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Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase

(*Escherichia coli*/colicin E1 DNA replication/phage λ DNA)

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ABSTRACT **Novobiocin and coumermycin are known to inhibit the replication of DNA in *Escherichia coli*. We show that these drugs inhibit the supercoiling of DNA catalyzed by *E. coli* DNA gyrase, a recently discovered enzyme that introduces negative superhelical turns into covalently circular DNA. The activity of DNA gyrase purified from a coumermycin-resistant mutant strain is resistant to both drugs. The inhibition by novobiocin of colicin E1 plasmid DNA replication in a cell-free system is partially relieved by adding resistant DNA gyrase. Both in the case of colicin E1 DNA in *E. coli* extracts and of phage λ DNA in whole cells, DNA molecules which are converted to the covalently circular form in the presence of coumermycin remain relaxed, instead of achieving their normal supercoiled conformation. We conclude that DNA gyrase controls the supercoiling of DNA in *E. coli*.**

Novobiocin and the related drug coumermycin are preferential inhibitors of DNA replication in intact *Escherichia coli* cells (1, 2). In toluenized cells of *E. coli*, these drugs inhibit replicative DNA synthesis but not repair synthesis (3, 4). They are also effective inhibitors in cell-free systems for the replication of colicin E1 (Col E1) DNA (5) and of phage φX174 replicative form DNA (6), but they do not inhibit the synthesis of the complementary strand of φX174 single-stranded DNA (6). Coumermycin-resistant mutants of *E. coli* have been isolated, and the mutation has been mapped near the *dnaA* locus (2).

An enzyme, DNA gyrase, that introduces negative superhelical turns into double-stranded closed circular DNA, has recently been purified from *E. coli* (7). In this paper, we show that DNA gyrase activity *in vitro* as well as *in vivo* is specifically inhibited by coumermycin and novobiocin.

MATERIALS AND METHODS

Bacterial Strains. Coumermycin-resistant *E. coli* mutants used for the present study were isolated by a two-step procedure. The starting strain, *E. coli* NT525 *pnp rns end strA*, was constructed by recombination between EB5004 *end* (8) and a *thy* derivative of SK107 *pnp rns strA polA*$^+$, the parental strain of SK108 *polA* (9), provided by S. Kushner. Strain NT525, like most other *E. coli* K12 strains, is quite resistant to novobiocin (grows on tryptone agar containing 600 μg/ml of novobiocin) because of low permeability to the drug (W. G. Coleman, personal communication). Strain NI708, with increased sensitivity to novobiocin, was constructed by mating NT525 with strain CL2, an HfrH derivative which carries a mutation conferring increased permeability to a number of drugs (W. G. Coleman, personal communication). Mutants of NI708 with various extents of increased resistance to novobiocin were isolated but all the isolates tested turned out to be resistant due to reduced permeability. The selection was therefore repeated with coumermycin, which is apparently indifferent to the permeability barrier for novobiocin (unpublished results). Among several spontaneous coumermycin-resistant mutants of NI708 which were tested, all gave resistant extracts for the *in vitro* Col E1 DNA replication system. One of these mutants, NI741, was chosen for further work because of its good growth. This strain apparently has a partial reversion of the permeability mutation of NI708. The *cou*^{R} mutation of NI741 was transferred, by phage P1 cotransduction with *dnaA*$^+$, to strain CRT46 *dnaA* (10). The resulting strain, NI748, was used as a source for purification of drug-resistant DNA gyrase. DNA gyrase sensitive to both novobiocin and coumermycin was isolated from N99 *recB*$_{21}$ (7). Strain N1071(λ*ind*$^-$) (11) was used for experiments of superinfection by phage λ, and strain YS1 (8) for testing supercoiling of Col E1 DNA in extracts.

Of these strains, the coumermycin-resistant isolates NI741 and NI748 are able to grow in liquid culture containing 60 μg/ml of coumermycin; growth of the other strains is blocked by 15 μg/ml of the drug.

Chemicals. Novobiocin was obtained from Sigma Chemical Co. Samples of coumermycin A$_1$ (referred to as coumermycin throughout this paper) were gifts from W. F. Minor (Bristol Laboratories) and J. Davies (University of Wisconsin). Sources of other materials have been described previously (7, 8, 12).

Methods. Procedures for the assay of Col E1 DNA replication (8, 12, 13), and for the purification and assay of DNA gyrase (7), have been described before. DNA gyrase from the coumermycin-resistant strain NI748 was purified by the same method. Fraction IV enzyme was used for all experiments.

DNA Preparations. Relaxed and intracellularly supercoiled Col E1 DNA samples were prepared as described (7, 8). Relaxed covalently-circular phage λ DNA was prepared by sealing with DNA ligase as described (7).

RESULTS

Inhibition of DNA gyrase activity by novobiocin and coumermycin

For visualizing the activity of DNA gyrase, we have made use of the difference in mobility between relaxed and supercoiled closed-circular Col E1 DNA on agarose gel electrophoresis (7, 14). As is shown in Fig. 1, intracellularly supercoiled Col E1 DNA (Fig. 1a) has roughly twice the mobility of relaxed closed-circular Col E1 DNA (Fig. 1b). When relaxed closed-circular Col E1 DNA was incubated with DNA gyrase purified from the coumermycin-sensitive strain N99 *recB*, most of the DNA was converted to a form with the increased mobility of supercoiled Col E1 DNA (Fig. 1c). This reaction was progressively inhibited by increasing concentrations of novobiocin (Fig. 1d–h). At low drug concentrations (0.3–1.0 μg/ml), intermediate DNA species were seen which had lesser degrees of supercoiling (15); at 3 μg/ml of novobiocin the reaction was totally inhibited. By contrast, the activity of DNA gyrase purified from the coumermycin-resistant strain NI748 was unaffected by all

Abbreviation: Col E1, colicin E1.

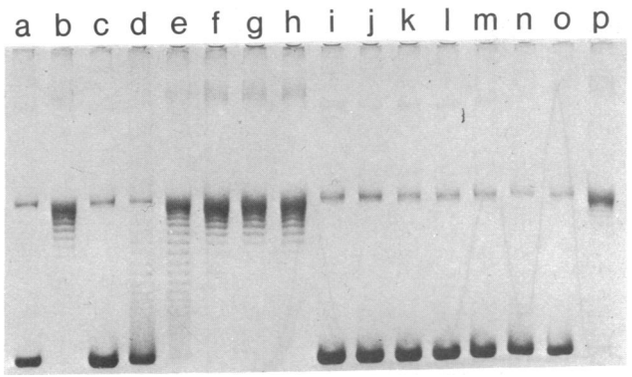

FIG. 1. Novobiocin sensitivity of DNA gyrase from sensitive and resistant *E. coli* strains. Channel (a) intracellularly supercoiled Col E1 DNA; (b) relaxed Col E1 DNA; (c–h) relaxed Col E1 DNA incubated with DNA gyrase purified from strain N99 $recB_{21}$ (0.65 μg) and varying concentrations of novobiocin; (i–n) relaxed Col E1 DNA incubated with DNA gyrase purified from strain NI748 (0.40 μg) and varying concentrations of novobiocin. Novobiocin concentrations were: (c) and (i), none; (d) and (j), 0.3 μg/ml; (e) and (k), 1 μg/ml; (f) and (l), 3 μg/ml; (g) and (m), 10 μg/ml; (h) and (n), 30 μg/ml. In (o) and (p), 0.33 μg of DNA gyrase from strain N99 *recB* and 0.20 μg of DNA gyrase from strain NI748 were mixed and assayed in the absence (o) and presence (p) of 3 μg/ml novobiocin.

these levels of novobiocin (Fig. 1i–n). Fig. 1o and p show that the resistant enzyme cannot confer novobiocin resistance on sensitive DNA gyrase; a mixture of the two enzymes showed evidence of more than 50% inhibition by novobiocin. Coumermycin also inhibited DNA gyrase activity (data not shown); this drug was effective at roughly 10-fold lower concentrations than novobiocin. DNA gyrase purified from N99 *recB* was inhibited more than 90% by 0.3 μg/ml of coumermycin; DNA gyrase from the resistant mutant was unaffected by concentrations of the drug up to 1 μg/ml.

Inhibition of *in vitro* Col E1 DNA replication by novobiocin

Replication of Col E1 DNA in an extract of a drug-sensitive strain (NT525) was inhibited more than 90% by 1 μg/ml of novobiocin while replication in an extract of a resistant strain (NI741) was relatively unaltered (Table 1). Replication of endogenous DNA in toluenized cells was likewise sensitive to novobiocin in strain NT525 but resistant in strain NI741 (data not shown). As in the assay of DNA gyrase activity, coumermycin was effective at a severalfold lower concentration (0.2 μg/ml) than novobiocin in inhibiting replication of Col E1 DNA in extracts (data not shown).

Experiments were also carried out to test the stimulation of novobiocin-inhibited Col E1 DNA replication by resistant DNA gyrase. As shown in Table 1, addition of DNA gyrase purified from the resistant strain NI748 to an extract of strain NT525 led to a stimulation of Col E1 DNA replication in the presence of novobiocin, while no such stimulation was seen when DNA gyrase from the sensitive strain N99 *recB* was added. However, the extent of stimulation was small. It appears most likely that the presence of sensitive DNA gyrase in the extract inhibits the activity of the resistant enzyme in the presence of novobiocin. In agreement with this interpretation, addition of DNA gyrase from strain N99 *recB* to a resistant extract resulted in marked inhibition of Col E1 DNA replication in the presence of novobiocin (Table 1). As noted above, a parallel effect has been seen directly in assays of DNA gyrase; in the presence of novobiocin, addition of sensitive DNA gyrase inhibits the activity of novobiocin-resistant DNA gyrase (Fig. 1p and unpublished data).

Table 1. Inhibition of Col E1 DNA replication by novobiocin and complementation by resistant DNA gyrase

Source of extract	Additions	dTMP incorporated (pmol)
NT525	None	3.8
	Sensitive DNA gyrase	3.9
	Resistant DNA gyrase	3.0
	Novobiocin	0.4
	Novobiocin + sensitive DNA gyrase	0.3
	Novobiocin + resistant DNA gyrase	1.1
NI741	None	3.4
	Sensitive DNA gyrase	3.4
	Resistant DNA gyrase	3.7
	Novobiocin	3.1
	Novobiocin + sensitive DNA gyrase	2.0
	Novobiocin + resistant DNA gyrase	3.7

Extracts were prepared as described from strain NT525 (coumermycin-sensitive) and from strain NI741 (coumermycin-resistant) (8), except that the cells were cultured at 30°. The procedure for the assay of Col E1 DNA replication was essentially as described (12). The reaction mixtures (30 μl) contained 10 μg/ml of intracellularly supercoiled Col E1 DNA, 25 μM each of four deoxyribonucleoside triphosphates (dNTPs), 400 μM ATP, 200 μM each of other ribonucleoside triphosphates (rNTPs), 1 mM NAD, 41 mM potassium phosphate at pH 7.1, 33 mM KCl, 25 mM $(NH_4)_2SO_4$, 8 mM $MgCl_2$, 60 μM dithiothreitol, 60 μM Na_3EDTA, 330 μg/ml of bovine plasma albumin, 10% (vol/vol) glycerol, and 2 mM spermidine. The reaction mixture contained 10 μl (100 μg of protein) of extract. Specific radioactivity of [α-^{32}P]dTTP was 500 cpm/pmol. DNA gyrase (fraction IV) was concentrated by precipitation with ammonium sulfate and was resuspended at a concentration of 1–2 mg/ml in 50% (vol/vol) glycerol, 0.15 M potassium phosphate at pH 6.8, 2.5 mg/ml of bovine plasma albumin, 0.5 mM Na_3EDTA, and 0.5 mM dithiothreitol. Novobiocin-sensitive DNA gyrase (from strain N99 $recB_{21}$) and novobiocin-resistant DNA gyrase (from strain NI748) were added in the amounts of 5.3 μg and 6.3 μg per assay, respectively. The final concentration of novobiocin was 1 μg/ml. After incubation for 60 min at 30°, acid-insoluble radioactivity was measured.

Effect of coumermycin on supercoiling of DNA in *E. coli* cells and extracts

If DNA gyrase is responsible for the supercoiling of closed-circular DNA in *E. coli* and in the Col E1 replicating system, then it should be possible to interfere with supercoiling by the use of coumermycin. Figs. 2 and 3 show that this expectation is borne out.

In the experiment of Fig. 2, Col E1 DNA previously nicked by pancreatic DNase was incubated with a cell extract. Sealing of the DNA by DNA ligase present in the extract produced covalently circular DNA, whose superhelix density could be analyzed by equilibrium centrifugation in a CsCl-ethidium bromide density gradient (16). In the absence of coumermycin, such an incubation (Fig. 2A) gave rise to a Col E1 DNA species whose position in the gradient, and thus its superhelix density, differed only slightly from that of an intracellularly supercoiled Col E1 DNA marker. In the presence of coumermycin (Fig. 2B) the DNA still was sealed, but its superhelix density now was that of the fully relaxed Col E1 DNA marker. In the presence of nicotinamide mononucleotide (NMN), a specific inhibitor of DNA ligase (17), no sealing occurred (Fig. 2C), while an incubation with NMN followed by a second incubation in the presence of coumermycin and enough NAD to permit sealing (Fig. 2D) again produced a fully relaxed DNA species. This last variation was a control against the possibility that the superhelix

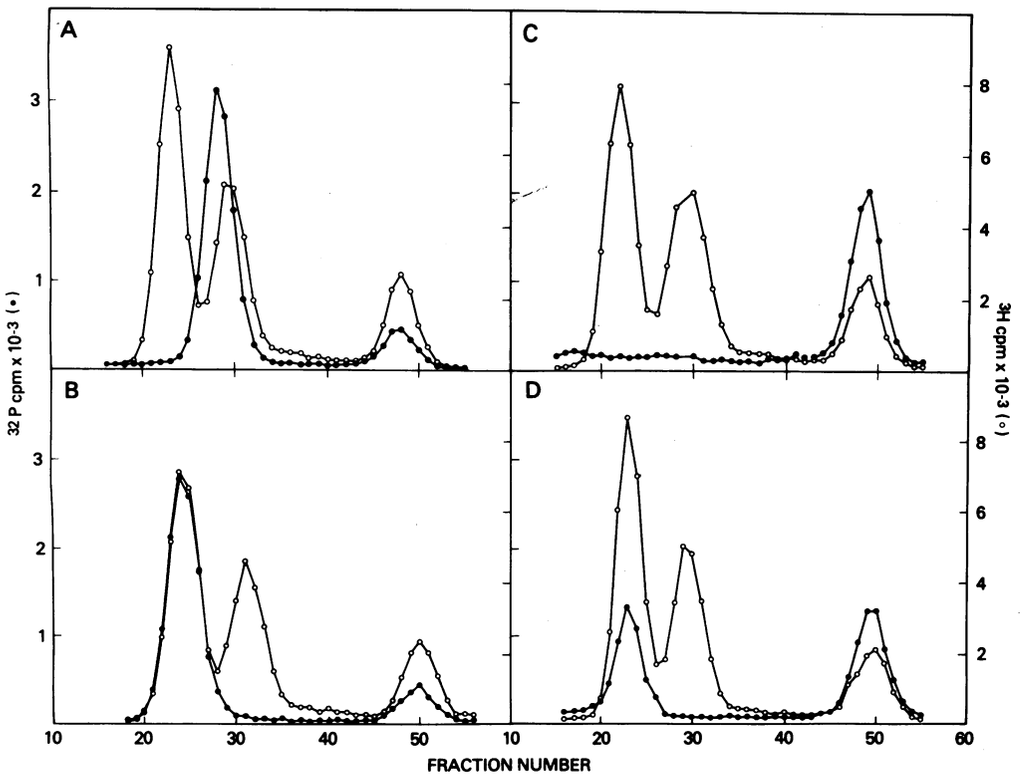

FIG. 2. Superhelix density of Col E1 DNA sealed in cell extracts in the absence or presence of coumermycin. ^{32}P-Labeled closed-circular Col E1 DNA (approximately 30 Ci/mol of P) was treated with DNase I (5 ng/ml) for 10 min at 30° in 20 mM Tris·HCl (pH 8.0) and 5 mM $MgCl_2$. The reaction was terminated by adding 10 mM Na_3EDTA. Approximately 80% of the DNA was converted to open-circular molecules which were isolated by sucrose density gradient centrifugation (8). The DNA was then pelleted by centrifugation in a Spinco SW 50.1 rotor at 35,000 rpm for 15 hr at 5° and suspended in 50 mM potassium phosphate at pH 7.4 and 50 mM KCl. The DNA was incubated with an extract of YS1 cells (12) in various conditions. The standard reaction mixture (100 μl) contained 33 μl of a cell extract, 10 μl of ^{32}P-labeled open-circular DNA (approximately 15,000 cpm), 25 mM potassium phosphate at pH 7.4, 67 mM KCl, 7.5 mM $MgCl_2$, 200 μM ATP, and various supplements as indicated. Rifampicin (5 μg/ml) was always added to block Col E1 DNA synthesis in the extract (8). Incubations were for the times indicated below at 30°. After termination of the reactions by adding Na_3EDTA (20 mM) and sodium dodecyl sulfate (0.25%), the reaction mixtures were incubated with proteinase K (E. Merck, 500 μg/ml) for 1 hr at 37°. The samples were mixed with a CsCl solution (final density 1.55 g/ml) containing 0.33 mg/ml ethidium bromide and ^{3}H-labeled Col E1 DNA preparations (8) that contained intracellularly supercoiled, relaxed closed-circular and open-circular molecules. The final volumes were 7.4 ml. The samples were centrifuged at 35,000 rpm for 60 hr at 15° in a Spinco Type 40 rotor. Approximately 70 fractions were collected and the radioactivity was measured. Sample A was incubated in the presence of 5 mM NAD for 20 min, sample B with 5 mM NAD and 0.5 μg/ml of coumermycin for 20 min, and sample C with 2 mM NMN for 20 min. Sample D was first incubated for 20 min with 2 mM NMN and then incubated for 20 min after addition of 0.5 μg/ml of coumermycin and 5 mM NAD. The peaks of the reference [^{3}H]DNAs are, from the left, relaxed closed-circular, supercoiled, and open-circular molecules.

density of DNA in the extract might partly be controlled by proteins which bound slowly to the DNA.

This experiment has also been carried out with novobiocin instead of coumermycin, with the same results (data not shown).

An experiment to examine supercoiling in intact *E. coli* cells was carried out by phage λ infection (Fig. 3). When phage λ superinfects a cell already lysogenic for λ, most of the superinfecting DNA is converted to a covalently circular form (18). In the experiment of Fig. 3, a lysogenic *E. coli* strain was superinfected with λ in the presence or absence of coumermycin, and the DNA was analyzed by CsCl–ethidium bromide density gradient centrifugation. Superinfection by phage λ in the absence of coumermycin (Fig. 3C) resulted in a λ DNA species whose position in the gradient matched that of the intracellularly supercoiled Col E1 DNA marker. Alternatively, superinfection in the presence of coumermycin, with incubation for either 5 min or 30 min (Fig. 3A and B), yielded covalently circular λ DNA that was almost fully relaxed. The DNA sealed in the presence of coumermycin (Fig. 3A and B) can be estimated to have a superhelix density 15% of that of normal superinfecting λ DNA, when compared with a reference sample of relaxed covalently circular λ DNA (Fig. 3D) [by the analysis of (16)].

These experiments lead us to conclude that DNA gyrase is responsible for catalyzing supercoiling of extrachromosomal DNA in *E. coli*.

DISCUSSION

The present experiments show that novobiocin and coumermycin act by inhibiting the supercoiling of DNA which is catalyzed by DNA gyrase. This seems to be the biologically significant action of the drugs, because the growth of *E. coli* cells and Col E1 DNA replication in extracts both become drug-resistant as the result of a mutation which also renders DNA gyrase activity resistant to both drugs. The identification of one mutation as being responsible for these properties is strengthened by the fact that coumermycin-resistant growth and coumermycin resistance of DNA gyrase are cotransduced by phage P1, and both are closely linked to the *dnaA* locus.

Inhibition of DNA gyrase, in cell extracts and in whole cells, leads to a total or almost total disappearance of the supercoiling normally found as a property of covalently circular DNA iso-

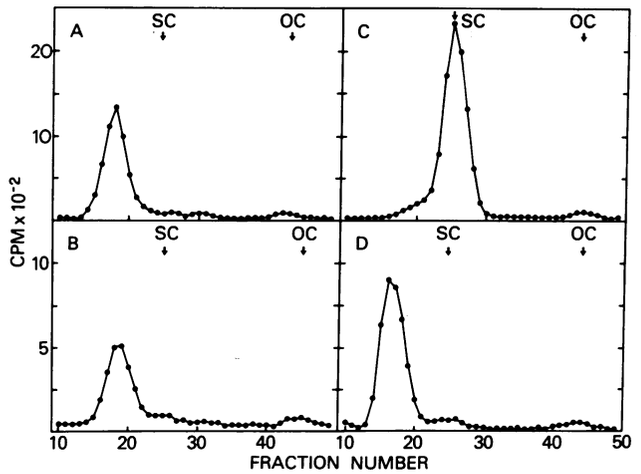

FIG. 3. Superhelix density of superinfecting phage λ DNA in λ-lysogenic cells in the absence or presence of coumermycin. Ten milliliter cultures of *E. coli* N1071 (λind⁻) were grown at 37° to a density of 2 × 10^8 cells per ml in a medium containing 10 mg/ml of Bacto-tryptone (Difco), 5 mg/ml NaCl, 2 mg/ml maltose, 5 mM $MgCl_2$, and 0.05 M Tris·HCl at pH 7.5. Cells were pelleted and resuspended in the same volume of fresh medium, and infected in the presence or absence of coumermycin with ^{3}H-labeled λ CI-857 Sam_7 at a multiplicity of 12 phage per cell. Coumermycin was added to a final concentration of 100 μg/ml from a 25 mg/ml solution in dimethyl sulfoxide; an equal volume of dimethyl sulfoxide was added to the control culture. After further incubation at 37° as specified below, the cultures were chilled, washed once by centrifugation, and again centrifuged. The pellets were resuspended in 0.5 ml of 20% sucrose, 50 mM potassium phosphate at pH 7.5, 0.1 M NaCl, and 10 mM NaN_3. The cells were lysed by incubation with 0.8 mg/ml of lysozyme for 5 min at 0°, followed by addition of sodium lauroyl sarcosinate to a final concentration of 5 mg/ml. After further addition of proteinase K (E. Merck) to a final concentration of 100 μg/ml, the incubation was continued for 30 min at 30°. The samples were centrifuged in CsCl-ethidium bromide and fractionated as described in the legend to Fig. 2 (no reference DNA was added at this stage). All material with density greater than the band of viscous chromosomal DNA (which also contained λ DNA which had not become covalently circular) was pooled and recentrifuged under the same conditions, this time including a small amount of ^{14}C-labeled Col E1 DNA, containing both intracellularly supercoiled and open-circular molecules, as a density reference. Sample A: coumermycin was added at zero time and phage λ was added 2 min later; the incubation was stopped at 7 min. Sample B: the incubation was stopped at 32 min; otherwise the same as A. Sample C: dimethyl sulfoxide was added at zero time and phage λ was added 2 min later; the incubation was stopped at 12 min. Sample D: no phage was added; after lysis, 0.6 μg of relaxed covalently circular [^{3}H]λ DNA was added. The arrows mark the position of supercoiled (SC) and open-circular (OC) marker Col E1 [^{14}C]DNA. The proportion of superinfecting λ DNA which became covalently circular was 50–70% in all samples.

lated from these systems (Figs. 2 and 3). The simplest interpretation of these results is that covalently circular DNA within *E. coli* cells is normally maintained in a negatively supercoiled state of strain by the action of DNA gyrase, and that the supercoiling found as a property of DNA isolated from the cells reflects this condition. It appears that the superhelix density of DNA in *E. coli* is altered only slightly, if at all, by the binding of other molecules to DNA.

Relaxed covalently circular DNA has previously been observed in *E. coli* as a transient newly replicated species of certain plasmids (19, 20). The following results obtained with the cell-free Col E1 DNA replication system offer a possibility of explaining the appearance of such intermediates. It was shown that open-circular molecules of Col E1 DNA with a nick or gap at the termination point of replication in the newly synthesized strand are the precursors of newly formed supercoiled molecules, and that the process of formation of closed circular molecules is inhibited by NMN (21). The formation of relaxed covalently closed DNA may represent a situation where newly sealed covalently circular DNA can be transiently captured in a relaxed state, before DNA gyrase can act on it. A similar situation may exist in the replication of mitochondrial DNA (22). The transient formation of relaxed covalently circular molecules may also occur as a result of nicking which is not coupled to replication.

DNA gyrase appears to be an essential component of numerous systems for the replication of circular DNA in *E. coli*, as evidenced by the variety of such systems which are inhibited by novobiocin and coumermycin. It is not clear in all these systems whether a supercoiled DNA template is required for initiation of DNA synthesis, for chain elongation, or for both. In the case of the cell-free replication of φX174-RFI DNA, a supercoiled DNA substrate is required to permit the endonucleolytic action of φX174 A protein, which in turn is required before DNA synthesis can be initiated (23). On the other hand, in the cell-free replication of Col E1 DNA, for which no plasmid-coded functions are needed (12), there is no evidence for a requirement of previous endonucleolytic action for initiation of replication. Although Col E1 DNA synthesis was strongly inhibited in the sensitive extract by novobiocin or coumermycin, it is not known at which early step DNA replication was arrested. Because coumermycin was able to prevent further replication of previously formed replicative intermediates of Col E1 DNA (data not shown), elongation of Col E1 DNA chains apparently requires a supercoiled DNA template.

If intracellular DNA is kept in a supercoiled strained condition, then other processes besides its replication are likely to be affected. It is known, for example, that transcription of supercoiled DNA is altered from that of relaxed circular DNA (24). It is also known that *in vitro* integrative recombination of λ DNA (25, 26) requires a supercoiled DNA substrate (K. Mizuuchi, M. Gellert, and H. A. Nash, manuscript in preparation). DNA gyrase must be involved in the formation of these supercoils, since integrative recombination of relaxed covalently circular λ DNA in this system is inhibited by novobiocin, while supercoiled λ DNA escapes the inhibition.

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