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Molecular Nature of R-Factor Deoxyribonucleic Acid Isolated from *Salmonella typhimurium* Minicells

RONALD J. SHEEHY,[1] ANDERSON PERRY, DAVID P. ALLISON, AND ROY CURTISS III[2]

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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In earlier reports it was shown that a variety of extrachromosomal elements harbored in the *Escherichia coli* minicell producer segregate into the minicells. We show in this report that the fi$^+$ R factor R-100-1 (derepressed derivative of R-100) similarly segregates into minicells produced by a *Salmonella typhimurium* strain. Four distinct classes of covalently closed circular deoxyribonucleic acid molecules are found in minicells derived from the R$^+$ *Salmonella* minicell producer. The sum of the average molecular weights or contour lengths of the circular molecules in two of the classes is equal to the average molecular weight or contour length of those in a third class. The data suggest that R-100-1 dissociates into resistance determinants (i.e., genes that specify the molecules that confer resistance) and the resistance transfer factor (i.e., genes responsible for the transferability of the R factor to a recipient). In contrast, only one molecular species is found in minicells derived from the R$^+$ (R-100-1) *Escherichia coli* minicell producer. The fourth size class consists of small covalently closed circles (minicircles), which were originally found in the R$^-$ *Salmonella* minicell producer and are shown in this report to be enhanced in number in R$^+$ *Salmonella* minicells.

Resistance factors (R factors) are extrachromosomal elements with two outstanding functions. They render their host resistant to a variety of antibiotics, and they promote the transfer of genes conferring resistance to antibiotics to strains that lack them. On the basis of genetic data, E. S. Anderson (1) first proposed that "under suitable conditions" many R factors dissociate physically into resistance (r) determinants, i.e., genes that specify the molecules that confer resistance, and the resistance transfer factor (RTF), i.e., genes responsible for transferability of the R factor to a recipient. Recently, Anderson's initial observations in *Salmonella typhimurium* (29AST) have been confirmed by further genetic experiments (2) and by molecular studies (19).

R-factor dissociation has also been observed in *Proteus mirabilis* harboring the fi$^+$ R factors R-1 (6, 12), R-6 (6), and R-100 (27), the latter being referred to by some authors as either R-222 (11, 21) or NR1 (27). In *Proteus*, the fi$^+$ R factors R-1 and R-6 have been reported to consist of three independent species of covalently closed circular deoxyribonucleic acid (DNA) with molecular weights of 65×10^6, 55×10^6, and 9.5 to 12×10^6 (6). Studies on R-1 and R-6 indicate that the species with molecular weight 55×10^6 is the RTF, and the component with molecular weight 9.5 to 12×10^6 represents the r genes (7, 30). In contrast to these results in *Proteus*, R-1 and R-6 in *Escherichia coli* are manifested as a single molecular species (5) with a molecular weight of 65×10^6 (7, 29). The single species of R-factor DNA observed in *E. coli* apparently represents a composite of the 55×10^6 species and the 9.5 to 12×10^6 species found in *Proteus*. Similar studies have been performed on *P. mirabilis* harboring R-100, in which R-100 has been shown to dissociate into two components with molecular weights of 50 to 54×10^6 and 10 to 12×10^6 (11, 20). However, a single molecular species with a molecular

[1] Present address: Department of Microbiology, The Public Health Research Institute of New York, 455 First Avenue, New York, N. Y. 10009.

[2] Present address: Department of Microbiology, The University of Alabama in Birmingham, Birmingham, Ala. 35294.

weight of 64 to 68 $\times$ 10^6 has been reported for R-100 in *E. coli* (21, 27) and in *Serratia marcesens* (27). The relative sizes and functions of the different R-100 components are comparable to the previously described components of the R-1 and R-6 factors.

In *E. coli* and *S. marcesens*, R-factor replication is under stringent (11) control and is coordinated with host cell division, and R-factor DNA makes up about 4% of the total extractable DNA (6, 21). In contrast, when R factors are present in *Proteus*, R-factor DNA may constitute up to 50% of the total extractable DNA (22). In addition, the numbers of copies of the three molecular species of R-100 in *Proteus* depend on both the stage of the bacterial growth cycle and the presence or absence of antibiotics in the culture (22, 26). Two models have been presented to explain the control of R-100 replication in *Proteus*. Rownd et al. (26) have suggested that the RTF and the r determinants dissociate and reassociate to provide a novel mechanism for regulating the number of r determinants. In contrast, Punch and Kopecko (22) have proposed that the number of individual R-factor components is subject to differential regulation of replication by positive and negative effectors.

It has been shown that a variety of extrachromosomal elements harbored in the *E. coli* minicell producer segregate into the minicells (13, 14, 17, 24). We show in this report that an fi^+ R factor, R-100-1 (derepressed derivative of R-100), similarly segregates into minicells derived from a strain of *S. typhimurium*, and that R-100-1 probably dissociates into RTF and r determinant components in this strain.

MATERIALS AND METHODS

Bacterial strains and R factors. The *S. typhimurium* minicell-producing strain (χ1313) was isolated by Tankersley (W. L. Tankersley, master's thesis, University of Tennessee, Knoxville, Tenn., 1970). It is prototrophic and is lysogenic for three temperate phages that infect *S. typhimurium* LT 2 (χ9) (28). One of these phages is serologically related to phage P22. The R factor R-100-1, a derepressed derivative of R-100 (10), which confers resistance to streptomycin (Str), spectinomycin (Spc), chloramphenicol (Chl), sulphonamide (Sul), and tetracycline (Tet), was obtained from Y. Nishimura (Department of Biology, Osaka University, Toyonaka, Osaka, Japan). The R-factor-containing *Salmonella* minicell-producing strain (χ1332) was isolated after a 12-h mating period of χ1266 (*E. coli* K-12 R-100-1/*his* str^s) and *Salmonella* χ1313 at 35 C in Luria broth by plating on minimal media (8) containing glucose (0.5%), Str (200 μg/ml), Spc (100 μg/ml), Tet (25 μg/ml), and Chl (25 μg/ml). The *S. typhimurium* strain isolated (χ1332) was maintained at 4 C on Penassay agar slants containing Chl (25 μg/ml) and Str (200 μg/ml). The R^+ *E. coli* minicell producer used in these studies was χ1100 (R-100-1/*thr leu lacY minA gal minB* str^r *thi*).

Culture conditions and isolation of minicells. Log-phase cultures of minicell-producing strains were inoculated 1:100 into 30 ml (or multiples thereof) of a minimal salts solution (8) supplemented with Casamino Acids (1.5%), glucose (0.5%), and, when required, thiamine hydrochloride (2 μg/ml). Such cultures were incubated at 37 C in a New Brunswick rotary shaker cabinet. After about 2 h, 5 to 20 μCi of tritiated thymidine ([^{3}H]dThd) and 200 μg of adenosine were added per ml, and cultures were further incubated at 37 C.

Minicells were harvested from cultures after approximately 12 h of growth by centrifugation at 10,000 rpm in a Sorvall SS34 rotor at 4 C for 15 min. The pellet containing minicells and cells was suspended in 9 ml of buffered saline with gelatin (BSG) (8) by Vortex mixing. Samples (3 ml) were layered over 35 ml of sterile 5 to 20% (wt/vol) linear sucrose (made up in BSG) gradients and centrifuged at 4,000 rpm for 15 min in a Spinco SW27 swinging-bucket rotor in a Beckman L3-50 ultracentrifuge. Minicell bands were withdrawn from the gradients with a syringe, slowly diluted with cold BSG, and pelleted by centrifugation at 15,000 rpm for 15 min in a Sorvall SS34 rotor. Pellets were suspended in cold BSG, absorbancy at 620 nm was measured, and samples were assayed for contaminating bacterial cells by plating. After one sucrose gradient, the contaminating cells were present at ratios of 1 cell per 10^2 to 10^3 minicells (partially purified minicells). Purified minicell preparations (1 cell per 10^4 to 10^5 minicells) were obtained after a second sucrose gradient centrifugation. It should be noted that overnight cultures of the *Salmonella* strain have a higher proportion of smaller cells that contaminate the minicell preparations than do overnight cultures of the *E. coli* minicell producer χ925. We have found, however, that minicells from log-phase cultures of the *Salmonella* strain can be purified by two successive sucrose gradient centrifugations to the same level of purity achieved for minicell preparations from the *E. coli* strain (1 cell per 10^6 minicells).

Alkaline sucrose, neutral sucrose, and ethidium bromide-CsCl centrifugation. The procedures for lysis of minicells and sedimentation analysis of [^{3}H]dThd-labeled DNA by alkaline sucrose, neutral sucrose, and ethidium bromide-CsCl (EtBr-CsCl) gradient centrifugation were as previously described (28). Lysates from purified minicells were used for neutral and alkaline sucrose centrifugations and lysates from partially purified minicells for EtBr-CsCl centrifugation. Recovery of radioactivity placed on neutral and alkaline sucrose gradients was 95% or more due to use of glycerol-coated polyallomer tubes. Recovery was 85% or more from EtBr-CsCl gradients.

Electron microscopy. Covalently closed circles were isolated from minicells by dye buoyant-density centrifugation in EtBr-CsCl density gradients. Fractions in the highest density peak were pooled, ethidium bromide was extracted with isopropanol,

and the DNA was dialyzed against 0.5 M ammonium acetate buffer containing 1 mM ethylenediaminetetraacetic acid and adjusted to a pH of 6.0 with acetic acid. A substantial fraction of the covalently closed circles were converted to open circles during dialysis. The DNA was prepared for electron microscopy according to the protein monolayer technique (15) by mixing 0.1 ml of dialyzed DNA with 1 μliter of cytochrome *c* and spreading the mixture onto a 0.25 M ammonium acetate (*p*H 7.5) hypophase. For very small amounts of closed circular DNA, prepared by neutral sucrose gradient centrifugation, the microversion of the Lang spontaneous absorption method (16) was used. Samples were picked up on Parlodion-coated 400-mesh copper grids, stained with uranyl acetate (9), and shadowed with platinum at an angle of 6°. Electron micrographs were made with a Siemens Elmiskop I electron microscope (calibrated with a diffraction grating replica Fullam 54,864 lines per inch) at a magnification of ×3,870. Contour measurements were made by projecting the negatives on a screen, tracing the DNA molecules, and measuring lengths from the tracings with a curvimeter.

RESULTS

Alkaline sucrose gradient centrifugation of [^{3}H]dThd-labeled DNA in purified R$^+$ and R$^-$ Salmonella minicells. It has been demonstrated that when DNA is isolated from a variety of bacterial strains containing R factors a substantial fraction of the R-factor DNA consists of covalently closed circular (CCC) molecules (5, 20), which sediment faster in alkaline sucrose than do the corresponding single-strand circles or linear molecules. Figure 1 presents data obtained by alkaline sucrose gradient centrifugation of [^{3}H]dThd-labeled DNA from purified R$^+$ and R$^-$ *Salmonella* minicells. Both minicell preparations were adjusted to the same volume and absorbancy at 620 nm before lysis and centrifugation. The R$^+$ minicell preparation shows fast-sedimenting material between fractions 6 and 12, which represents CCC R-factor DNA (24). This fast-sedimenting material is absent in a similar preparation from the R$^-$ minicells. Single-strand circular R-factor DNA and its linear counterpart are present at the top of the gradient. In addition, a small cryptic DNA species (molecular weight about 2.5 × 10^6), which has been shown previously to be present in R$^-$ *Salmonella* minicells (28), also is present in the R$^+$ minicells and appears at the top of the gradient in both preparations. Thus, the segregation of R-factor DNA into minicells derived from the *Salmonella* strain, is similar if not identical to segregation of R-factor DNA into minicells derived from the *E. coli* K-12 minicell producer (17, 24).

Dye buoyant-density equilibrium centrifugation of [^{3}H]dThd-labeled DNA in R$^+$ and R$^-$ Salmonella minicells. Figure 2 presents data obtained by pycnographic separation of [^{3}H]dThd-labeled DNA extracted from R$^+$ and R$^-$ *Salmonella* minicells. The presence of CCC

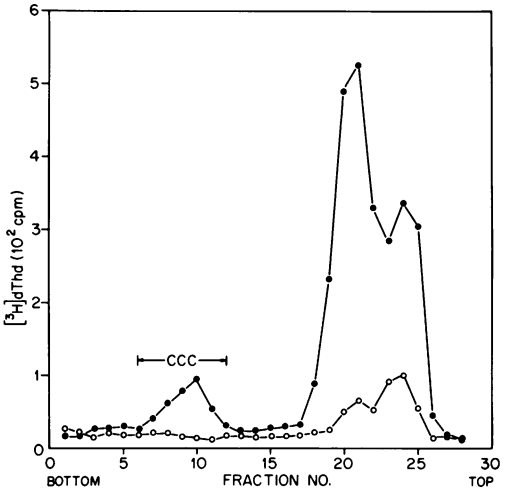

FIG. 1. *Alkaline sucrose gradient centrifugation of [^{3}H]dThd-labeled DNA from R$^+$ (●) and R$^-$ (○) Salmonella minicells. Salmonella minicells were labeled, purified, and lysed, and the DNA was sedimented in alkaline sucrose gradients as previously described (28).*

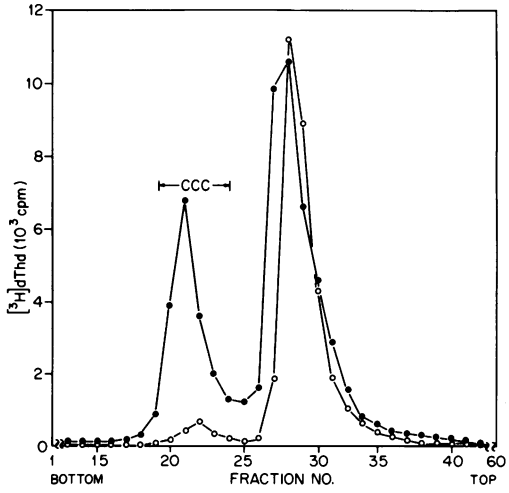

FIG. 2. *EtBr-CsCl density gradient centrifugation of [^{3}H]dThd-labeled DNA from R$^+$ (●) and R$^-$ (○) Salmonella minicells. The minicell-producing parents were grown and labeled as described in Materials and Methods. EtBr-CsCl centrifugation was performed as previously described (28).*

DNA in the minicells is further substantiated by this technique, since CCC DNA bands at a higher density than open circular (OC) DNA with a nick in one strand or linear DNA when centrifuged to equilibrium in EtBr-CsCl density gradients (23). Closed circular plasmid DNA appears in fractions 19 to 24, and OC and linear DNA in fractions 25 to 33. The difference in the amount of radioactivity in the CCC peak of R^+ and R^- minicell preparations of the same volume and absorbancy at 620 nm is attributed to the presence of CCC R-factor DNA and the corresponding enhancement in production of a small cryptic CCC element, which also segregates into R^+ minicells. Two cryptic plasmids have been found in the R^- minicell preparation, one that rarely segregates (less than 1 in 300 minicells) into the minicells (molecular weight about 130×10^6) and one that frequently segregates (10 copies per minicell) (molecular weight about 2.5×10^6) (28).

Electron microscopy of DNA obtained from R^+ S. typhimurium and R^+ E. coli minicells. CCC DNA was obtained from a pool of the four to five fractions at the peak of the highest density band after dye buoyant-density gradient centrifugation of [^{3}H]dThd-labeled DNA from "partially purified" R^+ minicells. DNA was prepared for electron microscopy, and contour lengths were measured as described in Materials and Methods. Four distinct size classes of DNA were found in preparations from *S. typhimurium* minicells, whereas only one size class was detected in a similar preparation from *E. coli*. In *Salmonella*, molecules with an average contour length of 26.3 μm (molecular weight about 51×10^6) were designated as Class I (Table 1, Fig. 3A). These molecules were of the same relative size as those found in *E. coli*, also designated Class I (Table 1). Class II (Table 1, Fig. 3B) consists of molecules with an average contour length of 19.9 μm (molecular weight about 39×10^6). Class III (Table 1, Fig. 3C) consists of molecules with an average contour length of 5.7 μm (molecular weight about 11×10^6). The sum of the average molecular weights or contour lengths of the molecules in Classes II and III closely approximates the average molecular weight and contour length of the molecules in Class I. These data suggest that molecules in Class I represent a composite of molecules in Classes II and III. In an examination of 33 randomly selected electron micrograph fields, there were a total of 309 molecules representative of Classes I, II, and III. Of the 309 molecules (CCC and OC), 94 were measurable (OC); and of the 94, 55.3% were in Class I, 34.7% were in Class II, and 10% were in Class III. In addition, 1,968 molecules with an average contour length of 1.31 μm (molecular weight about 2.5×10^6), designated Class IV (Table 1, Fig. 3A–C), were counted in the 33 electron micrographs.

Comparison of [^{3}H]dThd-labeled DNA in R^+ and R^- Salmonella minicells by neutral sucrose gradient centrifugation. Figure 4 presents data from neutral sucrose gradient centrifugations of [^{3}H]dThd-labeled DNA from R^+ and R^- minicells. Both minicell preparations were adjusted to the same volume and absorbancy at 620 nm before lysis and centrifugation. Sedimentation coefficients for the DNA in Fig. 4 were obtained by cosedimentation with marker [^{14}C]dThd-labeled M13 phage DNA (28*S*) and using the direct proportion relationship of distance sedimented to the sedimentation coefficient (4).

The 20*S* DNA extracted from the R^+ minicells accounts for 47% of the [^{3}H]dThd counts on the gradient and represents a threefold increase over similar sedimenting DNA extracted from the R^- minicells. We therefore conclude that the 20*S* material is enhanced in R^+ minicells as compared to R^- minicells. An *S* value of 20 corresponds to a molecular weight of 2.8×10^6, according to the formula of Bazaral and Helinski (3), which relates sedimentation coefficients to molecular weights for the similar-sized ColE1 plasmid. This value is in general agreement with the molecular weight calculated from contour length (Table 1).

CCC DNA sediments 1.25(32) to 1.5(6) times faster than OC DNA in neutral sucrose, and OC DNA sediments 1.14 times faster than linear DNA (32). From the above data the theoretical *S* value for linear duplex DNA can be calculated. The *S* value thus calculated can be used to calculate the molecular weight for linear duplex DNA according to the formula of Studier (31). Therefore, R-factor CCC DNA with sedimentation coefficients of 64*S* to 53*S* (Fig. 4) have molecular weights of 58×10^6 to 33×10^6, which is in general agreement with molecular weights calculated from contour lengths for Class I and II molecules (Table 1). DNA molecules sedimenting between 48*S* and 38*S* probably represent the OC form of Class I and II molecules. The presence of twisted circles (CCC) or open circles in this regard was verified by electron microscopy.

Only a very small number of Class III molecules were found in the electron micrographs, so they would not have given a distinguishable peak on the gradient (Fig. 4). In addition, the theoretical *S* values for CCC molecules of mo-

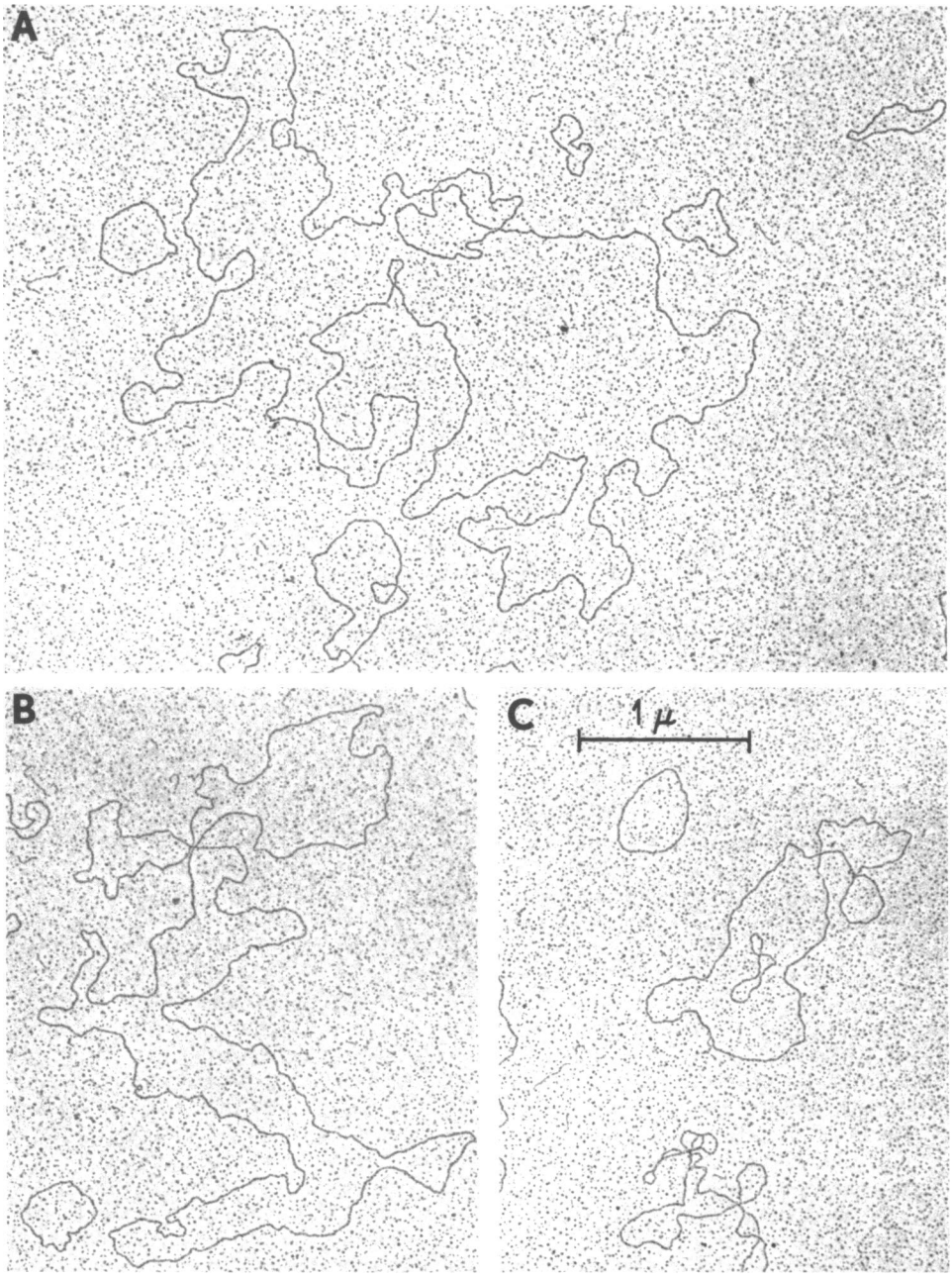

FIG. 3. *Electron micrographs of circular DNA isolated from partially purified Salmonella minicells on EtBr-CsCl density gradients, as described in the legend to Fig. 2. The DNA was prepared for electron microscopy as described in Materials and Methods. (A) OC DNA molecule about 26.3 μm; (B) OC DNA molecule about 19.9 μm; (C) OC DNA molecule about 5.7 μm. Small (about 1.3 μm) open circles are present in A, B, and C.*

lecular weights 10×10^6 to 12×10^6 are 33.6 to 36.4*S* (3), for OC molecules 25.8 to 28.0*S*, and for linear molecules 22.6 to 24.5*S*. The absence of distinguishable peaks of radioactively labeled DNA sedimenting at these *S* values further substantiates the above conclusion.

TABLE 1. *Contour lengths and molecular weights of circular DNA isolated by dye buoyant-density gradient centrifugation from S. typhimurium and E. coli minicells*

Strain	Class	Contour length (μm ± standard deviation)	No. molecules measured	Molecular weight[a] (10^6)
S. typhimurium χ1332 (R-100-1)	I	26.3 ± 1.0	52	51.5
		24.8 (min)	3	48.7
		28.8 (max)	2	56.6
	II	19.9 ± 0.8	32	39.0
		18.3 (min)	2	35.9
		22.3 (max)	1	43.7
	III	5.7 ± 0.5	10	11.2
		4.8 (min)	1	9.5
		6.2 (max)	3	12.3
	IV	1.3 ± 0.1	216	2.5
		1.2 (min)	5	2.2
		1.7 (max)	4	3.4
E. coli χ1100 (R-100-1)	I	28.2 ± 0.3	20	55.2
		27.1 (min)	1	53.1
		29.5 (max)	1	57.8

[a] Calculated on the basis of a mass of 1.96×10^6 daltons per μm (18).

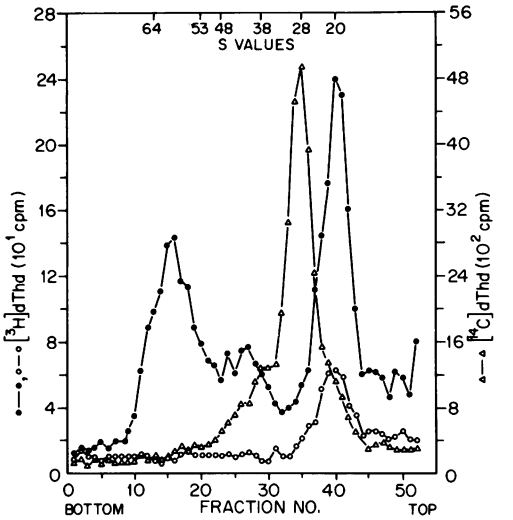

FIG. 4. *Neutral sucrose gradient centrifugation of [3H]dThd-labeled DNA from S. typhimurium minicells. The minicell-producing parents were cultured and harvested as described in Materials and Methods. Neutral sucrose gradient centrifugation was performed as previously described (28). R^+ S. typhimurium minicells, ●; R^- S. typhimurium minicells, ○; [14C]dThd-labeled marker M13 phage DNA (28S), △.*

DISCUSSION

The production of minicells by a strain of *S. typhimurium* (χ1332) has been used in this study to investigate the molecular nature of a resistance transfer factor (R-100-1). Minicells are easily separated from the cells that produce them, normally lack chromosomal DNA, but often contain extrachromosomal DNA so they are useful for the isolation and characterization of any DNA that they contain.

We have presented data which show that when the *S. typhimurium* minicell producer contains R-factor DNA it is often segregated into the minicells. The segregation of extrachromosomal elements into minicells derived from *Salmonella* is therefore similar, if not identical, to the segregation of a variety of extrachromosomal elements into minicells derived from the *E. coli* minicell producer.

R-100, the repressed parent of R-100-1, has been extensively studied (22, 26). It dissociates into its component parts (RTF and r determinants) in *Proteus* but exists as one composite molecule in *E. coli* (20, 27). The isolation of CCC DNA from R^+ (R-100-1) *Salmonella* minicells and subsequent analysis of this DNA by electron microscopy revealed four distinct size classes of circular DNA molecules, with average molecular weights (in daltons) as follows: (I) 51 $\times 10^6$, (II) 39 $\times 10^6$, (III) 11 $\times 10^6$, and (IV) 2.5 $\times 10^6$. The sum of the molecular weights of the DNA in Classes II and III closely approximates the molecular weight of the DNA in Class I. We suggest that molecules in Class I represent the composite R factor. This suggestion is based on the close approximation of the molecular weight of Class I molecules with the single species of R-100-1 DNA molecules found in minicells derived from *E. coli* (χ1100). The reported molecular weight of the r determinant of R-100 is 10 to 12 $\times 10^6$ (11, 20). The molecules in Class III are in this molecular weight range, and therefore are suggested to be r determinants. Accordingly, the molecules in Class II represent the RTF component. The difference between the molecular weight of the RTF from R-100-1 identified here (39 $\times 10^6$) and that of the RTF from R-100 identified in *Proteus* (52 $\times 10^6$) (11, 20) is accounted for by a deletion in the RTF component of R-100-1. R-100-1 was isolated as a derepressed derivative of R-100 after mutation induction by ultraviolet irradiation (10).

Since about 75% of the minicells harvested from stationary-phase cultures are produced in the last two generations, our data suggest that in late-log-phase cultures of *Salmonella* harboring R-100-1 that are cultured in the absence of antibiotics, 55% of the CCC R-factor DNA

molecules occur as the composite R factor, 35% as the RTF and 10% as the r determinant. In *Proteus* harboring R-1 and R-6, the composite R-factor component is reported to exist in negligible amounts in stationary-phase cultures but to be present in significant amounts in log-phase cultures (6, 22). In *Salmonella* minicells, however, the detection of a greater percentage of composite than RTF molecules in late log phase could indicate that dissociation is incomplete at this growth stage, or that dissociation is regulated only to produce a certain amount of r-determinant and RTF molecules, or both. The small number of presumptive r-determinant molecules detected in proportion to RTF molecules is also perplexing. Nonrandom segregation, changes in the proportion of CCC, OC, and linear forms for different size elements (since only CCC molecules were isolated on EtBr-CsCl gradients and characterized), and effects of replication, association, and/or dissociation after the minicells are produced make the final percentage of any component isolated from minicells difficult to interpret.

We have obtained similar results indicating dissociation of the I-like plasmid R-64-11 to those reported for R-100-1 and have also conducted preliminary studies with both plasmids that suggest that antibiotics during growth of the cultures stimulates R-factor dissociation. It is evident, however, that the presence of the cryptic minicircular DNA in *Salmonella* minicells (28) and the increased amount of this type of DNA in R^+ minicells makes reaching definitive conclusions using this system difficult. We are therefore endeavoring to "cure" the *Salmonella* minicell-producing strain of its cryptic plasmids and temperate phages. If successful, this "cured" strain should be extremely useful for studies on the molecular biology and genetics of plasmids in a pathogenic species.

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