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R Factor Deoxyribonucleic Acid in Chromosomeless Progeny of *Escherichia coli*

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The deoxyribonucleic acid (DNA) of resistance (R) factor 222 carried by *Escherichia coli* strain P678-54 was found in the normally chromosomeless progeny (minicells) of that strain. The entry of the R222 DNA into minicells appears to be via segregation at the time of their formation from normal cells. The R222 DNA can replicate in minicells although the extent of its replication appears to be limited. An analysis of the R222 DNA structure indicates that it exists in minicells as double-stranded linear, open circular, and twisted circular monomers (molecular weight, about 6.2×10^7 daltons). The monomers visualized by electron microscopy are 31.0 ± 0.5 μm in length. An examination of the effect of acridine orange on the replication of R222 and colicin E1 DNA indicates the dye intereferes with plasmid DNA replication.

Previous work has shown that colicin factor E1 (Col E1) deoxyribonucleic acid (DNA) can segregate into and replicate in the otherwise chromosomeless progeny (minicells; ref. 1) of the *Escherichia coli* K12 strain P678-54 (11, 12). The present work was undertaken to determine if minicells produced by strain P678-54 carrying the resistance (R) factor 222 (22) carry the R222 DNA. If such were the case, a relatively simple method for studying R factor DNA in *E. coli* would be available.

The results of this study indicate that R222 DNA is found in minicells, and the method is a useful one for studying that DNA.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. A thymine-requiring strain *E. coli* P678-54 (1) was isolated and its R factor-carrying derivative P678-54 $(R222)^+$ made by transferring R factor 222 (22) to it from a strain of *Salmonella typhimurium* LT2 provided by S. E. Luria. The transfer was accomplished by incubating both strains together overnight in nutrient broth at 37 C without shaking and was followed by a 1 to 20 dilution in nutrient broth with an additional incubation of 3 hr at 37 C. Selection was for P678-54 that had acquired multiple drug resistance. Whereas this R factor originally conferred resistance to streptomycin, sulfanilamide, tetracycline, and chloramphenicol (22), the R factor did not confer resistance to chloramphenicol at concentrations of 20 μg/ml. [P678-54 $(Col\ E1)^+$ was previously described (11).] Phage P1, used as a source of DNA as a sedimentation marker, was grown and radioactively labeled as previously described (11).

Media, solutions, and reagents. Tris(hydroxymethyl) aminomethane (Tris)-Casamino Acids-glucose (TCG) is 0.1 M Tris, *p*H 7.4; 0.4% Casamino Acids or vitamin-free Casamino Acids, Difco Laboratories Inc., Detroit, Mich. (for ^{3}H-thymidine labeling); and 0.5% glucose per liter. Supplemented TCG contained 15 μg/ml of threonine and leucine, 1 μg/ml of vitamin B_1, and 1 to 15 μg/ml of thymine as required. Saline citrate buffer (SSC) contains 0.15 M NaCl and 0.015 M sodium citrate, *p*H 7.4. Sucrose solutions were made with SSC. Acridine orange made to 2 mg/ml in distilled water was kept refrigerated for up to 1 week in the dark. An ethidium bromide solution contained 700 μg/ml in 0.1 M sodium phosphate buffer, *p*H 7.0. Solutions of 5-bromouracil, 2 mg/ml; 5-fluorodeoxyuridine, 2 mg/ml; and deoxyadenosine, 12.5 mg/ml were each prepared in sterile distilled water. ^{3}H-thymidine, 18.3 Ci/mmole, was from New England Nuclear Corp., Boston, Mass. Cesium chloride was obtained from the Harshaw Chemical Company and ethidium bromide from Sigma Chemical Co., St. Louis, Mo.

Growth of cells and minicell purification. The growth of cells in medium and subsequent purification of minicells are identical to those previously described (11). In suspensions of 10^{11} purified minicells per ml, about 1,000 viable cells per ml were found.

Isolation of DNA. DNA was isolated from minicells by the procedure described (11) with the exception that the extracts were treated with self-digested Pronase (37 C for 4 hr) at a final concentration of 1 mg/ml for up to 1 hr at 37 C prior to phenol extraction.

Sucrose gradient-velocity centrifugation. Sucrose gradient-centrifugation was performed in a model SW39 swinging-bucket rotor for 2 to 4 hr at 73,000 $\times$ *g* or in a model SW27 rotor at 95,000 $\times$ *g* for 3 hr, at 20 C in a Spinco model L2 ultracentrifuge. Fractions were collected from the bottom of the tube by drop collection (11). Phage P1 DNA [ref. 10; molecular weight, 6×10^7 daltons and a calculated sedimentation coefficient of 43.3 using Studier's equation ($S^0_{20,w} = 0.0882\ M^{0.346}$), ref. 18] was used as a sedimentation marker.

Cesium chloride buoyant density gradient centrifugation. A 5-ml solution of cesium chloride of appropriate density was prepared by adding DNA in SSC to solid cesium chloride and spun at 80,000 × *g* for 48 hr at 15 C in a Spinco 50 Ti fixed-angle rotor in a Spinco model L2 ultracentrifuge. Fractions were collected from the bottom of the tube by drop collection. Densities were determined by refractive index with an Abee-3L refractometer (11). *Escherichia coli* DNA was used as a density marker.

Cesium chloride-ethidium bromide buoyant density gradient centrifugation. To 4.33 g of solid cesium chloride was added 1.33 ml of ethidium bromide (700 μg/ml) and DNA in SSC to bring the refractive index of the final solution to 1.3920. This mixture was spun at 80,000 × *g* for 48 hr at 15 C in a Spinco 50 Ti fixed-angle rotor in a Spinco model L2 ultracentrifuge. Fractions were collected from the bottom of the tube by drop collection.

Radioactive labeling of DNA and the determination of radioactivity. The methods are identical to those previously described (11).

RESULTS

Demonstration of R222 DNA in minicells. It was previously demonstrated that purified preparations of minicells derived from strain P678-54 did not incorporate ^{3}H-thymidine into DNA, whereas minicells derived from P678-54 (Col E1)$^+$ incorporated ^{3}H-thymidine into the DNA of the replicating colicin factor which had entered the minicells via segregation during minicell production (11, 12). The presence of replicating R222 DNA in minicells should also be demonstrable by first showing that minicells derived from P678-54 (R222)$^+$ and not those from P678-54 incorporate ^{3}H-thymidine into DNA and then by showing that the labeled DNA has properties similar to those reported for R factor DNA (5, 6, 14; Tompkins et al. Bacteriol. Proc., p. 61, 1970).

Minicells purified from cultures of P678-54 and P678-54 (R222)$^+$ were incubated in the presence of ^{3}H-thymidine for 2 hr, and the radioactive DNA was purified (11). The P678-54 (R222)$^+$ minicells were found to contain radioactive DNA, whereas P678-54 minicells did not. The ^{3}H-minicell DNA was examined by cesium chloride and cesium chloride-ethidium bromide buoyant density gradient centrifugation, sucrose gradient-velocity sedimentation, and electron microscopy. Although no significant difference was noted in the densities of such minicell DNA and *E. coli* cellular DNA in cesium chloride, significant amounts (Fig. 1) of minicell DNA were relatively denser than *E. coli* DNA in cesium chloride-ethidium bromide gradients as would be expected if covalently closed circular molecules were present (16).

The radioactive minicell DNA, when examined by sucrose gradient-velocity sedimentation, produced a complex sedimentation pattern (Fig. 2). The presence of major peaks with sedimentation coefficients of about 77*S* and 46*S* are regularly reproducible as is a partially resolvable fast sedimenting shoulder of the 46*S* peak which has a sedimentation coefficient of about 52*S*. The sedimentation coefficients are the averages of eight separate experiments. The amount of label in the 52*S* region varies from experiment to experiment and may exceed that found at the 46*S* position. One problem in resolving the 52*S* shoulder of the 46*S* sedimenting peak has been the presence of material with a sedimentation coefficient of about 56*S*. In five different experiments a partial

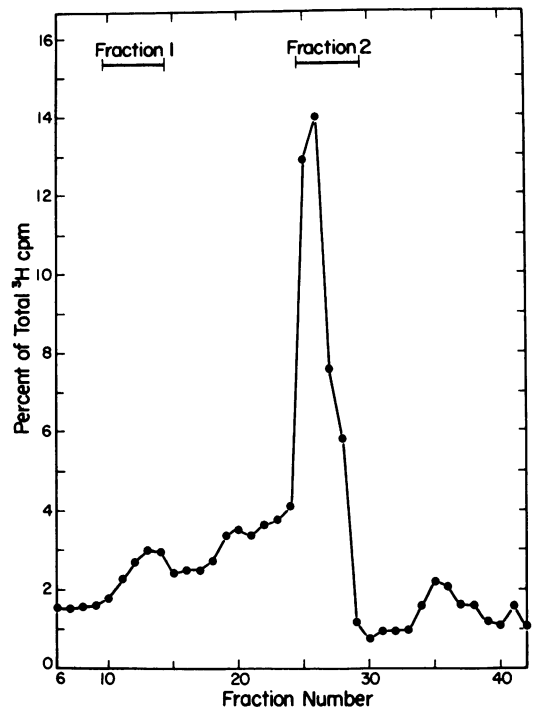

FIG. 1. *Analysis of replicated minicell DNA by cesium chloride-ethidium bromide density gradient centrifugation. Minicells purified from 4 liters of logarithmic phase P678-54 (R222)$^+$ or P678-54 cells grown in supplemented TCG medium were shaken for 2 hr at 37 C in supplemented TCG medium containing 1 μg of thymine per ml, 250 μg of deoxyadenosine per ml, 5 μCi of ^{3}H-thymidine per ml. The minicells were lysed by using the lysozyme-sarkosyl method (11) and treated with 1 mg of self-digested Pronase per ml before extracting the DNA with phenol (11). The DNA was mixed with cesium chloride-ethidium bromide and centrifuged; the gradients were fractionated as described in the text. Fractions (10 μliters) were counted, and appropriate fractions were pooled for further experiments. ^{3}H-DNA (●), 10,302 counts/min. Since no peaks in counts (fewer than a total of 200 counts/min) were found in the material isolated from P678-54 minicells, the data are not plotted.*

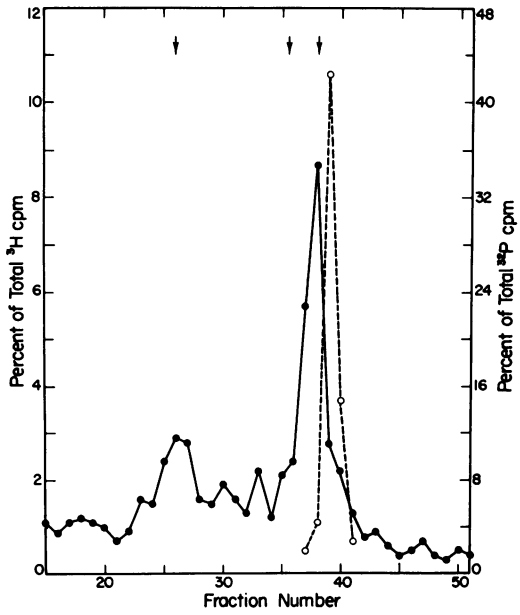

FIG. 2. *A sucrose gradient velocity sedimentation analysis of DNA synthesized in P678-54 (R222)⁺ minicells. DNA from minicells purified and labeled as described in Fig. 1 was prepared. A 0.5-ml sample of DNA was layered on a 17-ml 5 to 20% sucrose SSC gradient and centrifuged in a Spinco SW27 rotor at 95,000 × g for 3 hr at 20 C in a Spinco model L2 ultracentrifuge; the gradient was fractionated. The total number of fractions was 56. ³H-minicell DNA (●), 20,854 counts/min; ³²P-P1 DNA (○), 4,783 counts/min. The positions of a 46S and 76S peak are noted as is the 52S region of the gradient.*

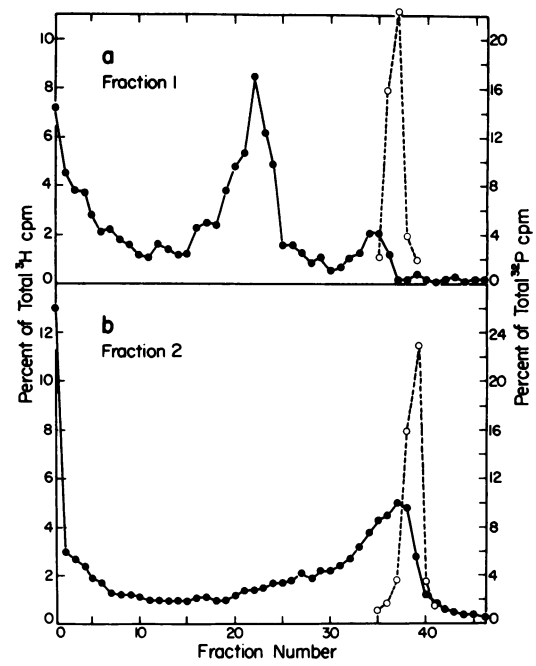

FIG. 3. *Sucrose gradient velocity centrifugation of DNA from Fig. 1. The pooled fractions 1 and 2 from Fig. 1 were dialyzed against SSC, concentrated to 0.4 ml, layered with ³²P-P1 DNA as a sedimentation marker on 17-ml 5 to 20% sucrose SSC gradients and spun for 3 hr at 95,000 × g in a Spinco SW27 rotor at 20 C in a Spinco model L2 ultracentrifuge. (a) Fraction 1; total fractions, 56.5. ³H-DNA (●), 3,471 counts/min; ³²P-P1 DNA (○), 1,698 counts/min. (b) Fraction 2; total fractions, 57. ³H-DNA (●), 34,844 counts/min; ³²P-P1 DNA (○), 1,700 counts/min.*

or complete resolution of a variable-sized peak, sedimenting with *S* values of 56.0, 56.9, 56.8, 57.5, and 56.5, has been noted. Numerous attempts have been made to determine the nature of that DNA and other DNA which appears in small amounts between the 53 and 70*S* positions of the sucrose gradient (*see* Fig. 2). Those results are still preliminary.

The radioactive material obtained from the region of the cesium chloride-ethidium bromide density gradient (fraction 1, Fig. 1) was analyzed for its sedimentation properties in a 5 to 20% SSC-sucrose gradient (Fig. 3a). That DNA principally sediments as a 77*S* peak provides evidence that the 77*S* peak (Fig. 2) probably represents covalently closed circular molecules. The DNA (fraction 2, Fig. 1) that banded in a position interpretable as containing double-stranded linear and open circular DNA sediments as a broad peak skewing from about the 47*S* position in the sucrose gradient towards the faster sedimenting region (Fig. 3b).

In Fig. 4 it is shown that only DNA from the 75-82*S* region of the sucrose gradient containing P678-54 (R222)⁺ minicell DNA shows significant rapid renaturation (45%) after alkali denaturation as is expected for covalently closed circular molecules. All marker *E. coli* DNA in the same samples underwent the expected 0.016 g/cm³ density shift characteristic of denaturation. It is concluded from the results (Fig. 1, 3, and 4) that the P678-54 (R222)⁺ minicell DNA sedimenting in the 77*S* region of the sucrose gradients represents the only significant covalently closed circular DNA in the 43 to 82*S* region of the gradients.

Electron micrographs of the purified but unfractionated DNA obtainable from P678-54 (R222)⁺ minicells were taken to further elucidate its nature (Fig. 5). Large numbers of open circular, twisted circular, and fewer linear molecules were observed in the preparation studied. Measurements of 11 open circular molecules gave a length of 31.0 ± 0.5 μm which corresponds to a linear molecule (molecular weight, about 6.2 × 10^7 daltons) with a calculated sedimentation value of about 44*S* (18). The previously reported

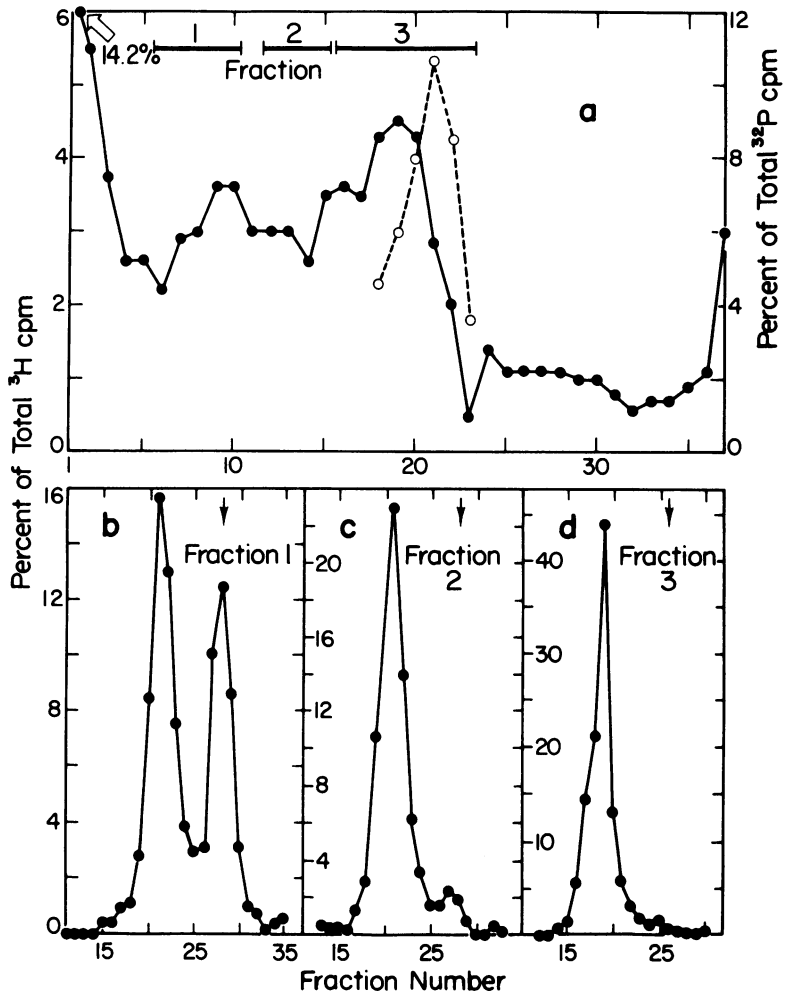

FIG. 4. *Analysis of replicated minicell DNA to determine its ability to rapidly renature after alkali denaturation. Purified minicells (10^{11}/ml) from E. coli P678-54 (R222)⁺ containing fewer than 1,000 viable cells/ml were incubated in ³H-thymidine, and the DNA was extracted as previously described (11). (a) DNA was concentrated with Carbowax 6000 to 0.25 ml, layered on a 4.5-ml 5 to 20% sucrose SSC gradient with ³²P-P1 DNA used as a sedimentation marker, and centrifuged at 20 C for 4 hr in a Spinco SW39 rotor at 73,000 × g in a Spinco model L2 ultracentrifuge. Total fractions, 37; ³H-DNA, (●); ³²P-P1 DNA, (○). Portions (0.01 ml) of fractions were examined for radioactivity, after which fractions 6-10, 12-15, and 16-23 were pooled, dialyzed for 2.5 hr against a 0.7 M NaCl, 0.05 M $Na_2HPO_4 \cdot 12H_2O$ solution (pH 7.0). Each pooled sample was mixed with ³²P-E. coli DNA, alkali denatured by being rapidly brought to pH 12.3 by using 1.1 M NaOH, and held for 8 to 10 min at 0 C; the samples were rapidly brought to pH 8.4 with a solution of 0.7 M Tris-hydrochloride in 0.3 M HCl (11, 17). The DNA was mixed with cesium chloride and brought to a refractive index of 1.414; the gradient was spun at 80,000 × g for 40 hr. The effect of alkali denaturation and rapid renaturation on ³H-minicell DNA density was measured. All of the ³²P-E. coli DNA was denatured in these experiments. (b) Fraction 1a; ³H-DNA (●), 3,661 counts/min (c) Fraction 2a; ³H-DNA (●), 4,429 counts/min. (d) Fraction 3a; ³H-DNA (●), 4,932 counts/min. The arrows mark the original position of the undenatured R222 DNA.*

size of R222 DNA as determined by other methods is about 6×10^7 daltons (6) which corresponds to the value obtained here.

The finding of open circular and linear double-stranded DNA molecules by electron microscopy, whose linear form has a calculated sedimentation coefficient of 44*S* (18), and the finding in sucrose gradients of a 46*S* peak that often has a prominent, fast sedimenting, partially resolvable shoulder are considered to support the interpretation that the 46*S* peak represents the linear molecules observed and that the material in the partially

FIG. 5. *Electron micrograph of DNA isolated from minicells derived from P678-54 (R222)⁺. The isolation and preparation of DNA and electron microscopy were done precisely as previously described (11, 15). Open circular molecules (31.0 ± 0.5 μm) and twisted circular molecules are shown.*

resolvable 52*S* shoulder represents the open circular molecules (7). This interpretation is further supported by the observation that significant amounts of material sedimenting slower than 46*S* are not seen. The 77*S* twisted circular DNA, the only significant twisted circular DNA identified in the SSC-sucrose gradients which sediments about 1.6 times faster than the 46*S* peak, sediments in a position at the ionic strength used that is consistent with its being the twisted circular form of linear 46*S* DNA (2, 19).

As no radioactive minicell DNA was found unless R222 was carried by P678-54 and as the analysis of that DNA (Fig. 1–5) showed that it had properties different from *E. coli* DNA and similar to R222 DNA, it is assumed the DNA in the P678-54 (R222) minicells is R222 DNA. The manner by which the R222 DNA entered the minicells from the parental cell will be covered in the Discussion.

On the extent of replication of R222 DNA in minicells. Experiments involving 5-bromouracil density labeling have been conducted to determine if R222 DNA undergoes one and possibly two complete rounds of replication in minicells as Col E1 DNA does (11). The results shown in Fig. 6 indicate that replication over a 2-hr period did not lead to the appearance of significant amounts of DNA that had shifted through a half heavy to a heavy position though many replicating molecules had undergone significant amounts of replication as is demonstrated by the density shift. The results of several such experiments lasting as long as 3 hr indicate that most R222 DNA is not undergoing more than one full round of replication under the conditions used.

Acridine orange inhibition of R222 and Col E1 DNA synthesis in minicells. It has been reported that the sex factor (F) of *E. coli* can be efficiently eliminated from its host by growing the cells in the presence of acridine orange (8). The maintenance of Col E1 in its host is reportedly unaffected by the presence of acridine orange (3), whereas that of R factor is only slightly affected (20, 21). As indirect evidence indicates that the effect of acridine orange on the F factor is the inhibition of its replication (9, 23), the effect of that dye on R222 and Col E1 DNA replication was determined. The experiment performed involved labeling plasmid DNA in minicells with ^{3}H-thymidine for approximately 20 min, removing the radioactive label, and adding 5-bromouracil with or without acridine orange. Continuing replication of the labeled plasmid DNA in the presence of 5-bromouracil should result in an increase in its density, whereas inhibition of replication by acridine orange should interfere with such a density shift. The results (Fig. 7) indicate that the replication of both Col E1 and R222 DNA is inhibited by the presence of

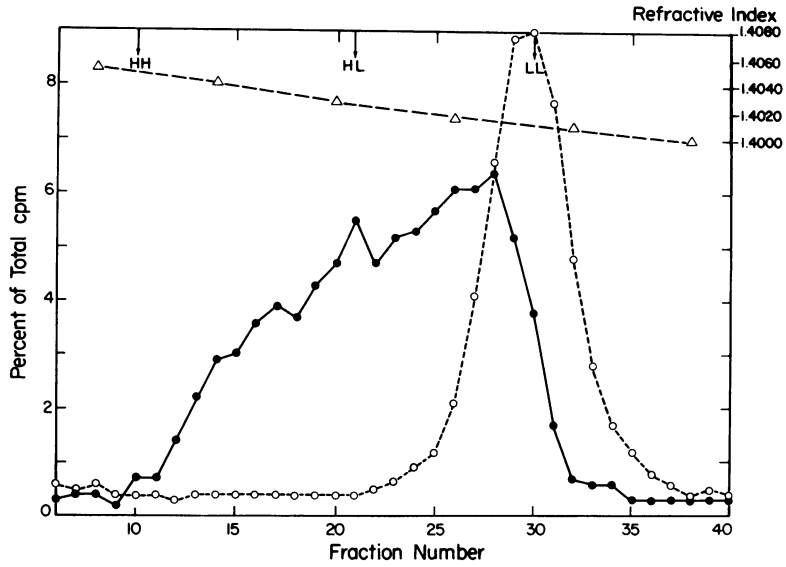

FIG. 6. *Cesium chloride density gradient analysis of DNA replicated in minicells in the presence of 5-bromouracil. Purified minicells derived from P678-54 (R222)$^+$ containing 1300 viable cells/ml were shaken at 37 C in supplemented TCG medium containing 10 μg of 5-bromouracil per ml, 15 μg of fluorodeoxyuridine per ml, 250 μg of deoxyadenosine per ml, and 50 μCi of ^{3}H-thymidine per ml for 120 min. The DNA was extracted and analyzed for an increase in density in a cesium chloride density gradient made with an original density of 1,720 g/cm^3. ^{3}H-5-bromouracil minicell DNA (●), 21,025 counts/min; ^{32}P-E. coli DNA (○), 10,089 counts/min. The positions for full heavy (HH), half heavy (HL), and light (LL) DNA are noted.*

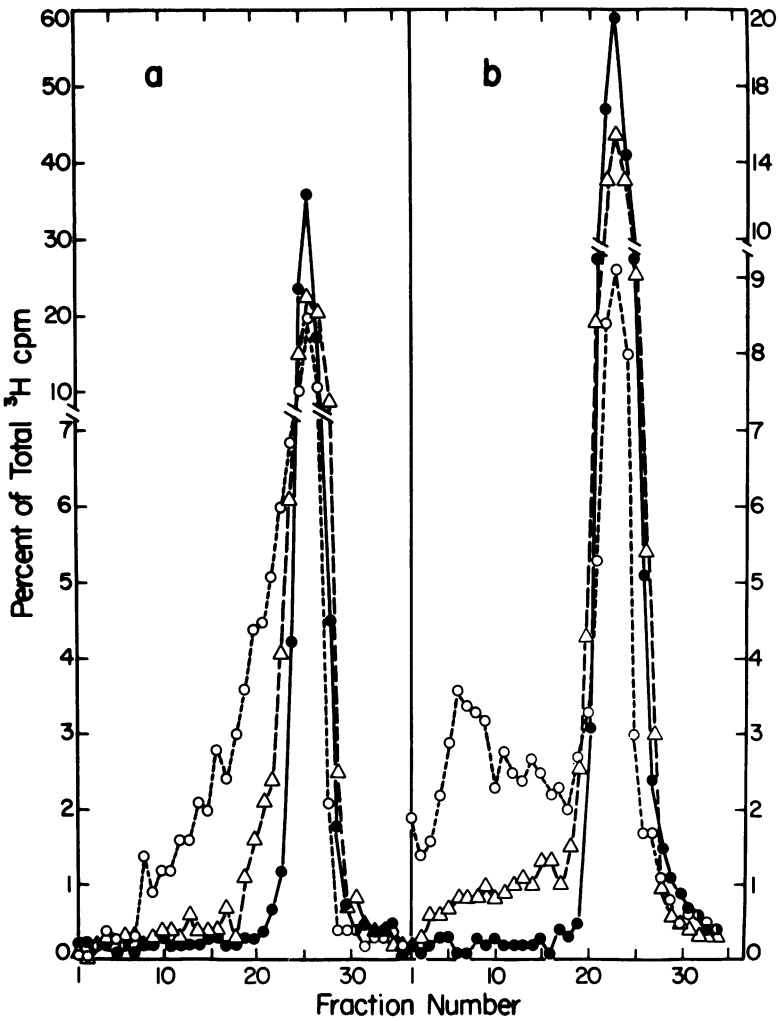

FIG. 7. *Effect of acridine orange on plasmid DNA replication in minicells. Minicells carrying either R factor R222 derived from P678-54 (R222)⁺ or Col El factor derived from P-678-54 (Col El)⁺ were incubated for 20 min in supplemented TCG medium (pH 7.9) containing thymine, 1 μg/ml; ³H-thymidine, 10 μCi/ml; deoxyadenosine, 250 μg/ml. They were then washed free of ³H-thymidine and either lysed immediately or transferred to supplemented TCG medium containing 10 μg of 5-bromouracil per ml, with or without 50 μg of acridine orange per ml. The 5-bromouracil-containing samples were incubated for an additional hour. DNA was extracted from the three different samples and spun in cesium chloride density gradients made to a density of 1.720 g/cm³. (a) R222-³H-DNA labeled for 20 min in minicells with no further treatment (●), 8,691 counts/min; or further incubated with 5-bromouracil with no acridine orange (○), 7,417 counts/min, or with 50 μg of acridine orange per ml (△), 6,553 counts/min. (b) Col El-³H-DNA labeled for 20 min in minicells with no further treatment (●), 13,164 counts/min; or further incubated with 5-bromouracil with no acridine orange (○), 14,845 counts/min, or with 50 μg of acridine orange per ml (△), 10,374 counts/min.*

acridine orange. If the per cent of the radioactive DNA that displays a density shift in the presence of 5-bromouracil without acridine orange is considered to represent the maximal continuing replication (100%), then in the presence of 50 μg/ml of acridine orange it was found that 75% of either the R222 or Col E1 DNA which replicated in the control was inhibited from replicating or was possibly degraded after replication. In the same medium with the same acridine orange concentration fewer than 1% of R222 carrying P678-54 were cured of R222, and fewer than 0.1% of Col E1 carrying strains were cured of Col E1. The implication of these results will be discussed later.

DISCUSSION

The presence of R222 DNA in the minicells may either be due to the segregation of the R222 genome into the forming minicells or to the conjugal transfer of the genome. Two observations support the interpretation that R222 segregates into minicells. (i) In previous reports of conjugal transfer of DNA to minicells significant amounts of single-stranded DNA were detected on cesium chloride density gradient centrifugation (4). No single-stranded DNA in minicells has been observed in these studies. (ii) The growth conditions used in this work, which involve vigorous agitation of the cultures in TCG medium, were found to reduce the level of conjugal transfer of R222 to normal cells to less than one donor cell in 10^5. As suspensions of about 10^{12} minicells per ml were used in preparing DNA for electron microscopy, if DNA were transferred by conjugation to minicells, no more than about 10^7 minicells per ml would be expected to contain R factor. The finding that most R factor DNA did not undergo much more than one round of replication indicates there should still have been about 10^7 R222 DNA molecules per ml of solution. As the concentrations of DNA in the electron microscope samples were reported to be greater than 0.1 μg/ml as estimated from the numbers of molecules observed (M. Fuke, *personal communication*), there were at least 10^9 DNA molecules per ml (molecular weight, 6×10^7 daltons). This value is about 100 times greater than that expected for the simple conjugal transfer of R222 to minicells using the efficiencies noted for the conjugal transfer of the R222 to normal cells.

In recent years a number of studies have been successfully directed toward examining the structure of R factor DNA in *Proteus mirabilis* and more recently in *E. coli* (5, 14, S. N. Cohen and C. A. Miller, Bacteriol Proc., p. 61, 1970; L. Tompkins et al., Bacteriol Proc., p. 61, 1970). The results reported here are consistent with those works.

In a paper (13) that appeared during this manuscript's preparation, the observation of the presence of radioactive DNA in minicells derived from an R222-carrying strain and not from a strain not carrying R222 was noted and the interpretation made of the segregation of R222 DNA into minicells. The data presented here provide some necessary data that both characterize such minicell DNA as R222 DNA and permit the contention that its entry into minicells is most probably by segregation.

Regarding the mechanism by which acridine orange cures cells of F factor, the significant though incomplete inhibition of R222 and Col E1 replication by acridine orange without causing significant curing of cells suggests that the effect of the dye on F factor replication may be more complete than that found for R222 or Col E1. A second possibility is that the distribution process of F to progeny cells (which may depend on the total number of replicating F factors per cell) is more sensitive to the same degree of replication inhibition found for R factor and Col E1. Answers to this problem should shed considerable light on the behavior of extrachromosomal genetic elements in *E. coli*.

It seems reasonable to conclude that the segregation of R factor DNA into minicells occurs and that the minicell system appears to permit a fuller study of what may be a rather complicated behavior of the R factor molecule.

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