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Nature of Col E_1 Plasmid Replication in *Escherichia coli* in the Presence of Chloramphenicol

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The colicinogenic factor E_1 (Col E_1) in *Escherichia coli* continues to replicate by a semiconservative mechanism in the presence of chloramphenicol (CAP) for 10 to 15 hr, long after chromosomal deoxyribonucleic acid (DNA) synthesis has terminated. Following CAP addition, the rate of synthesis of plasmid DNA gradually increases to an extent dependent on the medium employed. Within 2 to 4 hr after the addition of CAP, replication in a glucose-Casamino Acids medium approaches a maximum rate representing approximately eight times an average rate which would be required for a net doubling of DNA per cell in one generation. The number of copies of Col E_1 DNA molecules that accumulate under these conditions approaches about 3,000 copies per cell, representing a 125-fold increase over the normal level of 24 copies per cell. The system is particularly convenient for studying the mechanism of DNA replication.

The colincinogenic factor E_1 (Col E_1) in *Escherichia coli* has certain features which make it an attractive system for studies on the nature and control of deoxyribonucleic acid (DNA) replication. It is a relatively small plasmid (4.2×10^6 daltons) that can be isolated as a covalently closed supercoiled circular duplex molecule (2, 3, 20). Recently it has been found that when protein synthesis is inhibited, by either amino acid starvation or treatment with chloramphenicol (CAP), the plasmid continues to replicate while the chromosomal DNA replicates only to a point consistent with completion of rounds of replication (4; Clewell and Helinski, J. Bacteriol., *in press*). In this communication, results are presented dealing with a more detailed analysis of the nature of Col E_1 replication during the exposure of cells to CAP. Included are studies on the rate and extent of replication as well as a determination of the semiconservative nature of the duplication process.

MATERIALS AND METHODS

Materials. Reagents and sources were as follows: Brij 58, from Emulsion Engineering, Inc.; sodium deoxycholate (DOC) from Difco Laboratories; Sarkosyl NL30 (sodium dodecyl sarcosinate) from the Geigy Chemical Corp.; egg white lysozyme, Pronase, bromouracil, and ethidium bromide from Calbiochem; CAP from Parke, Davis and Co.; CsCl (optical grade) from Schwartz Bioresearch, Inc.; thymine-*methyl*-^3H (11.7 Ci/mmole), thymidine-*methyl*-^3H (6.7 Ci/mmole), and thymine-2-^{14}C (55.8 mCi/mmole) from New England Nuclear Corp.

Bacteria and media. *E. coli* strains JC411thy$^-$ (Col E_1) (6) (auxotrophic for methionine, leucine, histidine, arginine, and thymine obtained from D. Kingsbury) and CR34(Col E_1) (auxotrophic for threonine, leucine, vitamin B_1, and thymine) (17) were used in this study. Each strain harbors the Col E_1 plasmid DNA which was transferred by conjugation from *E. coli* K-30. An M9 medium (as described in reference 9) containing thymine (1-4 μg/ml), B_1 (2.5 μg/ml), and required amino acids (50 μg/ml) or 0.5% Casamino Acids (Difco) was employed for most of this work. In one case, Difco antibiotic medium no. 3 (Penassay Broth) was utilized (thymidine rather than thymine isotope was used in this case in order to get better incorporation of isotope). Cell growth was at 37 C and was followed by measuring turbidity in a Klett-Summerson colorimeter.

Preparation of cleared lysate. The lysing procedure has been described in detail previously (7, 8). It essentially involves the lysis of ethylenediaminetetraacetic acid (EDTA)-lysozyme spheroplasts with a detergent mixture of Brij 58 and DOC. Centrifugation of the crude lysate for 25 min at 48,000 × *g* pellets about 95% of the total DNA, leaving the plasmid DNA in the supernatant fluid. This supernatant fluid is referred to as cleared lysate. The latter can be frozen (−70 C) until ready for further examination.

Preparation of Sarkosyl lysates. Cells were harvested and resuspended in 1 ml of 25% sucrose [0.05

M tris(hydroxymethyl)aminomethane (Tris), pH 8.0]. Lysozyme was added [0.2 ml of a 5 mg/ml solution in TES (0.03 M Tris-0.005 M Na_2EDTA-0.05 M NaCl)], and after 5 min at 25 C, 0.4 ml of 0.25 M EDTA was added. After another 5 min at 25 C, Pronase was added (0.2 ml of a 5 mg/ml solution in TES, predigested at 37 C for 30 min). After 5 min, lysis was brought about by the addition of 1.8 ml of 2% Sarkosyl (in TES). At this point the crude lysate can be frozen (−70 C).

Preparative buoyant density-equilibrium centrifugation. Centrifugation was performed on a Beckman model L3-50 ultracentrifuge in a type Ti-50 fixed-angle rotor at 44,000 rev/min at 15 C for 60 hr. Each polyallomer centrifuge tube contained 7.5 g of CsCl and 6 ml of sample in TES. When dye was included, 8 ml of sample containing 1.5 mg of ethidium bromide was mixed with 7.5 g of CsCl. The remaining space in the centrifuge tube was filled with mineral oil. At the end of the run, the bottom of the tube was punctured and 0.2- to 0.3-ml fractions were collected.

Sucrose density gradients. Sucrose density gradient centrifugations, dropwise fractionation of the gradients, and the counting of radioisotopes were carried out as described in detail previously (8). When cleared lysates were sedimented, the gradients contained 0.55 M NaCl, 0.005 M Na_2 EDTA, and 0.02 M Tris, pH 8.0. Samples pooled from buoyant density gradients were sedimented on gradients containing similar levels of Na_2 EDTA and Tris, pH 8.0, but with 0.05 M NaCl.

Medium shifts. Medium shifts were carried out by one of two methods involving either centrifugation or filtration of cells. In the former case, cells were: (i) pelleted by centrifugation in an International model PR-6 centrifuge at 5,000 rev/min for 5 min at 25 C; (ii) washed by resuspending and centrifuging in twice the original volume of medium salts; (iii) resuspended in new fresh M9 medium (prewarmed to 37 C). The entire procedure required 12 to 15 min. Cell growth during the shift was minimal, as evidenced by less than a 10% increase in turbidity immediately following the shift.

The other method of medium shift involved collecting the cells on a membrane filter (Millipore, HA, 0.45 μm), washing with 20 ml of medium salts, and resuspending the cells in the new medium. The latter procedure required about 5 min.

Counting of radioisotopes. Samples were prepared and counted as previously described (8) in a Beckman LS250 liquid scintillation system.

RESULTS

A culture of *E. coli* JC411thy$^-$(Col E_1) was grown for several generations in glucose minimal medium containing ^{14}C-thymine to label uniformly all cellular DNA. The cells were then pelleted, washed, and resuspended in a similar medium containing ^{3}H-thymine in place of ^{14}C-thymine. A portion of this suspension served as a control and was allowed to undergo one division before being rapidly chilled in an ice bath. CAP was added to the remainder, after which samples were removed 1, 2, and 4 hr later and chilled on ice. All samples were then lysed by the Brij 58-DOC procedure (*see above*). Samples of the crude lysates were spotted on filter papers and counted to determine the ratio of ^{3}H to ^{14}C. Since Col E_1 DNA is usually a very minor component of the total DNA in crude lysates [plasmid normally represents less than 2% of the total DNA (3, 8)], the ratio of ^{3}H to ^{14}C here reflects the extent of replication of chromosomal DNA. The lysates were then centrifuged to obtain "cleared lysates" (see Materials and Methods), which were then analyzed on sucrose density gradients as shown in Fig. 1 to determine the ratio of ^{3}H to ^{14}C in Col E_1 DNA. The plasmid DNA is centered in fractions 14 to 17, typical of supercoiled Col E_1 DNA under these conditions (Clewell and Helinski, J. Bacteriol., *in press*). The rather broad low-molecular-weight peak is of unknown origin but apparently represents labeled thymine incorporated during the most recent round of replication. This is suggested by the fact that there is mainly ^{3}H in the peaks and very little, if any, ^{14}C. This material may represent degradation products of recently synthesized DNA.

The extent of replication of both plasmid and chromosomal DNA is depicted in Fig. 2. It is clear that, although the addition of CAP allowed chromosomal DNA to continue to an extent representing slightly more than half of that represented by the control culture (which was allowed to double its DNA content), the plasmid not only continued to replicate but gradually increased its rate of replication. From the slopes of the lines in Fig. 2, it is evident that the average rate of Col E_1 replication between the second and fourth hours was almost three times that observed for the first hour after chloramphenicol addition, and about twice the average rate that would be necessary to produce a normal doubling of plasmid DNA during a normal division cycle (i.e., as judged by comparison to the ^{3}H to ^{14}C ratio of the one-generation control cells). It is seen also that for about two generation equivalents (doubling time in this medium is 75 min), Col E_1 accumulation paralleled the theoretical logarithmic increase that would be expected if cells were growing logarithmically.

Figure 3 shows the results of an experiment identical to that of Fig. 2 except that Casamino Acids (0.5%) were present in the medium. In this case Col E_1 replication increased with time to a greater extent than that seen in minimal medium; and here the replication

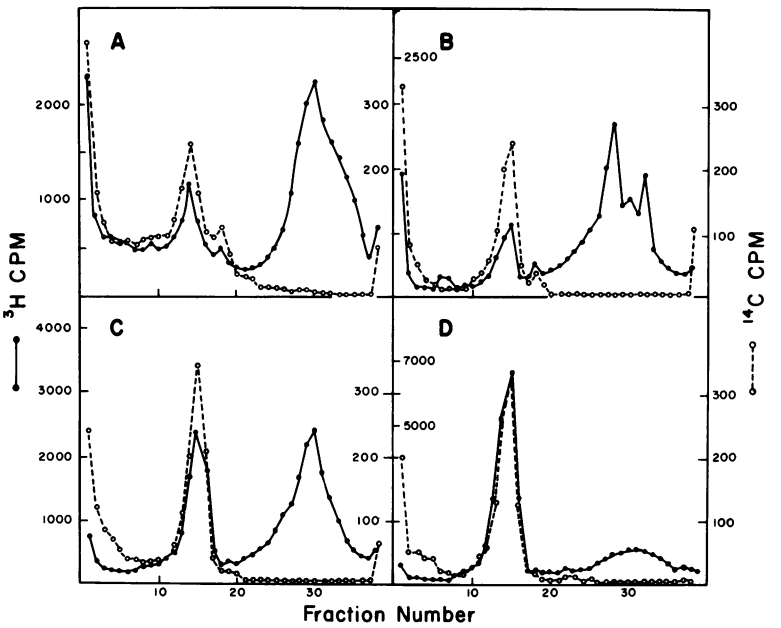

FIG. 1. *Sedimentation analyses of cleared lysates prepared from cells treated with chloramphenicol (CAP). A culture (50 ml) of E. coli JC411thy⁻ (Col E_1) was grown in glucose minimal medium containing ^{14}C-thymine (1.6 μCi per 3.6 μg per ml) at 37 C for several generations in log phase. The cells were then shifted to a similar medium (83 ml) containing ^{3}H-thymine (2.4 μCi per 5 μg per ml) in place of ^{14}C-thymine. Immediately the cells were divided into two portions. One portion (20 ml) was allowed to continue until the turbidity had doubled, at which point growth was terminated by rapid chilling in an ice bath. To the remaining portion, CAP was added to a concentration of 170 μg/ml. While incubating at 37 C, samples (20 ml) of the latter culture were removed and quickly chilled on ice at 1, 2, and 4 hr after the medium shift. Cleared lysates were prepared from cells, diluted twofold with TES, and 0.2-ml samples were centrifuged (from right to left) through 5 to 20% sucrose density gradients in an SW50.1 rotor (15 C) at 48,000 rev/min for 135 min. (A) Parallel control in which cells were shifted into medium identical (with the exception of the isotope) to the preshift medium for one generation (75 min). (B), (C), and (D) represent cells shifted to a medium containing CAP for 1, 2, and 4 hr, respectively.*

rate between hours 2 and 4 exceed the "normal" doubling time (50 min in this case) by approximately eightfold. The rate of accumulation of Col E_1 parallels the theoretical logarithmic increase for about four generation equivalents.

It is important to point out that the phrase "normal doubling time," as used here, refers to a value corresponding to the synthesis involved in a net doubling of the number of Col E_1 DNA molecules per cell (i.e., 24 copies) per generation. Although the overall rate of synthesis may increase exponentially during one generation, it can, for the sake of making comparisons, be represented by a value corresponding to a linear rate of synthesis which is constant throughout one division cycle. To follow plasmid replication for longer periods of time, CAP (150 μg/ml) was added to a log culture (100 ml) growing in M9 Casamino Acids medium containing ^{14}C-thymine (1.6 μCi per 3.6 μg per ml), and subsequently samples (20 ml) were removed (and put on ice) at 0, 4, 9, 20, and 26 hr. Lysates were prepared by the Sarkosyl procedure (see Materials and Methods) and centrifuged to equilibrium in CsCl density gradients containing ethidium bromide. The gradients were photographed with an ultraviolet (UV) lamp to promote ethidium bromide-mediated fluorescence of the DNA bands (Fig. 4). The higher-density DNA band (lower band) represents covalently closed circular DNA (18). The gradients were then fractionated, and radioactivity was determined in samples of the fractions (Fig. 5).

The UV photographs (Fig. 4) of the glucose-Casamino Acids-grown cells, which compare the DNA in cells that have not been exposed to CAP with that of cells that have been exposed for various lengths of time, clearly indicated that Col E_1 DNA (the lower band), which is not readily apparent at zero time, gradually approaches a level which is comparable to that of the chromosomal DNA (the

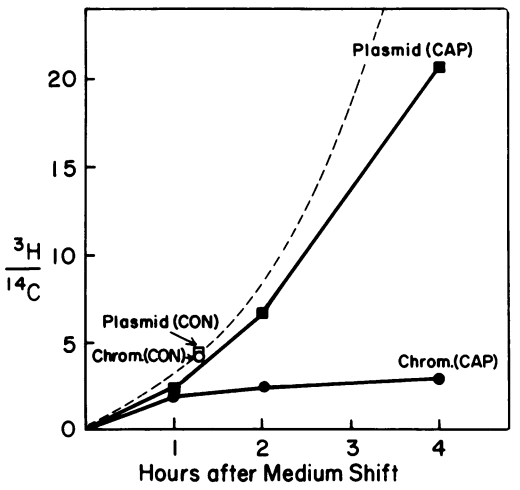

FIG. 2. *Extent of replication of plasmid and chromosomal DNA after treatment of cells in glucose-minimal medium with chloramphenicol. The counts-per-minute ratios of ^3H to ^{14}C represent the values obtained in the experiment depicted in Fig. 1. Corrections have been made regarding the base line level of counts within the peaks. The values obtained for the chromosome were based on the ratio of ^3H to ^{14}C counts in the crude lysate. CAP, cells treated with chloramphenicol; CON, control without chloramphenicol for one generation. Dashed line represents the theoretical increase in DNA that would arise during normal logarithmic cell growth.*

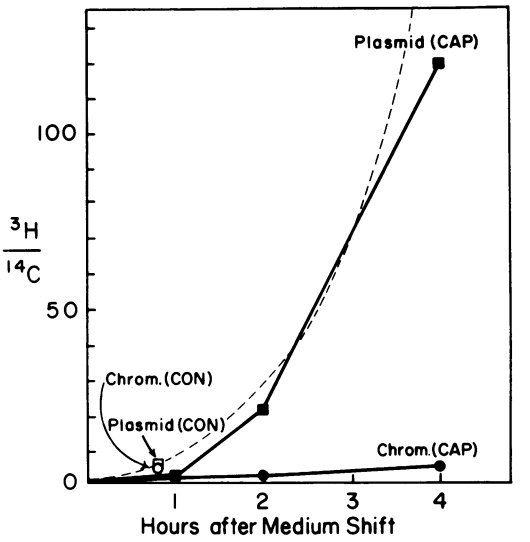

FIG. 3. *Extent of replication of plasmid and chromosomal DNA after treatment of cells in glucose-Casamino Acids medium with chloramphenicol. This experiment was carried out in essentially the same manner as the experiment in Fig. 1 and 2, with the exception of the medium employed. Normal doubling time in this medium, however, is 50 min. CAP, cells treated with chloramphenicol; CON, control without chloramphenicol for one generation. Dashed line represents the theoretical increase in DNA that would arise during normal logarithmic cell growth.*

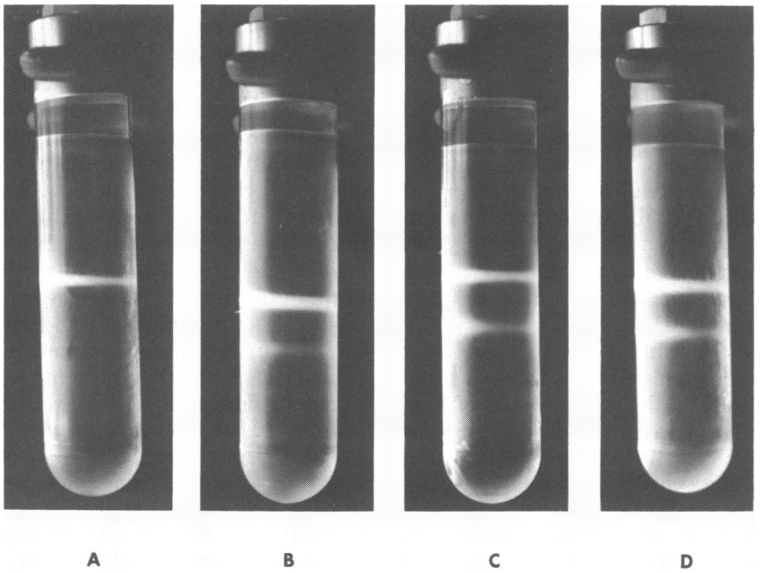

FIG. 4. *Photographs of UV-illuminated dye-buoyant density gradient of Sarkosyl lysates prepared from cells treated for varying lengths of time with chloramphenicol. (A), (B), (C) and (D) represent cells treated for 0, 4, 9, and 20 hr, respectively. Upper band represents chromosomal DNA; lower band represents covalently closed circular DNA.*

upper band). The positions of the UV-absorbing bands correspond well with peaks of radioactivity in the fractionated gradients (Fig. 5), indicating that incorporation of radioactive thymine into Col E_1 DNA relates to a net increase in mass and not simply a repair process.

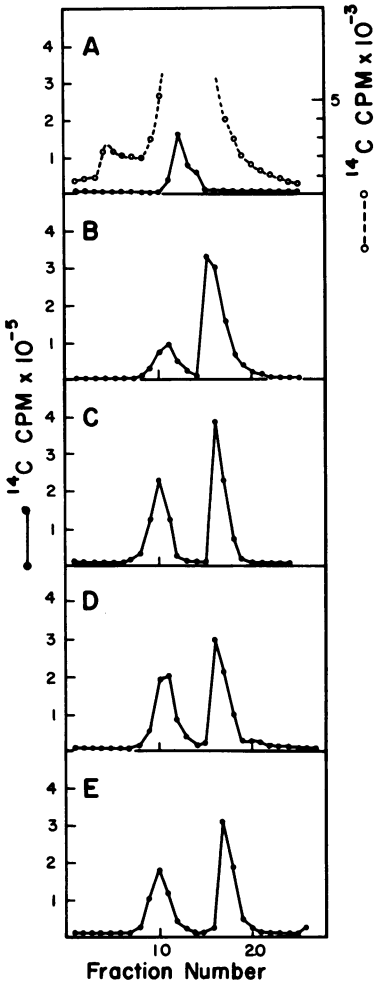

FIG. 5. *Dye-buoyant density centrifugation of Sarkosyl lysates from cells treated with chloramphenicol (CAP) for increasing lengths of time. The fractionation profiles shown correspond to those gradients pictured in Fig. 4 (density increases from right to left). In addition, the fractionation profile of a 26-hr treatment (from the same experiment) is shown. (A), (B), (C), (D), and (E) correspond to CAP treatments of 0, 4, 9, 20, and 26 hr, respectively. Fractions of equal volume were collected only in the region of the gradients where it was visually observed that the DNA was present. A portion (0.05 ml) of each fraction was counted. In the case of A, the counts were plotted also on a scale different from the others so that the higher-density satellite peak could be resolved.*

The Col E_1 DNA band, when analyzed (after pooling and dialyzing fractions) on neutral sucrose density gradients behaves as normal 23*S* material and also contains a small about (5%) of a 31*S* substance (Fig. 6) presumably representing dimer molecules (either circular or catenated dimers, or both). Dimers are generally not found to any extent in *E. coli* (3) except in certain abnormal situations (11), although oligomers are present in relatively high percentages when the plasmid is harbored in *Proteus mirabilis* (2, 12). The small amount of 17*S* DNA probably represents some circular Col E_1 DNA that has acquired a nick (see reference 2).

Beyond 4 hr in CAP it is reasonable to assume that the level of chromosomal DNA remains constant (see Fig. 2 and 3), in which case the amount of plasmid DNA can be measured relative to the level of chromosomal DNA. Figure 7 portrays these values for this experiment as well as for two similarly performed experiments carried out in a minimal medium (M9 glucose plus required amino acids) and a rich broth. These data indicate that after 3 to 4 hr of exposure to CAP, replication of Col E_1 DNA occurs linearly for sev-

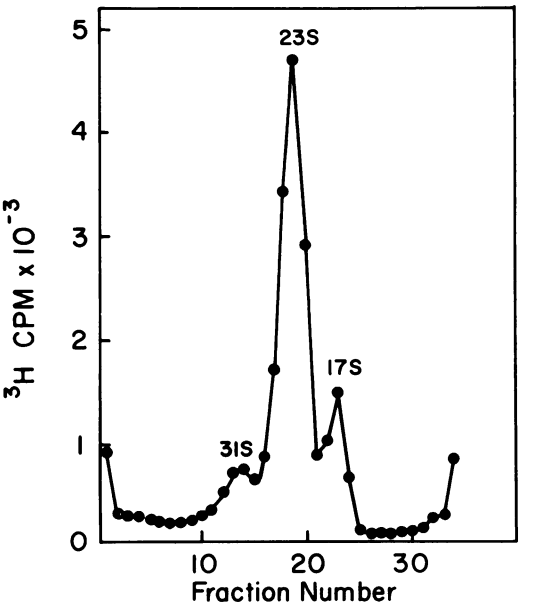

FIG. 6. *Sedimentation analysis of pooled fractions corresponding to covalently closed circular DNA isolated by dye-buoyant density centrifugation. The sample corresponds to combined fractions obtained from the experiment of Fig. 5. A 0.2-ml sample was centrifuged (from right to left) through 5 to 20% sucrose density gradients in an SW50.1 rotor (15 C) at 48,000 rev/min for 90 min.*

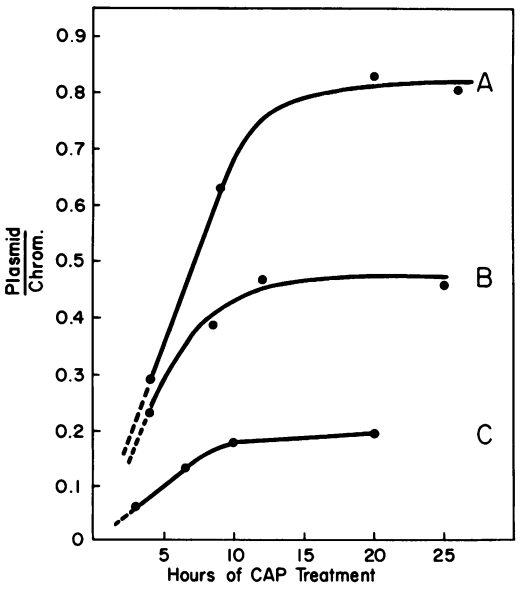

FIG. 7. *Extent of Col E_1 replication during treatment with chloramphenicol (CAP) in three different media. (A), (B), and (C) correspond to cells grown in glucose-Casamino Acids, Difco Penassay Broth (antibiotic medium no. 3), and glucose-minimal medium, respectively. After 3 to 4 hr in CAP, chromosomal DNA no longer replicates, and it is assumed here to remain at a constant level beyond this time. Thus the chromosomal DNA is used as a normalizing factor in estimating the amount of plasmid DNA present. The experiment corresponding to A represents the experiment of Fig. 5. The ratio of plasmid to chromosomal DNA was estimated on the basis of the areas enclosed in the two peaks of the dye-buoyant density gradients (see Fig. 5). Relative areas were determined by weighing pieces of paper cut to the dimension of the areas enclosed by the peaks. The data of B and C were calculated in the same manner.*

eral more hours and then levels off. In the case of glucose-Casamino Acids-grown cells, leveling off occurs at a point representing about 82.5% of the level of chromosomal DNA. Assuming the size of a chromosomal genome equivalent is 2.5×10^9 daltons (10), we can estimate that there are 2.1×10^9 daltons of plasmid DNA per chromosomal genome equivalent. Since Col E_1 DNA has a molecular weight of 4.2×10^6, this corresponds to 500 copies per chromosomal genome equivalent. It was determined on the basis of cell counts (using the Petroff-Hauser bacteria counter) and DNA analyses [by the method of Burton (5)] that log cells growing in this medium contain approximately four (actually 3.75 ± 0.30) chromosomal genome equivalents of DNA per cell. Since normally there are about 24 copies of Col E_1 DNA per cell independent of the number of chromosomal equivalents (Clewell and Helinski, J. Bacteriol., *in press*), there are in this case six copies of plasmid DNA per chromosomal equivalent. Under conditions of protein synthesis inhibition, cell mass increases only slightly (10–15%), and the amount of chromosomal DNA increases by about 50% (15; and see Fig. 2 and 3). Thus, if we assume that after extended CAP treatment there is an average of six chromosomal genome equivalents per cell, this would correspond to $6 \times 500 = 3{,}000$ copies of Col E_1 DNA per cell (see Table 1), representing about a 125-fold increase per cell over the normal level.

In the case of cells grown in Difco Penassay Broth, the relative amount of plasmid DNA to chromosomal DNA leveled off at a point representing about 47.5% that of the chromosomal DNA (also see Table 1). Cells in this medium have a normal doubling time of 30 min (as compared to 50 min for glucose-Casamino Acids-grown cells) and might be expected, on the basis of experiments performed on other strains of *E. coli* and *Salmonella*, to have a

TABLE 1. *Rate of synthesis and extent of accumulation of plasmid DNA in the presence of chloramphenicol*

Medium	Avg no. of Col E_1 DNA molecules synthesized per minute during maximum rate of synthesis[a]		Final no. of copies of Col E_1 DNA	
	Per chromosomal genome equivalent	Per cell[b]	Per chromosomal genome equivalent	Per cell[b]
A. Glucose-Casamino Acids	0.68	4.1	500	3000
B. Broth	0.55	3.3	283	1698
C. Glucose minimal	0.20	0.7	119	440

[a] "Maximum" rate of synthesis refers to that rate of synthesis which is achieved after the first 3 to 4 hr of exposure to chloramphenicol and corresponds to the time related to the linear regions of the curves shown in Fig. 7. Rates were calculated by determining the difference between the number of Col E_1 copies at two arbitrarily chosen time points and dividing by the time elapsed between the two points.

[b] Based on presence of six chromosomal genome equivalents per cell for A and B and 3.7 chromosomal genome equivalents for C (or 1.5 times the number of chromosomal genome equivalents per cell during normal log-phase growth).

greater cell mass and more chromosomal DNA per cell (10, 16). Efforts to confirm this in our K-12 strain indicated (reproducibly) that this is not the case for these cells and that the cell mass and amount of DNA per cell in Penassay Broth is indistinguishable from that of the glucose-Casamino Acids-grown cells. The reason for this is not clear but could simply reflect the nature of this particular strain. Thus, the fact that the leveling off of plasmid replication in broth-grown cells at a level approximately half that of Casamino Acids-grown cells is apparently not related to a difference in the amount of chromosomal DNA per cell. More likely, Col E_1 replication in these (broth-grown) cells had become somewhat dependent on one or more nutrients in the broth, present in very low amounts, which were exhausted relatively quickly. In the presence of CAP, the cell could not compensate with new enzyme synthesis, and thus replication terminated.

In the case of cells in minimal medium (normal doubling time is 75 min), replication of plasmid DNA continued for about 10 hr but leveled off at a point representing only 20% of the level of chromosomal DNA. This result is probably simply a reflection of the relatively poor medium. The number of chromosomal genome equivalents per logarithmically growing cell was found to be 2.45 ± 0.35 (the corresponding number of copies of Col E_1 is shown in Table 1).

As indicated in Table 1, the replication rates during the time of linear increase in plasmid DNA (i.e., after 3 to 4 hr in CAP) indicate again that Col E_1 DNA replicates at rates significantly faster than that which occurs under normal cell growth conditions. In the case of glucose-Casamino Acids-grown cells, a normal doubling of Col E_1 DNA corresponds to the synthesis of 24 copies in 50 min and may be represented as occurring at an average rate of 0.48 copies per min. Beyond 4 hr in CAP the rate is 4.1 per min per cell or slightly more than an eightfold increase in the average rate. This is approximately the same relative rate that we obtained between hours 2 and 4 in the shorter-term experiment of Fig. 3. It is thus apparent that upon addition of CAP to cells in this medium, replication of Col E_1 occurs logarithmically for a time period representing about four generation equivalents before becoming linear.

The following experiment was performed to determine whether Col E_1 replication in the presence of CAP occurs by a semiconservative process. A 45-ml culture of CR34(Col E_1) cells was grown for several generations (in log phase) in a glucose-Casamino Acids medium containing ^{14}C-thymine (0.67 μCi per 1.5 μg per ml). CAP (150 μg/ml) was added, and after 4 hr the cells were shifted to 48 ml of medium containing bromouracil (BU) (200 μg/ml) and ^{3}H-thymine (50 μCi per 1.2 μg per ml) in a molar ratio of 110 to 1 (BU to thymine). The new medium also contained chloramphenicol at the same concentration as was present in the preshift medium. Samples (20 ml) were removed and rapidly chilled on ice after 2 and 4 hr. Crude lysates were prepared by the Sarkosyl procedure and centrifuged to equilibrium in CsCl density gradients. The results are shown in Fig. 8. The sample representing cells incubated for 2 hr in BU exhibits one rather broad ^{3}H peak, which contains also some ^{14}C-labeled DNA. The material in this peak has a higher density than unreplicated ^{14}C-DNA

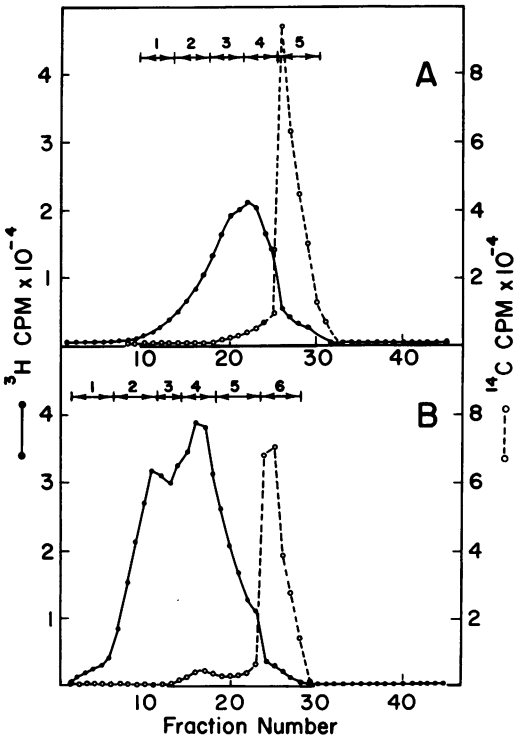

FIG. 8. *CsCl-buoyant density gradient centrifugation of Sarkosyl lysates prepared from cells exposed to chloramphenicol and bromouracil. Cells prelabeled with ^{14}C-thymine and exposed to chloramphenicol for 4 hr were shifted to a medium containing ^{3}H-thymine (in place of ^{14}C-thymine), bromouracil, and chloramphenicol (as described in text). (A) and (B) represent cells exposed to bromouracil for 2 and 4 hr, respectively (density increases from right to left). Arrows indicate fractions which were pooled and analyzed on sucrose gradients (see Fig. 9).*

(devoid of ^3H) and apparently represents hybrid (heavy:light) DNA. After 4 hr, a more dense DNA appears which is practically devoid of ^{14}C label and must therefore represent DNA with BU in both strands. Such a pattern is what one would expect as a result of semiconservative replication. The ^3H-labeled DNA is Col E_1 DNA, as was demonstrated by pooling fractions (as indicated) through regions of the gradient, dialyzing, and analyzing by sedimentation on sucrose density gradients (Fig. 9). The typical supercoiled 23*S* DNA is the major species in all pools except that representing unreplicated ^{14}C-labeled DNA, in which case larger chromosomal DNA is the predominant species. The amount of 17*S* open circular DNA seems greater than that normally seen. Nicking of covalently closed circular DNA could have resulted from nonspecific nuclease(s) activity before or during the lysis procedure. That this is a likely possibility is supported by the fact that in these experiments lysis of cells after exposure to BU was premature. That is, lysis occurred to some degree even before lysozyme was added to the cell suspension. Since no EDTA was present prior to this time, nucleases (activated by endogenous divalent cations) could have produced nicks in the DNA.

Material representing Col E_1 DNA that has undergone one round of replication is centered in pool 4 (of Fig. 8B). When the DNA in this pool was denatured by boiling for 20 min (this procedure nicks as well as promotes denaturation) and then was fractionated after alkaline CsCl centrifugation, the ^3H counts all appeared in a band of higher density than the ^{14}C-labeled band. When a similar analysis was performed on material in pool 2, representing twice-replicated Col E_1 DNA, there was only one radioactive peak and it contained almost entirely ^3H. These data indicate that Col E_1 replication in the presence of CAP is semiconservative.

DISCUSSION

The results presented above clearly indicate that, in the presence of a high level of CAP, *E. coli* (Col E_1) cells continue to replicate Col E_1 DNA by a semiconservative process long after chromosomal DNA synthesis has terminated. The rate of Col E_1 replication under these conditions depends somewhat on the medium used. Accumulation of plasmid DNA occurs logarithmically during the first few hours. After 2 to 4 hr of CAP treatment, cells in a glucose-Casamino Acids medium allow replication at a rate approximately eight times the normal rate. Replication continues for up to 10 to 15 hr following the addition of CAP, during which time the number of Col E_1 molecules per cell increases approximately 125-fold.

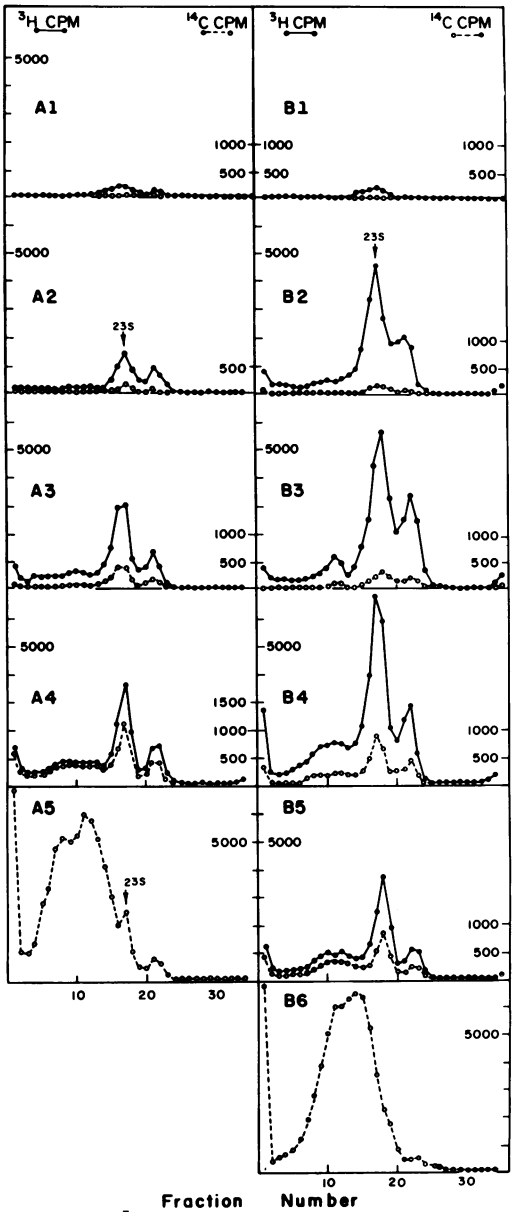

FIG. 9. *Sedimentation analyses of the pooled fractions indicated in Fig. 8. A1 through A5 represent pools 1 to 5, respectively, corresponding to cells exposed to bromouracil for 2 hr. B1 through B6 represent pools 1 to 6, respectively, corresponding to cells exposed to bromouracil for 4 hr. Samples were sedimented through 5 to 20% sucrose density gradients (from right to left) in an SW50.1 rotor (15 C) at 48,000 rev/min for 90 min.*

The reason for the increased rate of replication is not clear. One possibility is that each new plasmid DNA molecule is, under these conditions, immediately used as a template by replicating enzymes present in excess amounts, thus yielding a logarithmic increase in DNA synthesis until achievement of a maximum rate corresponding to saturation of the replicating enzymes. It is still not clear, however, why such replicating machinery, if it were present in such excess, should be more accessible to Col E_1 DNA during inhibition of protein synthesis. Perhaps the indirect inhibition of chromosomal DNA synthesis somehow frees replicating enzyme(s). The Kornberg DNA polymerase (polymerase I), an enzyme upon which the Col E_1 plasmid has been previously shown to be dependent (13), is normally present to the extent of about 400 molecules per cell (19) and may be involved in this phenomenon.

Another possibility is that there may always be an excess of available replicase, but that the level of certain other rate-limiting factors has become altered. For example, suppose that the overall rate of Col E_1 synthesis is regulated by the frequency of initiation. If initiation were negatively controlled through the interaction of the appropriate protein subunit(s) (e.g., a "repressor" substance which binds and inhibits an initiator substance), then conceivably a situation could arise (e.g., after extended CAP treatment) resulting in a breakdown of the normal maintenance of the repressed state due to abnormal dissociation of the regulatory components. Alternatively, an increased frequency of initiation could result from the accumulation of higher levels of "signal" activity which normally "triggers" initiation. In any case, a breakdown of the control of initiation would then make the maximum rate of synthesis dependent on other factors, such as, perhaps, the replicase level.

Lastly, there is always the unattractive possibility that a protein involved in Col E_1 replication continues to be synthesized in the presence of high levels of CAP and gives rise to accelerated Col E_1 synthesis. Such a possibility seems unlikely but should not be completely overlooked.

It has been reported that under conditions of amino acid starvation Col E_1 DNA immediately begins to replicate at a rate of two to three times the normal rate (4; Clewell and Helinski, J. Bacteriol., *in press*). Thus an acceleration of Col E_1 DNA synthesis in the absence of protein synthesis is brought on much more rapidly by amino acid starvation than by CAP treatment, although the mechanism by which acceleration occurs may be the same in both cases.

In certain bacteriophage systems, replication of phage DNA in the presence of high levels of CAP (100 μg/ml) has been shown to occur to some extent, but usually at rates which decrease rapidly with time (1, 14). Thus, in these systems, unlike the case of Col E_1, inhibition of protein synthesis by CAP impedes continuation of DNA replication.

The replication of Col E_1 DNA in the presence of CAP provides an extremely convenient and simple in vivo system for studying the mechanism of DNA replication. The Col E_1 DNA molecule can be isolated with ease and, if desired, in relatively large quantities. The system is currently being used in our laboratory to study the effects of various chemical compounds (antibiotics, etc.) on DNA replication as well as the mechanism by which these compounds act.

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