internal loop of varying size were observed: four such molecules are shown in Figure 4E–H. These forked-circular DNA molecules were not observed without prior DNase I or ethidium bromide treatment among several thousand molecules examined in the pooled fractions or in the samples of DNA that sedimented at the 23S and 17S positions in the original sucrose gradient (Figure 2a). These observations suggest that at least one form, if not the principal form, of a replicating ColEl molecule, extracted from intact *E. coli* cells, consists of a molecule with the parental strands covalently closed.

Neutral Sucrose Gradient Analysis of Replicating ColEl DNA. Samples of each of the pools 1–4 and 1′–4′ of the dye–buoyant density gradients described in Figure 3 were sedimented through neutral sucrose density gradients. As expected, virtually all of the material in pools 1 and 1′ sedimented as 23 S corresponding to supercoiled ColEl DNA (Figures 5a and 6a). In pools 2 and 2′, the majority of the ^{14}C-labeled DNA that sedimented as 23S supercoils probably was the result of contamination of each pool with material from pools 1 and 1′, respectively (Figures 5b and 6b). The ^{3}H-labeled DNA in pools 2 and 2′ was found to sediment as heterogeneous material ranging from 31 to 38 S and 26 to 38 S, respectively (Figures 5b and 6b). Since fragmented chromosomal DNA would not be expected to appear in pools 2 or 2′, it is likely that the majority of the material sedimenting ahead of the supercoiled peak of DNA is ColEl DNA that was extracted from the cell while undergoing a replication cycle. The overall similarity of the radioactivity profiles for the DNA synthesized in the absence or the presence of CM also supports the conclusion that the majority of the DNA represents plasmid DNA, since the level of chromosomal DNA synthesis after incubation of the cells in the presence of CM for 3.5 h is extremely low (Clewell and Helinski, 1972). In pools 3, 4, 3′, and 4′, a substantial amount of the ^{14}C-labeled DNA and a relatively smaller proportion of the ^{3}H-labeled DNA was found to sediment at the 23S position (Figures 5c,d and 6c,d). Apart from the material that sedimented at the 17S position (corresponding to open-circular DNA), the balance of the DNA in pool 3 sedi-

mented as a broad band at 36–38S (Figure 5c). The remaining DNA of pools 4, 3′, and 4′ similarly sedimented faster than the supercoiled ColEl DNA (23 S) (Figures 5d and 6c,d). These findings generally are consistent with the observations reported for the replicating forms of the papavoviruses, namely, that as a result of increasing extent of replication the circular ColEl DNA becomes less dense in an ethidium bromide–cesium chloride gradient and sediments more rapidly through neutral sucrose gradients. The failure to separate replicating forms of ColEl DNA into discrete size classes on the basis of neutral sucrose gradient sedimentation probably is the result of the incomplete separation of the forms on the basis of buoyant density after a single passage through the dye–buoyant density gradient.

Alkaline Sucrose Density Gradient Analysis of Nascent ColEl DNA. The sizes of the growing strands in replicating ColEl DNA were determined from sedimentation analysis of pools 2′, 3′, and 4′ through alkaline sucrose. The preparations from the cells incubated in the presence of CM were used in this case because after 3.5 h of CM treatment the ^{3}H pulse label was found previously to have been incorporated exclusively into ColEl DNA (Clewell and Helinski, 1972). In each case, greater than 90% of the ^{14}C prelabeled DNA pelleted, as expected, for covalently closed ColEl DNA. The remainder of the ^{14}C-labeled DNA separated into two peaks representing the circular single strand of ColEl and a unit size, linear, single-stranded molecule (Figure 7). In pool 2′, the ^{3}H-labeled DNA was distributed into broad bands of 5–6 S, 7–9 S, and 14–16 S material (Figure 7a) with approximately 50% of the total radioactivity appearing as 7–9S material. In pool 3′, the amount of 14–16S DNA was found to have increased relative to the smaller material (Figure 7b). A leading shoulder on the 14–16S peak, corresponding to the position of unit length linears also was observed (Figure 7b). Most of the nascent ColEl DNA in pool 4′ was found to sediment at the unit length linear and the 14–16S positions (Figure 7c). Smaller proportions of the 5–6S and 7–9S material were found. In none of the gradients was there observed ^{3}H-labeled nascent DNA that sedimented more rapidly than unit length linear ColEl marker DNA.

Reduction of the Superhelical Density During ColEl DNA Replication. Since the replicating ColEl molecules appear to contain no discontinuities in their parental strands, the unwinding of the parental strands without an apparent permanent swivel mechanism can follow either of two processes: a simple local unwinding without nicking the parent strands that would result in an increase in the superhelical density of the unreplicated portion, or single-strand cleavages followed by partial unwinding and resealing that would permit DNA synthesis to occur with no change or a limited increase in the superhelical density of the unreplicated portion. If replicating molecules were subjected to heating to remove the growing strands and then quickly cooled, in the former case, since no prior nicking of the molecule had occurred, the parent strands would renature and the reconstructed supercoiled DNA would have the same superhelical density as native, unreplicating supercoiled ColEl DNA molecules. In the latter case following this treatment, the renatured parent molecule would exhibit a more negative superhelical density due to the increased deficiency of topological turns following the continual nicking and unwinding that had taken place (Vinograd et al., 1968; Salzman et al., 1973). These renatured molecules would bind more dye than the reference supercoiled DNA and, hence, appear less dense than the supercoiled DNA in ethidium bromide–cesium chloride gradients.

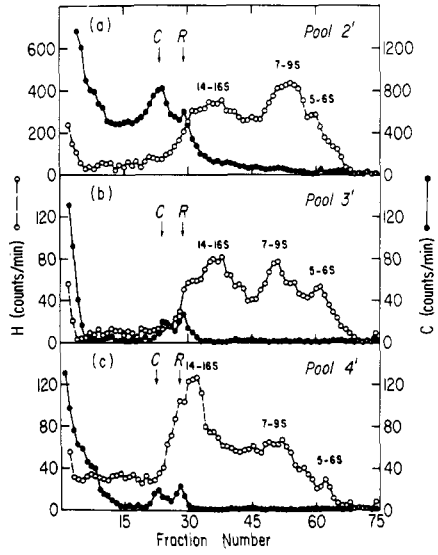

FIGURE 7: Alkaline sucrose density gradient sedimentation of nascent ColEl DNA from CM-treated cells. Pools 2′, 3′, and 4′ of Figure 3b were applied to 5 mL of a 5–20% sucrose gradient containing 1 M NaCl, 0.001 M EDTA, and 0.3 M NaOH, and centrifuged for 4 h at 50 000 rpm in a Beckman SW50.1 rotor. The samples were collected dropwise from the bottom. The C and R designations represent the positions in each gradient in circular (19 S) and linear, unit length (17.9 S) single-stranded ColEl DNA. The S values shown were determined from the positions of the C and R forms. (a) pool 2′; (b) pool 3′; (c) pool 4′.

Parental strands of ColEl DNA were labeled exclusively by an incubation of a culture (500 mL) of cells in the presence of CM for 90 min and then an additional incubation for another 90 min in the presence of CM and [^{3}H]thymine. Following a subsequent 30-min thymine-starvation treatment in the presence of CM and cAMP the cells were pulsed at reduced temperature with unlabeled thymidine to initiate replication. A sodium dodecyl sulfate cleared lysate was prepared and centrifuged through a neutral sucrose gradient. A single peak at the 23S position with a leading shoulder of DNA was found. The DNA sedimenting at 23 S, 26–28 S, and 28–31 S was purified separately by dye–buoyant density gradient equilibrium centrifugation. A sample of each preparation was then subjected to a 15-min period of heating at 100 °C and quick cooling. Heated and unheated samples were banded in an ethidium bromide–cesium chloride density gradient and the position of the material, with respect to an added supercoiled and open-circular reference DNA marker in each gradient, was examined. The position of the 23S pool after heat treatment did not change relative to the added reference ColEl DNA (Figure 8a,b). The material originally sedimenting at the 26–28S position in the sucrose gradient banded in an intermediate position in the ethidium bromide–cesium chloride gradient (Figure 8c). After heating and cooling, approximately 60% of the label was found as a broad band with a peak 3 fractions less dense than the reference open-circular molecules. The remaining 40% of the label banded one fraction more dense than the unheated sample (Figure 8d). The 28–31S material also banded in the intermediate position in the dye–CsCl gradient (Figure 8e) and was indistinguishable from the material depicted in Figure 8c. After heat denaturation, however, 60% of the label that shifted position was found at a peak position 5 fractions less dense than the reference open-circular ColEl (Figure 8f).

The finding that a substantial amount of the material in the original 26–28S and 28–31S peaks did not band at less dense positions in the dye–CsCl gradients following the heating and

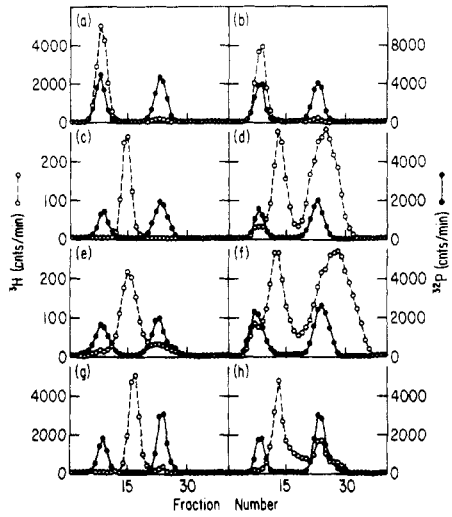

FIGURE 8: Superhelical density of parental strands of replicating ColEl DNA after alkali denaturation. At 90 min after CM was added to a 500-mL culture, the cells were incubated in the presence of [^{3}H]thymine and CM for 2.5 h. Following thymine starvation in the presence of CM and cAMP for 30 min, the DNA was extracted by the sodium dodecyl sulfate method either following a 30-s pulse with unlabeled thymidine (material represented in gradients a–f) or with no thymidine pulse (material represented in gradients g and h). Material extracted from the pulsed cells was purified once by sedimentation in a neutral sucrose gradient. The fractions from the gradient were combined into three pools (23 S, a and b; 26–28S, c and d; 28–31S, e and f) of different S values and subsequently each fraction was purified by ethidium bromide–CsCl density gradient centrifugation and analyzed by ethidium bromide–CsCl density gradient centrifugation after either no further treatment (a, c, and e) or after heating at 100 °C for 15 min and rapid cooling (b, d, and f), respectively. Catenated ColEl DNA, prepared from nonpulsed cultures as described in the text, also was analyzed by dye-buoyant density gradient centrifugation before (g) and after (h) heat denaturation. Reference ^{32}P-labeled supercoiled and open-circular ColEl DNA were added to each gradient.

cooling treatments suggested that DNA other than replicating ColEl was present in the preparations. Microscopic examination of the material depicted in Figure 8e revealed the presence of a large amount of catenated ColEl DNA. It was therefore necessary to independently examine the behavior of a purified preparation of catenated molecules upon the heat treatment. Since it was assumed that the catenates would be present in the CM-treated cells whether or not the replicating molecules were also present, a culture prepared in an identical manner to that described for the isolation of the replicating molecules, except that the pulse with unlabeled thymidine was omitted, was lysed and the ^{3}H-prelabeled material was purified in a dye–CsCl gradient and examined microscopically. More than 85% of the molecules that banded in the intermediate region in the dye-buoyant density gradient (Figure 8g) was observed as catenated ColEl molecules consisting of interlocked supercoiled DNA and open-circular DNA. Upon heating and cooling and subsequent dye-buoyant density gradient centrifugation, it was observed that 75% of the radioactivity shifted to a slightly more dense position (Figure 8h). The materials that banded in the open-circular region are most likely the linear single strands that would be dissociated from the open-circular portion of the catenated molecule.

This result supports the conclusion that the material in the samples containing the replicating plasmid DNA that did not shift to the less dense position after the heat treatment is catenated DNA found in each preparation. It is reasonable to conclude, therefore, that the material that did shift to less-dense positions following the heat treatment (Figures 8d,f) consists of supercoiled molecules that contain more negative superhelical density and, hence, band at a less dense position than native supercoiled ColEl DNA, consistent with the proposed progressive decrease in the overall superhelical density of the ColEl DNA molecule as replication proceeds (Salzman et al., 1973).

Discussion

In this study, it has been demonstrated that ColEl DNA, isolated in various stages of replication in the absence or presence of chloramphenicol from whole *E. coli* cells, consists of structures containing a supercoiled, unreplicated region and two open-circular branches of equal size that presumably contain the nascent DNA. Denaturation of the replicative intermediates and subsequent renaturation of the parental strands has revealed that the parental strands of the purified replicating molecules are covalently closed and that unwinding of the parental strands to permit nascent DNA synthesis is accompanied by a progressive reduction of the superhelical density. Because of the partial supercoiled structure and the added mass of the nascent DNA, these molecules sediment through neutral sucrose density gradients more rapidly than the native ColEl supercoiled DNA and bind more dye than the unreplicating supercoiled molecules and less or equal amounts of dye than the open-circular molecules, and, hence, are distributed both into the region between the supercoiled and open-circular DNA and the region containing the open-circular DNA in a dye–CsCl density gradient.

The finding of a substantial fraction of the total plasmid DNA as replicating ColEl DNA is due to the increased amount of synchronous ColEl DNA replication in cells following the thymine starvation and cAMP treatments. The addition of cAMP results in a two- to fourfold increase in the fraction of ColEl molecules per cell that initiate simultaneous replication (Katz and Helinski, 1974). Since this treatment permits the initiation of molecules not in the process of replication, it is likely that most of the material isolated following the treatment and the subsequent short [^{3}H]thymidine pulse contains the nascent DNA labeled from the site of the origin of replication. An evaluation of the fraction of molecules at various stages of replication can therefore be made by direct examination of the size distribution of the nascent DNA. Analysis of the various pools of replicating ColEl DNA by alkaline sucrose density gradient centrifugation revealed that the more mature replicating molecules (pools 3′ and 4′ of Figure 7) contained a greater fraction of the nascent DNA as unit length (17.9 S) and near unit length (14–16S) than the less extensively replicated molecules (pool 2′ of Figure 1) where at least 80% of the nascent DNA sediments as 5–6S and 7–9S fragments. The finding of discrete size distributions of the nascent DNA in the preparations containing either "young" or "mature" replicative forms suggests that at least one growing ColEl DNA strand in a molecule undergoing replication is produced discontinuously as short fragments and that covalent association between parental and nascent ColEl DNA does not occur. In an in vitro ColEl replicating system, Sakakibara and Tomizawa (1974a,b) have demonstrated a 6S nascent ColEl DNA fragment that appears to contain a short sequence of RNA at one end in an early covalently closed replicative intermediate. Single-stranded DNA fragments sedimenting as 5–23S molecules have been released also from replicative intermediates purified from minicells (Oka and Inselburg, 1975). Evidence has been obtained for an association of RNA with a portion of these fragments (Oka and Inselburg, 1975).

The replicating ColEl DNA molecules described in this

study appear to be similar in properties to those described in investigations employing minicell preparations (Inselburg and Fuke, 1971; Oka and Inselburg, 1975) and cell-free replication systems (Sakakibara and Tomizawa, 1974a,b). The findings of a covalently closed replicating structure as the principal replicate intermediate indicate that a permanent nick in a parental strand is not required for plasmid DNA replication and that ColEl DNA does not replicate in *E. coli*, either in the presence of chloramphenicol or its absence via a rolling-circle or Cairns forked-circle intermediate.

Further studies employing the molecules prepared in this investigation have revealed that ColEl DNA replicates unidirectionally from a unique origin (Lovett et al., 1974a). Employing as a reference point the unique site at which the *Eco* Rl restriction enzyme breaks the ColEl molecule, it was shown also that the site of the origin-terminus of replication is indistinguishable from the site of the nick in the induced relaxation complex of ColEl (Lovett et al., 1974b). Although this recent finding suggests a role for the relaxation complex in ColEl DNA replication, it was not possible to isolate replicative intermediates of ColEl in the form of sodium dodecyl sulfate inducible relaxation complex. It is possible that the replicative intermediates are associated with relaxation complex in vivo but unlike relaxation complex involving nonreplicative ColEl DNA, the relaxation proteins are dissociated from the replicating DNA on extraction. Alternatively, relaxation complex may be involved only in the initial stages of replication and, therefore, the replicative intermediates purified are free of relaxation proteins. It has been found previously that the completed covalently closed circular DNA products of ColEl replication became complexed with protein shortly after the replication cycle is completed (Katz et al., 1973).

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