*GENERATION OF HIGHER MULTIPLE CIRCULAR DNA FORMS IN BACTERIA**

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Circular DNA structures have been demonstrated for several colicinogenic (*Col*) factors, which are extrachromosomal genetic elements that determine the production of antibiotically active substances termed colicins. The *FColVColBtrycys* factor, a sex factor-*Col* factor,[1] is a closed circular DNA molecule with a molecular weight of 107×10^6.[2] The *Col* E factors (bacterial plasmids[3, 4]) are considerably smaller, consisting in *E. coli* of closed circular DNA molecules with molecular weights of 4.2×10^6 for *Col*E_1 and 5×10^6 for *Col*E_2 and *Col*E_3, respectively.[5, 6] *Col*E_1 DNA, isolated from a *Proteus mirabilis* strain that received this factor by conjugal transfer from an *E. coli* strain, consists of closed circular duplex molecules of three size classes that correspond to molecular weights of approximately 4.2×10^6, 8.5×10^6, and 12.7×10^6, respectively.[5, 6] These multiple-length circular forms of *Col*E_1 DNA have not been observed in any significant amount in *E. coli* strains carrying the *Col*E_1 factor.[7]

In this report, higher multiple circular DNA forms of *Col*E_1 are shown to be synthesized in response to protein synthesis inhibition by the addition in chloramphenicol, or by amino acid starvation. The patterns of pulse-labeling with radioactive isotope during the generation of the higher multiple circular forms indicate that the higher multiple forms do not arise simply by random nonreciprocal recombination events between pre-existing lower multiple circular DNA forms. A model is proposed to account for the duplication of the *Col*E_1 factor and the synthesis of higher multiple DNA forms of this factor.

Materials and Methods.—Bacterial strains: The colicinogenic *P. mirabilis* strain used in this study, *P. mirabilis* (*Col* E_1) *thy*$^-$, *try*$^-$, *arg*$^-$, which requires thymine, arginine, and tryptophan for growth, was isolated by treatment of the previously described[6] thymine-requiring strain, *P. mirabilis* (*Col* E_1) *thy*$^-$, with N-methyl-N′-nitro-N-nitrosoguanidine. The *E. coli* JC411 (*Col* E_1) strain has been previously described.[8]

Media and growth conditions: Reagents and media are the same as described previously,[6, 9] except that the phosphate and tris media were supplemented with 10 μg/ml of L-tryptophan. Cells were grown in either 25 ml or 1 liter of medium, depending upon whether the DNA was to be isolated by direct dye–buoyant density centrifugation or by the phenol technique. One liter of medium containing 1 mc of (^{3}H)-thymidine or 1 mc of (^{3}H)-thymine was inoculated with 0.5 ml of an overnight enriched nutrient broth[4] culture of a single colony isolate of the colicinogenic strain. A similar inoculum was used for 25 ml of medium containing 0.1 mc of (^{3}H)-thymidine. ^{32}P-labeling was accomplished by adding 5 mc carrier-free (^{32}P)H_3PO_4 per liter.

Chloramphenicol treatment and amino acid starvation: Proteus cells were grown in phosphate-buffered, casamino acids medium to 5×10^8 cells/ml. The cells were harvested by centrifugation in the cold, washed twice with cold TS (0.03 *M* tris, 0.05 *M* NaCl, pH 8.0), and transferred to (^{32}P)-labeled tris-buffered minimal medium (preincubated at 37°) containing 150 μg/ml chloramphenicol, or lacking tryptophan and casamino acids. After being shaken on a rotary shaker at 37° for 40 min, the cells were harvested by centrifugation in the cold.

DNA extraction and isolation of ColE$_1$ supercoiled DNA: DNA was isolated either by the phenol technique previously described,[5] or by direct dye–buoyant density centrifugation of cell lysates as described elsewhere,[7] except that sodium dodecyl sulfate (final concentration of 0.3%) was used instead of sarkosyl for the lysis of the *Proteus* strain. Conditions for centrifugation and for the collection and radioisotope counting of fractions are described elsewhere.[6] The *ColE*$_1$ DNA appeared as a heavy satellite peak, approximately 0.04 gm/cc more dense than the main chromosomal DNA peak.

For the isolation of *ColE*$_1$ DNA from DNA preparations purified by the phenol technique, a mixture of 400 μg DNA in 3 ml of dilute SSC (0.015 *M* NaCl, 0.0015 *M* sodium citrate, pH 7.0), 1.5 ml of ethidium bromide solution (1 mg/ml), 10.5 ml TES buffer, and 15 gm CsCl was added to each of eight centrifuge tubes of a Ti-60 fixed-angle rotor. The DNA concentration of purified DNA preparations was determined by Burton's procedure,[10] and the cellular content of DNA was determined by the procedure described by Lark *et al.*[11]

Sucrose gradient velocity centrifugation: Sucrose gradient centrifugation was performed using an SW65 rotor and a Spinco L-4 or L-2 preparative ultracentrifuge; 0.2 ml of a DNA sample in dilute SSC was layered on a 4.5-ml 5–20% linear sucrose gradient containing 0.1 *M* NaCl, 0.001 *M* ethylenediaminetetraacetate (EDTA), and 0.01 *M* tris, pH 8.0. The samples were centrifuged at 50,000 rpm for 90 min at 15°C. Ten-drop fractions were collected from the bottom of the tube directly onto filter papers and were counted as described earlier.[6]

Results.—Col factor content of cells grown with glucose and acetate as carbon sources: It has been previously demonstrated that the amount of DNA per *E. coli* cell is dependent on the carbon source used for the growth of the cells.[12] *E. coli* cells grown in an acetate medium contain an amount of DNA per cell corresponding to approximately one chromosome per cell, whereas with glucose as a carbon source, a level of DNA is found that is equivalent to approximately two chromosomes per cell.[13] The *Proteus* (*Col*E$_1$) strain was grown in phosphate-buffered minimal medium with 0.2 per cent glucose as a carbon source. The generation time in this medium was about 50 minutes. As shown in Table 1, approximately two chromosomes were found per cell for cells obtained either from the log phase or from the late stationary phase in this medium, assuming that the weight of a single chromosome in *P. mirabilis* is similar to that found for the *E. coli* chromosome (0.65×10^{-14} gm). Log- or late-stationary-phase *P. mirabilis* cells grown with 0.5 per cent acetate as a carbon source (3–4 hr generation time) contain an amount of DNA per cell that is equivalent to one chromosome per cell. Similar results were obtained for the *E. coli* JC411 (*Col*E$_1$) strain.

The percentage of supercoiled *Col*E$_1$ factor DNA in these preparations was determined by measuring the areas under the satellite DNA band and the chromosomal DNA band following dye-buoyant density centrifugation of SDS-lysates of the cells. Virtually all of the *Col*E$_1$ DNA is present as supercoiled DNA when the cells are lysed and the DNA is fractionated by this procedure.[7] The ratio of supercoiled *Col*E$_1$ DNA to chromosomal DNA in both the *P. mirabilis* and *E. coli* colicinogenic strains was found to be relatively constant regardless of the carbon source of the growth stage (Table 1). Thus, the cell modulates both the number of *Col*E$_1$ molecules and the number of chromosomal DNA molecules in response to a change in the carbon source.

Proportion of each of the various ColE$_1$ DNA multiple forms in cells grown on different carbon sources: It was of interest to determine whether conditions that

TABLE 1. *ColE$_1$ and chromosomal DNA content per cell.*

Strain	Stage of growth	Cells Grown in Acetate: DNA/cell [$\times 10^{14}$ gm]*	Cells Grown in Acetate: Per cent *ColE$_1$* DNA	Cells Grown in Glucose: DNA/cell [$\times 10^{14}$ gm]*	Cells Grown in Glucose: Per cent *ColE$_1$* DNA
E. coli JC411 (*ColE$_1$*)	Log	0.80	0.75	1.34	0.80
	Late stat.	0.75	0.90	1.28	0.85
P. mirabilis (*ColE$_1$*) *thy$^-$, try$^-$, arg$^-$*	Log	0.70	1.15	1.20	1.10
	Late stat.	0.62	1.23	1.12	1.00

* These values represent averages of two independent determinations.

decrease the amount of *Col*E$_1$ DNA per cell have any effect on the distribution of the various *Col*E$_1$ DNA forms. Cultures of the *Proteus* (*Col*E$_1$) strain were grown in phosphate-buffered minimal media, with 0.2 per cent glucose or 0.5 per cent sodium acetate as carbon sources. Tritium-labeled *Col*E$_1$ DNA was isolated by the direct dye–buoyant density centrifugation method. The fractions containing supercoiled *Col*E$_1$ DNA were pooled, dialyzed against dilute SSC, and further analyzed by sucrose gradient centrifugation. As already demonstrated,[6] the *Col*E$_1$ DNA isolated from *P. mirabilis* can be separated into three peaks in a sucrose gradient, representing, on the basis of size, monomer (23*S*; 4.2×10^6 mol wt), dimer (31*S*; 8.5×10^6 mol wt), and trimer (37*S*; 12.7×10^6 mol wt) forms. As shown in Figure 1*a* and *b*, there is no significant difference in the *Col*E$_1$ DNA pattern between cells grown to the log phase either in the glucose medium or in the acetate medium. When the cells were allowed to grow to a late stationary phase (2×10^9 cells/ml), a similar shift in the *Col*E$_1$ pattern is observed in both media (Fig. 1*d* and *e*). This effect can also be seen by pulse-labeling the cells during the late-log and early-stationary state. During this period, monomer *Col*E$_1$ DNA form is produced at a fourfold higher level than the dimer form. *Col*E$_1$ DNA isolated from *E. coli* JC411 (*Col*E$_1$) after growth in the glucose or acetate medium is essentially represented only by the 23*S* monomer form (Fig. 1*c*). The minor component that sediments more rapidly than the 23*S* form could represent a small amount of the dimer *Col*E$_1$ form. There is no observable difference in the *Col*E$_1$ DNA pattern when this colicinogenic *E. coli* strain was allowed to grow to a late-stationary phase (Fig. 1*f*).

The effect of chloramphenicol and amino acid starvation on the formation of the supercoiled ColE$_1$ DNA forms: The inhibition of protein synthesis by the addition of chloramphenicol or amino acid starvation of an *E. coli* amino acid–requiring strain has been reported to interfere with the initiation of chromosomal DNA synthesis.[14, 11, 15] It was of interest to determine the effect of these conditions on the formation of *Col*E$_1$ DNA. The *Proteus* (*Col*E$_1$) strain was treated with chloramphenicol as described in Figure 2*b*, and the *Col*E$_1$ DNA was isolated by direct dye–buoyant density centrifugation of duponol lysates and analyzed by sucrose gradient centrifugation. As shown in Figure 2*b*, chloramphenicol addition results in a significant increase in dimer, trimer, and higher multiple circular DNA forms over that of the control (Fig. 2*a*). Amino acid starvation (Fig. 2*c*) resulted in an even more striking increase in the higher multiple forms with very little monomer formation under these conditions. $S_{20,w}$ values of 42, 47, and 51 can be calculated for the supercoiled tetramer, pentamer, and hexamer forms,

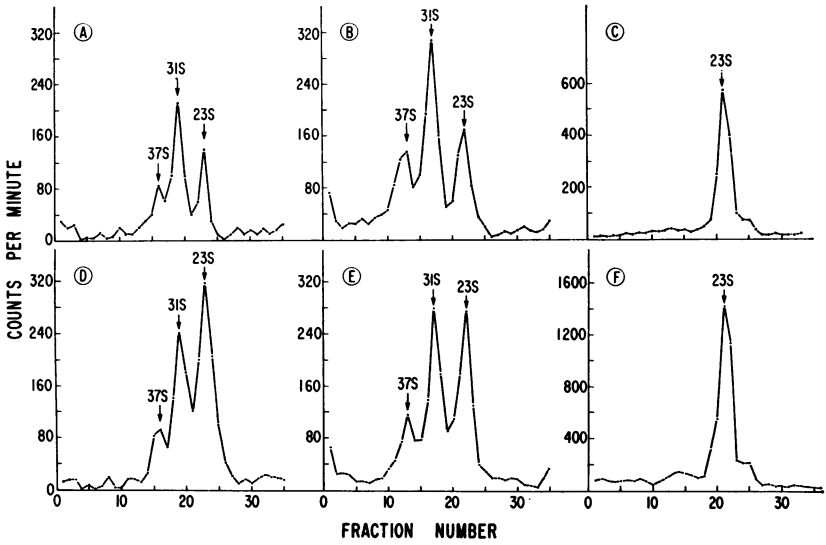

FIG. 1.—Sucrose gradient analyses of *Col*E$_1$ DNA isolated from *Proteus* (*Col*E$_1$) and *E. coli* JC411 (*ColE*$_1$) grown under various conditions. Approximately 1 μg of DNA from the heavy satellite band in an ethidium bromide–cesium chloride gradient was applied to a sucrose gradient and centrifuged as described in *Materials and Methods*. Fractions are numbered in the order collected from the bottom of the centrifuge tube.

(*A*) *Proteus* (*Col*E$_1$), log phase, glucose medium. (*B*) *Proteus* (*ColE*$_1$), log phase, acetate medium. (*C*) *E. coli* JC411 (*Col*E$_1$), log phase, glucose medium. (*D*) *Proteus* (*ColE*$_1$). stationary phase, glucose medium. (*E*) *Proteus* (*Col*E$_1$), stationary phase, acetate medium. (*F*) *E. coli* JC411 (*Col*E$_1$), stationary phase, glucose medium.

respectively, of *Col*E$_1$ DNA.[6] The sucrose gradient profiles presented in Figure 2 are not altered to any significant extent by prior treatment of the isolated *Col*E$_1$ DNA with alkali (pH 12.2), followed by neutralization and filtration on nitrocellulose filters. These conditions result in removal of DNA that is not in the covalently closed circular form.[6] The various multiple forms show a buoyant density in cesium chloride that is characteristic of the *Col*E$_1$ factor, and they are not observed in the corresponding noncolicinogenic *Proteus* strain treated under similar conditions. The amount of tritium incorporation during the 40-minute period of chloramphenicol treatment or amino acid starvation corresponds to an increase in *Col*E$_1$ DNA of approximately 50–75 per cent. Analyses by electron microscopy of the *Col*E$_1$ DNA isolated from an amino acid–starved *Proteus* culture also revealed a substantial proportion of the supercoiled DNA forms as higher multiples of the monomer form. Preliminary experiments indicate that these conditions do not lead to multiple DNA forms of *Col*E$_1$ in an *E. coli* (*Col*E$_1$) strain.

To examine the origin of the higher multiple forms of *Col*E$_1$ DNA, a double labeling experiment was carried out. *Proteus* (*Col*E$_1$) cells were grown to the logarithmic stage in 1 liter of tritium-labeled phosphate-buffered medium and then shifted to tris-buffered medium containing ^{32}P-inorganic phosphate. The cells were incubated in this medium in the presence of chloramphenicol or in the absence of amino acids for a 40-minute period. The DNA was extracted by the

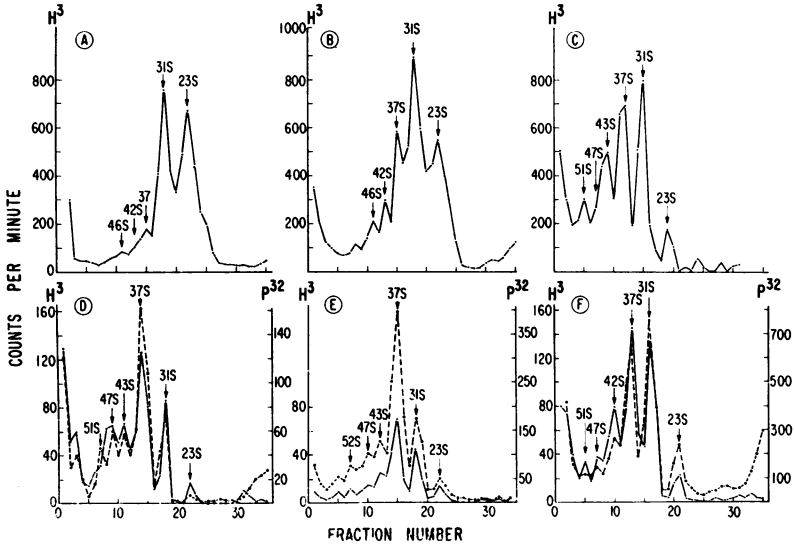

FIG. 2.—Sucrose gradient analyses of *Col*E_1 DNA isolated by dye–buoyant density centrifugation from *Proteus* (*Col*E_1) following treatment with chloramphenicol or amino acid starvation.

(*A*) Cells were grown to a log phase in 25 ml of phosphate-buffered minimal medium and then shifted to the same volume of a low-phosphate tris medium containing ^{3}H-thymine and grown for 40 min.

(*B*) Cells were grown as described in (*A*), but 150 μg/ml of chloramphenicol was added to the low-phosphate tris medium containing ^{3}H-thymine.

(*C*) Cells were grown as described in (*A*), except that the labeled low-phosphate tris medium was not supplemented with amino acids.

(*D*) Cells were grown to a log phase in 1 liter of phosphate-buffered minimal medium containing ^{3}H-thymine, and then shifted to the same volume of a low-phosphate tris medium containing (^{32}P) H_3PO_4 and 150 μg/ml of chloramphenicol. The cells were incubated for 40 min in the presence of chloramphenicol. *Solid line*, ^{3}H; *dashed line*, ^{32}P.

(*E*) Cells were grown to a log phase as described in (*D*) and then shifted to 1 liter of low-phosphate tris medium containing (^{32}P) H_3PO_4, but not supplemented with amino acids.

(*F*) Cells were grown to a log phase in 1 liter of phosphate-buffered minimal medium containing ^{3}H-thymine, and then shifted to the same volume of a low-phosphate tris medium containing ^{3}H-thymine but not supplemented with amino acids. After 40 min incubation in the absence of amino acids, the cells were shifted to 1 liter of the low-phosphate tris medium supplemented with amino acids and containing (^{32}P) H_3PO_4. Sucrose gradient centrifugation conditions are described in Fig. 1.

phenol procedure, and *Col*E_1 DNA was isolated by the dye–buoyant density centrifugation technique. Both treatments resulted in an increase in higher multiple circular DNA forms and a striking decrease in the monomer (23*S*) form (Fig. 2*d* and *e*). Once again there was an increase of 50–75 per cent in the *Col*E_1 DNA during this period. The higher multiple circular DNA forms are labeled with tritium as well as with ^{32}P, an indication that the higher forms arise by a mechanism that utilizes the pre-existing, tritium-labeled, lower circular DNA forms. The ratio of ^{32}P/^{3}H in the *Col*E_1 DNA forms varies with the higher multiple forms having a higher ratio than the monomer and dimer forms (in Fig. 2*d*, 1.29, 0.86, and 0.39 for the trimer, dimer, and monomer forms, respectively, and in Fig. 2*e*, 5.56, 3.89, and 3.93 for these forms, respectively). This

result is not in agreement with the formation of the higher multiple forms solely by a mechanism involving random recombination of pre-existing lower forms, since this mechanism should yield trimer DNA forms with a $^{32}P/^{3}H$ ratio that is no higher than the $^{32}P/^{3}H$ ratio of either the dimer or monomer forms.

To examine the question whether higher multiple DNA forms, once synthesized, are able to replicate, the *Proteus* (*Col*E_1) strain was grown to the logarithmic state in the presence of ^{3}H-thymine and starved for amino acids as described above in order to accumulate the higher multiple forms. The cells were then washed and resuspended in a ^{32}P-labeling tris-buffered medium, supplemented with tryptophan and arginine, and grown for two more generations to a late-stationary phase. The *Col*E_1 DNA was isolated and resolved into its components as described for the previous double-labeling experiment. As indicated by the ^{32}P-labeling pattern shown in Figure 2*f*, higher multiple forms appear to be synthesized during the late logarithmic growth period following the amino acid starvation. The $^{32}P/^{3}H$ ratio of the various *Col*E_1 forms is the highest for the monomer (11.9) and decreases for the higher multiple forms (6.5 for dimer, 4.1 for trimer, and 3.5 for tetramer), consistent with the duplication of the higher multiple forms that are synthesized during the amino acid starvation period and the previously observed preferential replication of the monomer, or lower circular DNA forms, during the late growth phase.

Discussion.—In several respects the *Col*E_1 factor exhibits properties not unlike that of the bacterial chromosome: (1) the *Col*E_1 factor is a circular DNA element, whereas circularity has been shown at least for the *E. coli* chromosome;[16] (2) the duplication and segregation of *Col*E_1 DNA involves a carefully regulated process that assures a relatively constant level of *Col*E_1 factor per cell population and this level can be modulated by a change in the carbon source in the growth medium; and (3) a sex factor will promote the transfer via conjugation of either the chromosome or the *Col*E_1 DNA to a recipient cell. In at least one respect, however, the control of *Col*E_1 duplication may differ from the process controlling chromosomal DNA synthesis. Although the inhibition of protein synthesis appears to affect the reinitiation of chromosomal DNA synthesis,[14, 11, 15] the above results indicate that chloramphenicol treatment or amino acid starvation does not prevent the reinitiation of *Col*E_1 DNA synthesis in *P. mirabilis* (assuming a similar rate of DNA synthesis for the *Col*E_1 factor and the chromosome).

Hudson and Vinograd[17] have proposed a recombination mechanism for the origin of multiple circular DNA forms in mitochondria. The following observations, each by itself insufficient to prove any one mechanism, taken together favor errors in duplication, rather than a recombination mechanism, for the origin of multiple forms of the *Col*E_1 DNA. (1) *Col*E_1 DNA is present essentially as the monomer form in its natural host *E. coli* but is found as multiple circular forms in the unnatural host *P. mirabilis*. (2) Lowering of the cellular content of *Col*E_1 DNA did not alter the proportion of the multiple forms. (3) Conditions which affect protein synthesis, such as chloramphenicol addition and amino acid starvation, induce the formation of higher multiple forms. (4) Radioisotope labeling of *Col*E_1 DNA during the formation of the higher multiple forms resulted in labeling patterns that are not consistent with the formation of higher circular

forms solely by a random recombination process involving a pre-existing pool of smaller circular DNA molecules.

A model to account for the generation of multiple circular forms by errors in the duplication process is illustrated in Figure 3. This model is consistent with the radioisotope-labeling experiments and is similar to the model proposed by Fulton[18] to account for the observed continuous transfer of chromosomal DNA during conjugation. The formation of higher multiple forms in *P. mirabilis* can be considered to result (in this organism) from an imbalance in the formation or concentration of the enzymes responsible for *Col*E$_1$ DNA duplication. The mechanism proposed in this model conceivably could also account for the concatenated structures reported as intermediates in the formation of T2[19] and λ[20] DNA. *Col*E$_1$ DNA molecules, similar in certain respects to the concatenated structures of bacteriophage DNA, have been observed in addition to the multiple

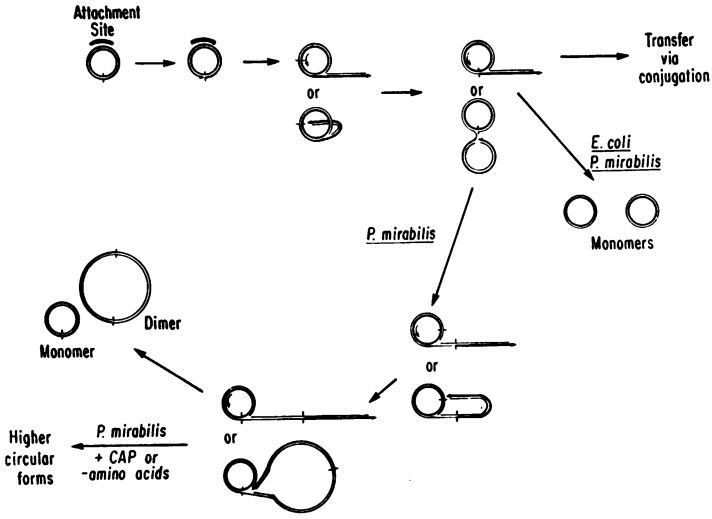

FIG. 3.—Model for the duplication of *Col*E$_1$ DNA and the formation of higher multiple circular DNA forms. Duplication of the *Col*E$_1$ DNA is considered to involve initially a break in one of the phosphodiester bonds of one of the two strands of the covalently closed double-stranded circle. It is proposed that this endonucleolytic cleavage occurs at a specific site, the initiation region for the duplication of the cyclic DNA, and conceivably generates a cohesive end for subsequent cyclization of the duplicated strand. Duplication involves the unwinding of the complementary strands with the formation of a "forked" structure. During this process one of the strands remains in the covalently closed cyclic form and serves as a template for the potential synthesis of an unlimited number of covalently linked complete sequences of the other strand. Either the covalently open strand has a "free" end or the end remains associated with the initiation point to generate structures similar to those observed by Cairns[16] for the replicating *E. coli* chromosome. After a complete sequence of the covalently open strand is duplicated, a second endonucleolytic cleavage in the initiation region releases the duplicated strand for cyclization. In *P. mirabilis* the second endonucleolytic cleavage occasionally does not occur until more than one complete sequence of *Col*E$_1$ DNA is duplicated, resulting in the formation of dimers, trimers, or higher circular forms. The failure to carry out the second endonucleolytic cleavage after the duplication of one length of circular DNA is considered to occur with increased frequency in *P. mirabilis* under conditions of inhibition of protein synthesis. The heavy lines represent parental DNA strands, and the light lines represent newly synthesized DNA strands.

circular forms of *Col*E_1 after chloramphenicol treatment, or to a lesser extent after amino acid starvation, of the colicinogenic *Proteus* cells. The utilization of the monomer *Col*E_1 DNA forms in the initial steps leading to the formation of these concatenated forms may account for the striking decrease of the monomer form in the population of supercoiled *Col*E_1 DNA molecules.

Summary.—The proportion of the various circular DNA forms of *Col*E_1 was determined after growth of *P. mirabilis* (*Col*E_1) cells under a variety of conditions. A model is presented for the duplication of circular DNA forms by a mechanism that can readily generate multiple circular DNA forms.

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