*A MINUTE CIRCULAR DNA FROM ESCHERICHIA COLI 15**

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In the course of preparing the intracellular replicative form of phage ϕX174 DNA (RFI),[1, 2] we discovered an even smaller duplex circular DNA. This new DNA, synthesized by uninfected as well as infected cells of *Escherichia coli*, may be either an episome or a plasmid.[3] For lack of evidence indicating its integration into the chromosome, we regard it provisionally as a plasmid. In this report we describe some of the physical properties of this DNA and its distribution among strains of *E. coli*. The function of this plasmid remains unknown.

Experimental Procedure.—Materials: Sources were as follows: CsCl (optical grade) was obtained from Harshaw; Brij 58, a nonionic detergent, from Atlas Chemical; Sepharose 4B from Pharmacia; egg-white lysozyme from Calbiochem; pancreatic DNase from Worthington; H^3-deoxythymidine from New England Nuclear Corp.; and ethidium bromide, a gift from Boots Pure Drug Co., Nottingham, England.

Methods: Cells were labeled with H^3-thymidine ($1–2 \times 10^7$ cpm/μmole) by growth in Fraser and Jerrel's glycerol-casamino acids medium;[4] cells labeled with P^{32} ($5–9 \times 10^8$ cpm/μmole) were grown in a medium described by Hershey,[5] supplemented with a mixture of the 20 natural amino acids. Cultures were harvested in the logarithmic phase at an A_{590} of 1.5–2.0.

Cytoplasmic DNA was released from cells by the following modification[6] of the Godson and Sinsheimer lysis procedure.[7] To about 5×10^{10} cells suspended in 1 ml of 25% sucrose, 0.05 *M* Tris HCl, pH 8.0, were added 0.2 ml of lysozyme (5 mg/ml in 0.25 *M* Tris HCl, pH 8.0) and 0.4 ml of 0.25 *M* Na_2EDTA. Conversion to spheroplasts was essentially complete after a 10-min incubation at 25°. The spheroplasts were ruptured by a further 10-min incubation at 25° with Brij added to a final concentration of 0.5%. Cellular debris and 90–98% of the total DNA were removed by sedimentation at $60,000 \times g$ for 15 min. At this stage, extracts may be analyzed for plasmid DNA by sucrose gradient sedimentation.

For the further purification of plasmid DNA, the supernatant fluid was extracted three times with redistilled phenol and the phenol removed by extraction with ethyl ether. The solution was concentrated to an A_{260} of 500 by rotary evaporation and then digested with pancreatic ribonuclease (0.3 μg/ml for 10 min at 37°) to the point where 10% of the RNA became acid-soluble. The RNA fragments were removed by filtration through a column of Sepharose 4B with a bed volume ten times that of the sample. The duplex covalently closed plasmid DNA was separated from circular duplexes bearing single-strand breaks and linear forms by banding in a solution of CsCl ($\rho = 1.57$ gm cm^{-3}) and ethidium bromide (100 μg/ml) for 36 hr or more at 40,000 rpm and 5° in the 50 rotor of the Spinco model L ultracentrifuge.[8] The last step was repeated if the product was less than 90% pure as judged by sedimentation in alkali. Ethidium bromide was removed by extraction with an equal volume of isopropanol or by passage through a Dowex-50 column.[8] The final material was dialyzed against 0.05 *M* potassium phosphate buffer, pH 7.2, and 1 mM EDTA. The yield of plasmid DNA from a 20-liter culture harvested at an A_{590} of 1.5 was about 0.4 μmole (expressed as nucleotide residues).

Sucrose gradient sedimentation of DNA samples was carried out as previously described.[9] The buffer contained: foı neutral low-salt sedimentation, 10 mM Tris HCl, pH 8.0, and 1 mM EDTA titrated to pH 8.0 with NaOH; for neutral high-salt sedimentation, 1 *M* NaCl in addition; and for alkaline high-salt-sedimentation, 0.8 *M* NaCl, 1 mM EDTA, and 0.3 *M* NaOH. Analytical determinations of buoyant density were made in

CsCl solution (ρ = 1.70 gm cm^{-3}) in the Spinco model E ultracentrifuge at 44,000 rpm. Kel-F centerpieces were employed in standard cells. Ultraviolet absorption photographs were taken at regular intervals and subsequently scanned with a Joyce-Loebl recording microdensitometer.

DNA was viewed with a Siemens Elmiskop 1A electron microscope using the protein film technique of Kleinschmidt *et al.*[10] as modified by Inman *et al.*[11] The hypophase was water. Magnification was determined from a diffraction grating of known line spacing.

Colicin 15 was assayed by the procedure of Mukai[12] with *E. coli* strain TAU[13] as the indicator and the colicin-resistant strain THU[14] as a control. The dose of UV light used to induce colicin production was 900 ergs mm^{-2}.

Results.—Duplex circular plasmid DNA: In cells of strain THU, infected with ϕX174, a form of DNA in addition to RFI of ϕX174 was discovered. This new DNA had a density in an ethidium bromide–CsCl gradient characteristic of closed circular duplex DNA and sedimented less rapidly than ϕX174 RFI. The ratios of sedimentation velocities in alkaline high-salt, neutral high-salt, and neutral low-salt sucrose gradients were 0.65, 0.76, and 0.71, respectively. DNA with these same sedimentation properties was also found in uninfected cells. Thus, this new DNA appears to be a plasmid form of host cell DNA. The ratio of the alkaline to neutral sedimentation rates for the plasmid DNA is several times that anticipated for a linear duplex[15] and is consistent with a closed circular duplex structure.[16] This supposition was tested by the action of pancreatic DNase. Purified plasmid DNA, sedimented to equilibrium in a CsCl gradient in the presence of ethidium bromide, yielded over 90 per cent of the DNA at a density value characteristic of ϕX174 RFI (Fig. 1*A*). When single-strand scissions were introduced with pancreatic DNase, the DNA shifted into the lighter peak occupied by an RFII marker (Figs. 1*B*, *C*). This effect of pancreatic DNase on the banding pattern strongly supports a closed circular duplex structure of the plasmid DNA.[8]

Contour length: The circular form of the plasmid DNA was also revealed by electron microscopy with the Kleinschmidt protein film technique (Fig. 2). The contour length was found to be 0.96 μ ± 0.13 (SD) for the plasmid DNA and 2.26 μ ± 0.13 (SD) for ϕX174 RFII included on the same grids. The two samples of DNA were mixed and spread together to provide an internal control for the variations in absolute length measured by electron microscopy.[17, 18] Based on a molecular weight of the ϕX174 RF of 3.4 × 10^6 daltons,[19] that of the plasmid DNA is calculated to be 1.45 × 10^6. The molecular length distributions shown in Figure 3, as well as the standard deviations, show that the length variation is similar for both types of DNA and thus that the cytoplasmic DNA has a rather homogeneous size distribution.

In a separate experiment in which no ϕX174 RFII standard was added, only small circles and no polymeric forms[8, 20, 21, 9, 22] were found among 93 molecules examined. Furthermore, when the cytoplasmic DNA was analyzed on an alkaline sucrose gradient, no peak of radioactivity (less than 0.3%) was detectable at the position anticipated for dimers.

Buoyant density: The buoyant densities of plasmid and T7 DNA were compared with that of poly dAT (ρ = 1.678)[23] as a standard. The plasmid DNA (ρ = 1.710) was 0.001 gm cm^{-3} lighter than the T7 DNA. Since T7 and *E. coli*

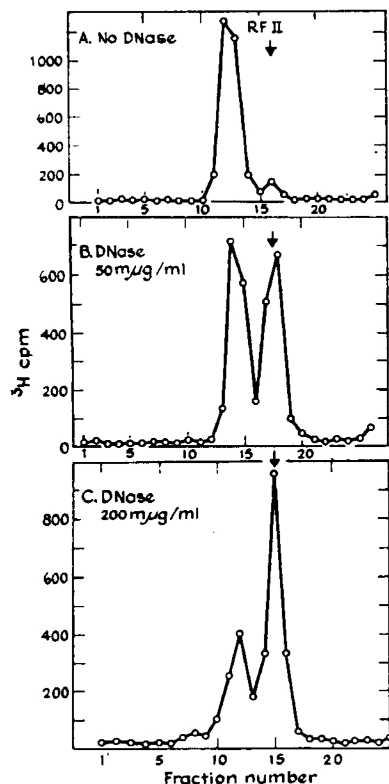

FIG. 1.—The effect of pancreatic DNase treatment on banding of plasmid DNA in a CsCl–ethidium bromide gradient. Samples were centrifuged in a 1.5-ml solution of CsCl (0.75 gm/ml), 5 mM EDTA, and ethidium bromide (100 μg/ml) at 45,000 rpm, 10°C, for 48 hr in the SB405 rotor of the International B60 ultracentrifuge. The arrows indicate the position of a marker of φX174 RFII DNA. The plasmid DNA was treated with DNase at the indicated concentrations for 60 min at 37°.

DNA have equal densities,[23] the calculated GC content[23] of the plasmid DNA is comparable to that of *E. coli* chromosomal DNA. The half-width of the plasmid DNA band was 0.005 gm cm^{-3}, which is in the expected range for a DNA of its size,[24] indicating a lack of extensive density heterogeneity.

Molecular weight: Since the relationship between sedimentation velocity and molecular weight for closed duplexes is complicated by the number of tertiary turns,[16] the sedimentation velocities of the open or "nicked" form of the plasmid DNA and of φX174 RFII were compared. To ensure that the pancreatic DNase was introducing only single-chain and not double-chain scissions into the P^{32}-labeled plasmid DNA, conditions were chosen which supported the conversion of at least 90 per cent of H^3-lableled φX174 RFI (in the same reaction tube) to the nicked circular RFII. The neutral and alkaline sedimentation velocities gave molecular weight values of 1.53–1.63 $\times 10^6$ daltons (Table 1), in good agreement with the value of 1.45 $\times 10^6$ obtained from the contour lengths.

Tertiary structure: To determine whether the closed molecules contain tertiary turns, the ratio of the neutral sedimentation rates of the closed and open forms of the plasmid DNA were compared with that for φX174 RF and with the published value for phage λDNA.[25] The presence of tertiary turns in a DNA increases its sedimentation coefficient.[16] For plasmid DNA, at both high and low ionic strengths, this ratio was greater than one, which indicates that the DNA contains tertiary turns under these conditions (Table 2).[26] The increase in the ratio of sedimentation velocities at low ionic strength was marked for λDNA, insignifi-

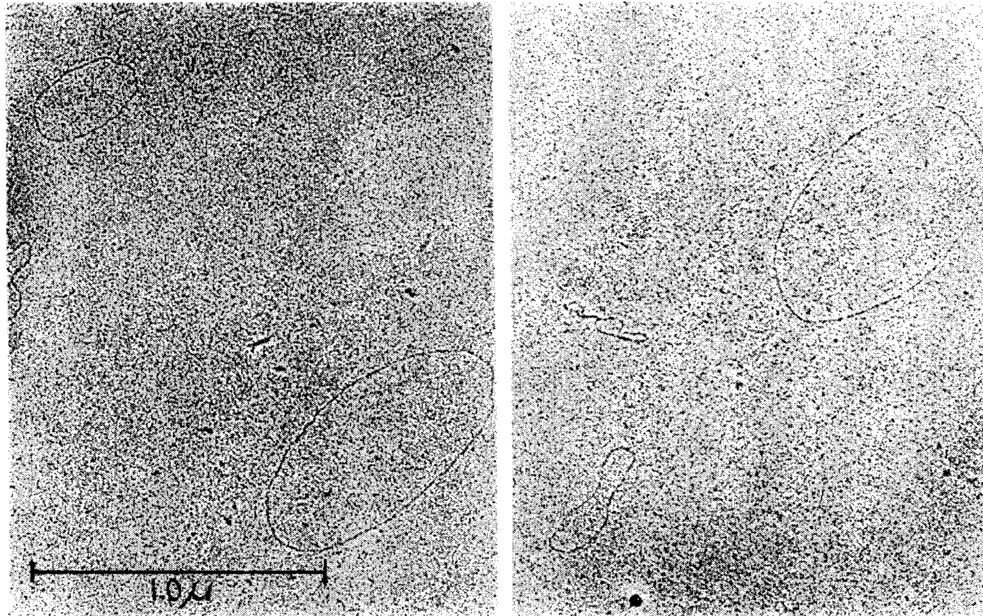

FIG. 2.—Electron micrograph of plasmid and φX174 RFII DNA. The smaller circles are the plasmid DNA, and the larger the viral RF.

cant for the plasmid, and intermediate for φX174 RF, suggesting that this ionic strength dependence is a function of molecular weight.

Number of plasmids per chromosome: This value was estimated from the fraction of the total DNA recovered as plasmid DNA in the supernatant after detergent lysis. Spheroplasts of strain THU, uniformly labeled with H^3-thymidine, were lysed with Brij, and the amount of plasmid DNA in the supernatant fluid was measured by sedimentation in an alkaline sucrose gradient with a marker of purified P^{32}-labeled plasmid DNA. Three such determinations gave percentage values for the plasmid DNA of 0.64, 0.77, and 0.80 of the total cellular DNA. Assuming a molecular weight of 2.8×10^9 for the resting *E. coli* chromosome,[27] and 1.5×10^6 for the plasmid DNA, there are, on the average, 12 to 15 copies of plasmid DNA per chromosome. Our estimate of the number of plasmids might be low because of breakage of DNA during handling, the possible natural occurrence of open forms (analogous to φX174 RFII), or incomplete extraction. The first two sources of error are probably not significant because single-stranded DNA of molecular weight 7×10^5 was not detectable by alkaline sucrose sedimentation of DNA from Brij-lysed cells. To determine whether plasmid DNA was sedimented with the host DNA after Brij lysis, the yield with this technique was compared to that after Sarcosyl lysis[28] and phenol extraction of the total cellular DNA. Both extraction procedures gave yields which differed by less than 50 per cent.

Occurrence of plasmids in various E. coli strains: The studies described up to this point were conducted with strain THU, a derivative of strain 15. Nine other strains of *E. coli* were also tested for the presence of the plasmid DNA

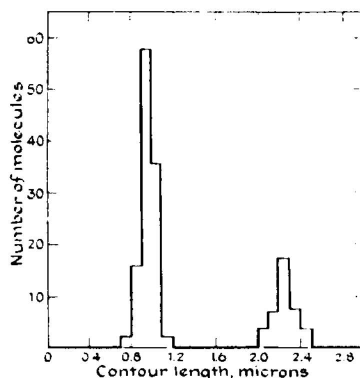

FIG. 3.—Distribution of contour lengths of plasmid DNA and ϕX174 RFII. Linear or aggregated molecules were not included in these measurements; the majority of the forms were circular.

(Table 3). H^3-thymidine-labeled cells were lysed with Brij, and the supernatant fraction was analyzed on an alkaline sucrose gradient (and in several cases also on a neutral sucrose gradient) with a marker of purified P^{32}-labeled cytoplasmic DNA from strain THU. All derivatives of strain 15 showed a similar peak of radioactivity coincident with the P^{32} marker. Figure 4*A* shows one such experiment. Also, the DNA from strain 15T–D3 was purified and its contour length determined. The length, 1.03 μ ± 0.09 (SD), was the same within experimental error as that determined for the plasmid from strain THU. In contrast to these results with derivatives of strain 15, no cytoplasmic DNA (<0.02% of the total DNA or <0.4 plasmid per chromosome) was detected in the strain K12 and B derivatives. One such experiment is shown in Figure 4*B*. It is possible that plasmids are synthesized by these strains but that extrachromosomal DNA is degraded or is not released by our lysis procedure. This possibility appears unlikely for the K12 strains, at least, since high yields of ϕX174 RFI can be obtained from these strains by this procedure. It therefore appears that, among the strains examined, the plasmid DNA is specific to strain 15.

Discussion.—All the derivatives of strain 15 studied possess the plasmid DNA

TABLE 1. *Molecular weight of plasmid DNA.*

	Buffers		
	Neutral high salt	Neutral low salt	Alkaline high salt
Relative sedimentation velocity of ϕX174 RFII to strain 15 plasmid DNA (open form)	1.32	1.29	1.35
Mol wt of plasmid DNA ($\times 10^{-6}$)	1.53	1.63	1.61

The sedimentation buffers are described in *Methods*. The ratio of sedimentation velocities is the average of 2–4 determinations and the average error is 1%. The molecular weights were calculated from the equation of Studier.[15]

TABLE 2. *Effect of single-strand scissions on sedimentation velocity of circular DNA.*

Relative sedimentation velocities of closed vs. open forms	*E. coli* plasmid	ϕX174 RF	Circular λDNA
High salt buffer	1.24	1.25	1.32*
Low salt buffer	1.26	1.37	1.67*

The sedimentation buffers are described in *Methods*.
* From Bode and Kaiser.[25]

TABLE 3. *Occurrence of plasmid DNA among ten strains of E. coli.*

Strain	*E. coli* subspecies	Reference	Colicin 15 production	UV-light sensitivity	Plasmid DNA
THU	15	14	−	*S*	+
TAU	15	13	+	*S*	+
TAU-c	15	29	−	*S*	+
TAU-bar	15	30	−	*R*	+
15T⁻	15	31	+	*S*	+
15T⁻D3	15	32	+	*S*	+
JG151	15	33	−	*T*	+
B3	B	34	−	*R*	−
HF4704	K12	35	−	*R*	−
HF4733	K12	36	−	*R*	−

All strains are thymidine auxotrophs; additional requirements are cited in the references. The lytic response to UV light was determined by irradiation of cells with 900 ergs mm^{-2} of UV light and subsequent turbidometric monitoring of growth. For sensitive (*S*) strains, there was about a fivefold decrease in optical density several hours after irradiation; for the tolerant (*T*) strain there was only a slight drop in turbidity; resistant (*R*) strains continued to grow. Colicin assays were performed on chloroform-treated cultures 6–8 hr after UV irradiation, the time of maximal clearing for sensitive strains. Strains listed as not producing colicin failed to do so even after a tenfold concentration. Strains THU, HF4704, and HF4733 were obtained from R. L. Sinsheimer;[35] TAU and TAU-bar from P. C. Hanawalt;[30] TAU-c and 15T⁻D3 from K. Matsubara;[32] 15T⁻ and JG 151 from A. Yudelevich;[34] and B3 from N. E. Melechen.[36]

even though some of these mutants were isolated as long as 14 years ago.[31] Moreover, the only common parent of these strains is strain 15 itself which was first described in 1944.[37, 38] This genetic stability suggests a mechanism for maintaining the plasmid in the population, perhaps an orderly segregation of plasmids to daughter cells. It is further possible that occasional cured strains are at a selective disadvantage or can be reinfected with the plasmid.

The plasmid DNA level of 15 copies per chromosome is high, especially since it was found in exponentially growing cells and thus represents a steady-state value.[39] By contrast there is only about one *F'lac*[40] *or FColVColBtrycys*[41] particle per chromosome. Multiple copies of a drug-resistance factor were found only after transfer from an *E. coli* donor to a *Proteus mirabilis* recipient.[42] If we assume that the many copies of plasmid DNA are genetically identical, a reasonable assumption in light of their uniform molecular weight and density homogeneity, then it is puzzling why the cell maintains such extensive genetic redundancy. Still a further question is the mechanism whereby the cell maintains the number of copies of the chromosome and the plasmid at such markedly different levels. The ease of identification of the plasmid DNA may make it a favorable system for study of this mechanism of replicon[40] control.

The failure to detect the plasmid in K12 or B led us to look at the properties of strain 15 that are not shared by B and K12. Some derivatives of strain 15 produce a particulate colicin (colicin 15) which is apparently a defective phage.[43] It is not likely that the small plasmid DNA codes for the defective phage because the virus appears to be large and thus would be expected to have a much larger DNA. Also, four of the strains analyzed, THU, TAU-bar, TAU-c, and JG151, do not produce colicin 15 but nevertheless contain the plasmid DNA (Table 3). Strain TAU-c was obtained from TAU under curing conditions[29] and specifically lost the ability to produce colicin while retaining its plasmid DNA. Strain JG151 has also lost two other unusual properties associated with strain 15: high sus-

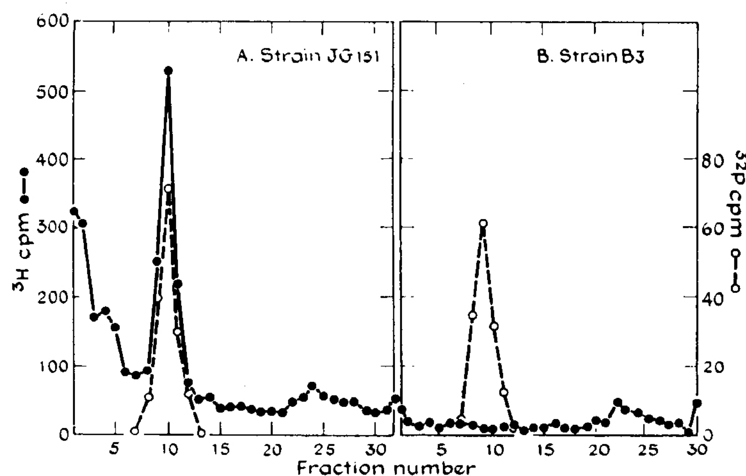

FIG. 4.—Neutral high salt-sucrose sedimentation of H^3-labeled DNA from strain JG151 (*A*) and strain B3 (*B*). P^{32}-labeled plasmid DNA from strain THU was included for reference.

ceptibility to thymineless death,[33] and the induction of a new methylase by UV irradiation.[44] The induction of lysis by UV irradiation is still another property of some derivatives of strain 15 that also is not necessarily correlated with the presence of the plasmid DNA (Table 3). Thus far we have been unable to ascribe any biological function to this DNA.

The strain 15 plasmid DNA is the smallest monodisperse DNA species thus far described,[8] and the smallest microbial DNA of any kind.[45] One of its strands could code for a polypeptide of only 75,000 in molecular weight and thus could contain very few cistrons. Its small size, relative abundance, ease of separation from chromosomal DNA, and its location in *E. coli* make it a convenient source of material for the investigation of DNA structure, replication, and function.

Summary.—Strains of *Escherichia coli* 15 contain a supertwisted, circular, plasmid DNA with a molecular weight of 1.5 million, the smallest known microbial DNA. There are about 15 plasmids per chromosome in the strain 15 cells but none was detected in strains of *E. coli* K12 or B.

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[1] Abbreviations: RFI and RFII denote the covalently closed and open duplex replicative forms of ϕX174 DNA, respectively; EDTA, ethylenediaminetetraacetate; Tris HCl, tris (hydroxymethyl)aminomethane HCl; poly dAT, polydeoxyadenylate-deoxythymidylate.

[2] Burton, A., and R. L. Sinsheimer, *J. Mol. Biol.*, **14**, 327 (1965).

[3] Jacob, F., and E. L. Wollman, *Sexuality and the Genetics of Bacteria* (New York: Academic Press, 1961).

[4] Fraser, D., and E. A. Jerrel, *J. Biol. Chem.*, **205**, 291 (1953).

[5] Hershey, A. D., *Virology*, **1**, 108 (1955).

[6] Komano, T., and R. L. Sinsheimer, *Biochim. Biophys. Acta*, **155**, 295 (1968).

[7] Godson, G. N., and R. L. Sinsheimer, *Biochim. Biophys. Acta*, **149**, 476 (1967).

[8] Radloff, R., W. Bauer, and J. Vinograd, these PROCEEDINGS, **57**, 1514 (1967).

[9] Goulian, M., and A. Kornberg, these PROCEEDINGS, **58**, 1723 (1967).

[10] Kleinschmidt, A. K., D. Lang, D. Jacherts, and R. K. Zahn, *Biochim. Biophys. Acta*, **61** 857 (1962).
[11] Inman, R. B., C. L. Schildkraut, and A. Kornberg, *J. Mol. Biol.*, **11**, 285 (1965).
[12] Mukai, F. H., *J. Gen. Microbiol.*, **23**, 539 (1960).
[13] Barner, H. D., and S. S. Cohen, *Biochim. Biophys. Acta*, **30**, 12 (1958).
[14] Stern, J. L., M. Sekiguchi, H. D. Barner, and S. S. Cohen, *J. Mol. Biol.*, **8**, 629 (1964).
[15] Studier, F. W., *J. Mol. Biol.*, **11**, 373 (1965).
[16] Vinograd, J., and J. Lebowitz, *J. Gen. Physiol.*, **49**, 103 (1966).
[17] Inman, R. B., *J. Mol. Biol.*, **25**, 209 (1967).
[18] Lang, D., H. Bujard, B. Wolff, and D. Russel, *J. Mol. Biol.*, **23**, 163 (1967).
[19] Sinsheimer, R. L., *J. Mol. Biol.*, **1**, 43 (1959).
[20] Clayton, D. A., and J. Vinograd, *Nature*, **216**, 652 (1967).
[21] Roth, T. F., and D. R. Helinski, these PROCEEDINGS, **58**, 650 (1967).
[22] Rush, M. G., A. K. Kleinschmidt, W. Hellman, and R. C. Warner, these PROCEEDINGS, **58**, 1676 (1967).
[23] Schildkraut, C. L., J. Marmur, and P. Doty, *J. Mol. Biol.*, **4**, 430 (1962).
[24] The half-width of the plasmid DNA band is 1.56 times that of ϕX174 RF, measured under the same conditions. The calculated ratio of molecular weights is therefore 2.43 (Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957)), in good agreement with that determined by electron microscopy.
[25] Bode, V. C., and A. D. Kaiser, *J. Mol. Biol.*, **14**, 399 (1965).
[26] J. Wang has found that the tertiary structures of ϕX174 RFI and plasmid DNA are relaxed by the same number of molecules of ethidium bromide per unit length of DNA (personal communication).
[27] Cairns, J., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 43.
[28] Young, E. T., II, and R. L. Sinsheimer, *J. Mol. Biol.*, **30**, 147 (1967).
[29] Isolated from strain TAU after two growth cycles in the presence of 35 μg/ml acridine orange, followed by thymidine deprivation.
[30] Hanawalt, P. C., *Nature*, **198**, 286 (1963).
[31] Barner, H. D., and S. S. Cohen, *J. Bacteriol.*, **68**, 80 (1954).
[32] Matsubara, K., A. Taketo, and Y. Takagi, *J. Biochem. (Japan)*, **54**, 225 (1962).
[33] Ishibashi, M., and Y. Hirota, *J. Bacteriol.*, **90**, 1496 (1965).
[34] Melechen, N. E., and P. D. Skaar, *Virology*, **16**, 21 (1962).
[35] Lindqvist, B. H., and R. L. Sinsheimer, *J. Mol. Biol.*, **28**, 87 (1967).
[36] A strain lacking endonuclease I isolated by P. H. Flanders.
[37] Roepke, R. R., R. L. Libby, and M. H. Small, *J. Bacteriol.*, **48**, 401 (1944).
[38] Roepke, R. R., *J. Bacteriol.*, **93**, 1188 (1967).
[39] The exposure of THU to 900 ergs mm^{-2} of UV light, sufficient to initiate cell lysis within 1 hr, did not cause any change in the amount of plasmid DNA.
[40] Jacob, F., S. Brenner, and F. Cuzin, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 329.
[41] Hickson, F. T., T. R. Roth, and D. R. Helinski, these PROCEEDINGS, **58**, 1731 (1967).
[42] Rownd, R., R. Nakaya, and A. Nakamura, *J. Mol. Biol.*, **17**, 376 (1966).
[43] Nomura, M., *Ann. Rev. Microbiol.*, **21**, 257 (1967).
[44] Yudelevich, A., and M. Gold, *Federation Proc.*, **26**, 450 (1967).
[45] Thomas, C. A., and L. A. MacHattie, *Ann. Rev. Biochem.*, **36**, 485 (1967).