

# Nalidixic acid resistance: A second genetic character involved in DNA gyrase activity

(*Escherichia coli*/DNA supercoiling/oxolinic acid/colicin E1 DNA replication)

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Communicated by Gary Felsenfeld, August 29, 1977

**ABSTRACT** ATP-dependent DNA supercoiling catalyzed by *Escherichia coli* DNA gyrase was inhibited by oxolinic acid, a compound similar to but more potent than nalidixic acid and a known inhibitor of DNA replication in *E. coli*. The supercoiling activity of DNA gyrase purified from nalidixic acid-resistant mutant (*nalA*<sup>R</sup>) bacteria was resistant to oxolinic acid. Thus, the *nalA* locus is responsible for a second component needed for DNA gyrase activity in addition to the component determined by the previously described locus for resistance to novobiocin and coumermycin (*cou*). Supercoiling of  $\lambda$  DNA in *E. coli* cells was likewise inhibited by oxolinic acid, but was resistant in the *nalA*<sup>R</sup> mutant. The inhibition by oxolinic acid of colicin E1 plasmid DNA synthesis in a cell-free system was largely relieved by adding resistant DNA gyrase.

In the absence of ATP, DNA gyrase preparations relaxed supercoiled DNA; this activity was also inhibited by oxolinic acid, but not by novobiocin. It appears that the oxolinic acid-sensitive component of DNA gyrase is involved in the nicking-closing activity required in the supercoiling reaction. In the presence of oxolinic acid, DNA gyrase forms a complex with DNA, which can be activated by later treatment with sodium dodecyl sulfate and a protease to produce double-strand breaks in the DNA. This process has some similarities to the known properties of relaxation complexes.

Previous work (1-3) has described an enzyme activity, DNA gyrase, that is responsible for the supercoiling of DNA in *Escherichia coli*. As isolated from extracts of *E. coli*, the enzyme introduces negative superhelical turns into covalently closed circular DNA in an ATP-dependent reaction; the hydrolysis of ATP presumably provides the free energy needed to accumulate mechanical strain energy in the DNA.

One genetic locus (*cou*), which determines resistance to coumermycin A<sub>1</sub> and novobiocin, has been identified as controlling the activity of DNA gyrase (2). The enzyme isolated from wild-type cells is inhibited by both these antibiotics, while DNA gyrase from a coumermycin-resistant mutant strain is unaffected. Intracellular DNA supercoiling is similarly blocked by coumermycin.

In this paper we report the involvement of a second genetic locus (*nalA*), which determines resistance to nalidixic acid and oxolinic acid (4, 5), in controlling DNA gyrase activity. These two drugs are inhibitors of DNA replication in *E. coli* (4, 5). They also inhibit replication in cell-free systems of colicin E1 plasmid (ColE1) DNA (6, 7) and of phage  $\phi$ X174 replicative form DNA (8), but they do not inhibit the synthesis of the complementary strand of  $\phi$ X174 single-stranded DNA (8). These properties are parallel to those described for coumermycin A<sub>1</sub> and novobiocin.

Nalidixic acid-resistant mutants of two classes have been identified and mapped (9). Mutations at one locus (*nalB*, 57 min

on the standard *E. coli* map) are responsible for low-level resistance and have been characterized as interfering with the permeability of the cells to nalidixic acid. Mutations at the other locus (*nalA*, map location 48 min) determine resistance to high levels of nalidixic acid; the function involved in the *nalA* locus has not been identified previously. The *nal* genes are widely separated from *cou* (map location 82 min). Mutants resistant to nalidixic acid are also resistant to oxolinic acid, a more powerful but structurally similar drug (5).

We show here that oxolinic acid interferes with DNA supercoiling in *E. coli* cells and inhibits the DNA supercoiling reaction catalyzed by purified DNA gyrase. A *nalA*<sup>R</sup> mutant, and DNA gyrase purified from it, are much less sensitive to these effects of the drug. DNA gyrase from the *nalA*<sup>R</sup> mutant is also able to restore oxolinic acid-inhibited synthesis of ColE1 DNA in a cell-free system.

Under modified assay conditions, DNA gyrase displays a nicking-closing activity capable of relaxing supercoiled DNA. This activity is inhibited by oxolinic acid, but not by novobiocin, and is resistant to oxolinic acid in enzyme from the *nalA*<sup>R</sup> strain. Incubation of DNA with DNA gyrase and oxolinic acid leads to formation of a complex which, upon treatment with detergent and a protease, produces double-strand breaks in the DNA.

## MATERIALS AND METHODS

**Bacterial Strains.** *E. coli* strains N99*recB*<sub>21</sub>, NT525, NI-748*cou*<sup>R</sup>, and N1071 ( $\lambda$ ind<sup>-</sup>) have been described (1, 2). Strain N4156 *polA end thy nalA*<sup>R</sup> (strain Nal<sup>RC</sup> of ref. 9) was sent by R. Sternglanz. N4158 is N4156 lysogenized with  $\lambda$ ind<sup>-</sup>. NI744, a spontaneous nalidixic acid-resistant mutant of NT525, was identified as a *nalA* mutant by cotransduction with *dnaF*. N3048*recB*<sub>21</sub>*recC*<sub>22</sub>*sbcB*<sub>15</sub>*end* is strain MO611 (10) cured of phage P1 by J. L. Rosner. Of these strains, N4156, N4158, and NI744 were able to grow in liquid culture containing 5  $\mu$ g of oxolinic acid per ml; growth of the other strains was blocked by 0.5  $\mu$ g of the drug per ml.

**Chemicals.** Oxolinic acid samples were gifts from J. D. Stein, Jr. (Warner-Lambert Research Institute, Morris Plains, NJ) and from W. Staudenbauer. Stock solutions were made up in dilute KOH. Proteinase K was obtained from E. Merck, and sodium dodecyl sulfate from BDH Chemicals. Homogeneous  $\omega$  protein, and antiserum directed against it, were gifts from J. C. Wang. Sources of other materials have been described (1, 2).

**Methods.** The previously described procedures for the assay of ColE1 DNA replication (6, 11, 12) were modified as described in the legends to Table 2 and Fig. 2. Wild-type DNA gyrase was purified either from strain N99*recB*<sub>21</sub> or from strain N3048 as described (1). DNA gyrase from the nalidixic acid-resistant strain N4156 was purified by the same method. The

Abbreviation: ColE1, colicin E1 plasmid.

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Table 1. Inhibition of  $\lambda$  DNA supercoiling in *E. coli* cells by oxolinic acid

<i>E. coli</i> strain	Oxolinic acid concentration, $\mu\text{g/ml}$	Fractional DNA supercoiling
N1071 $nal^S$	0	(1.00)
	50	0.68
	100	0.51
	200	0.35
N4158 $nal^R$	0	0.98
	50	0.98
	100	1.05
	200	1.05
$\lambda$ DNA sealed <i>in vitro</i>		(0)

Cultures of strains N1071 (oxolinic acid-sensitive) and N4158 (oxolinic acid-resistant), both lysogenic for  $\lambda ind^-$ , were grown and infected with  $^3\text{H}$ -labeled  $\lambda\text{Cl-857Sam}_7$  as described (2). Oxolinic acid, where used, was added 5 min before infection from a 25 mg/ml stock solution. Cells were incubated for 15 min at 37° after infection, and were then lysed as described (2). Centrifugation to equilibrium in CsCl/ethidium bromide was carried out in the presence of  $^{14}\text{C}$ -labeled relaxed, intracellularly supercoiled, and open-circular forms of ColE1 DNA added as markers. The technique was as described (2), except that a single cycle of centrifugation was used. A sample of  $\lambda [^3\text{H}]\text{DNA}$  sealed *in vitro* by DNA ligase was centrifuged in parallel as a reference standard for fully relaxed DNA. Fractional supercoiling was calculated (13) relative to the value determined for superinfecting  $\lambda$  DNA in strain N1071 in the absence of oxolinic acid.

assay of DNA gyrase activity was carried out as before. One unit of activity is defined as that amount of DNA gyrase that brings 50% of relaxed ColE1 DNA to the fully supercoiled position in agarose gel electrophoresis under the standard assay conditions. Since the assay is not linear in enzyme concentration (1, 3), the unit is only useful for comparison of activities near this extent of reaction. For experiments on ColE1 DNA replication, DNA gyrase purified by a revised method from strains N99 $recB_{21}$  and N4156 was used. After the previously described polymin fractionation and dialysis (1), enzyme was applied to a column of hydroxyapatite (Biogel HTP) with a bed volume of 0.25 ml/mg of protein and eluted with a gradient (10 bed volumes) of 0.05–0.5 M potassium phosphate (pH 7.5) containing 0.1 M KCl, 1 mM dithiothreitol, and 10% (wt/vol) glycerol. Active fractions, which eluted between 0.1 and 0.16 M phosphate, were pooled, dialyzed, and applied to a phosphocellulose column as described (1). The column was developed with a gradient (10 bed volumes) of 0.05–0.5 M potassium phosphate (pH 6.8) containing 1 mM EDTA, 5 mM dithiothreitol, and 10% (wt/vol) glycerol. Active fractions were pooled and concentrated by dialysis against dry Sephadex G-50.

**DNA Preparations.** Relaxed and intracellularly supercoiled ColE1 DNA samples and relaxed covalently circular phage  $\lambda$  DNA were prepared as described (1, 2).

## RESULTS

**Inhibition by Oxolinic Acid of DNA Supercoiling in *E. coli* Cells and in Purified DNA Gyrase Preparations.** Supercoiling of phage  $\lambda$  DNA, which becomes covalently circular upon superinfecting a lysogenic strain, can be used as a test for DNA gyrase function *in vivo* (2). In the experiment shown in Table 1, lysogenic *E. coli* strains were superinfected with phage  $\lambda$  in the presence or absence of oxolinic acid and the extent of supercoiling of the DNA was analyzed by equilibrium centrifugation in a CsCl/ethidium bromide density gradient. When the wild-type strain N1071 was superinfected with phage  $\lambda$  in the

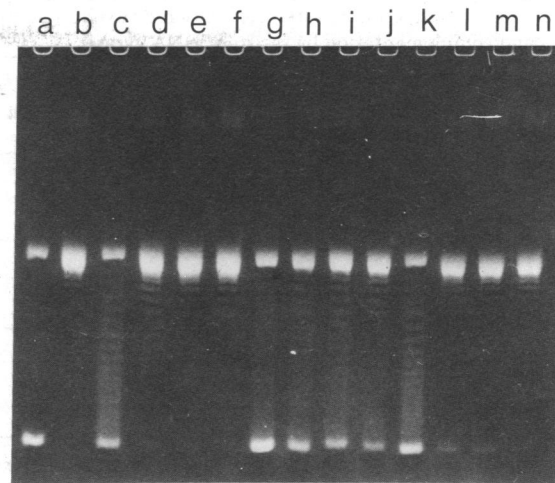


FIG. 1. Oxolinic acid sensitivity of DNA gyrase from sensitive and resistant *E. coli* strains. (a) Intracellularly supercoiled ColE1 DNA containing some open-circular DNA; (b) relaxed ColE1 DNA. The remaining channels display the results of incubating relaxed ColE1 DNA with DNA gyrase (approximately 1 unit) purified from the following strains: (c–f) N3048 (sensitive); (g–j) N4156 (oxolinic acid-resistant); (k–n) NI748 (coumermycin-resistant). Assays were carried out in the absence and presence of oxolinic acid as described (1), except that the concentration of  $\text{MgCl}_2$  was raised to 6 mM; for each enzyme, the oxolinic acid concentrations were, from left to right, 0, 25, 50, and 100  $\mu\text{g/ml}$ .

presence of increasing concentrations of oxolinic acid, supercoiling of the DNA was progressively decreased from the normal value found in the absence of drug. The partial degree of inhibition did not result in a slow increase of supercoiling with time. The extent of DNA supercoiling in cells pretreated with oxolinic acid did not change between 5 and 45 min after infection. By contrast, supercoiling in the oxolinic acid-resistant strain N4158 was unaffected at even the highest concentration of oxolinic acid.

A similar correlation with the genetic determinant for oxolinic acid resistance was obtained for the activity of purified DNA gyrase, as is shown in Fig. 1. Assay of DNA gyrase (fraction IV of ref. 1) from the oxolinic acid-sensitive strain N99 $recB_{21}$  in the absence of drug resulted in conversion of the relaxed ColE1 DNA substrate to the supercoiled form (Fig. 1c). The enzyme was progressively inhibited by increasing concentrations of oxolinic acid (Fig. 1d–f). The concentrations required were in the range used to inhibit DNA supercoiling *in vivo*; at 25  $\mu\text{g}$  of oxolinic acid per ml, there was roughly 80% inhibition. On the other hand, the activity of DNA gyrase from the oxolinic acid-resistant strain N4156 was only slightly inhibited by these concentrations of the drug (Fig. 1g–j). While the differential inhibition of the two enzymes was readily seen under the assay conditions previously described, at 1.6 mM  $\text{MgCl}_2$  (1), it was even more apparent at the higher magnesium ion concentration used in the experiment shown in Fig. 1. DNA gyrase purified from the coumermycin-resistant strain NI748 was as sensitive to oxolinic acid as enzyme from the wild-type strain (Fig. 1k–n). There appears to be no crossresistance between the two families of drugs inhibiting DNA gyrase.

**Complementation by DNA Gyrase of Oxolinic Acid-Inhibited ColE1 DNA Synthesis.** Synthesis of ColE1 DNA in a cell-free system derived from the oxolinic acid-resistant strain NI744 was only slightly inhibited by 50  $\mu\text{g}$  of oxolinic acid per ml, while synthesis in the corresponding system from the sensitive strain NT525 was inhibited by about 90% (Table 2). In the ammonium sulfate fractions used here, the products of

Table 2. Inhibition of ColE1 DNA replication by oxolinic acid and complementation by resistant DNA gyrase

Source of fraction	Additions	dTMP incorporated, pmol	% resistant activity
NT525 <i>nal</i> <sup>S</sup>	None	6.1	—
	Oxolinic acid	0.6	10
	Sensitive DNA gyrase	9.0	—
	Sensitive DNA gyrase + oxolinic acid	1.3	15
	Resistant DNA gyrase	6.5	—
	Resistant DNA gyrase + oxolinic acid	3.0	47
NI744 <i>nalA</i> <sup>R</sup>	None	4.0	—
	Oxolinic acid	2.4	60

Extracts were prepared as described (6) from strain NT525 (oxolinic acid-sensitive) and strain NI744 (oxolinic acid-resistant), except that the cells were cultured at 30°. Ammonium sulfate fractions were prepared from the extracts as follows. A solution of 20% streptomycin sulfate was added to the extract to give a final concentration of 4%. The supernatant solution obtained after centrifugation was adjusted to 40% saturation with saturated ammonium sulfate. After centrifugation, the pellet was discarded and the ammonium sulfate concentration of the supernatant solution was adjusted to 70% saturation. The pellet obtained after centrifugation was dissolved, in one-fifth the volume of the initial extract, in 33 mM potassium phosphate (pH 7.5)/100 mM KCl/1 mM dithiothreitol/6.7% sucrose. The fraction was passed through a Sephadex G-25 column equilibrated with the same buffer. The procedure for the assay of ColE1 DNA replication was essentially as described (11). The reaction mixtures (30  $\mu$ l) contained 10  $\mu$ g of intracellularly supercoiled ColE1 DNA per ml, 25  $\mu$ M each of dATP, dCTP, dGTP and [ $\alpha$ -<sup>32</sup>P]dTTP (400 cpm/pmol), 400  $\mu$ M ATP 200  $\mu$ M each of other ribonucleoside triphosphates, 1 mM NAD, 3 mM dithiothreitol, 30 mM potassium phosphate (pH 7.1), 13 mM KCl, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 8 mM MgCl<sub>2</sub>, 10% (vol/vol) glycerol, and 2 mM spermidine. The reaction mixtures contained 4  $\mu$ l (52  $\mu$ g of protein) of ammonium sulfate fraction. Oxolinic acid-sensitive DNA gyrase (from strain N99*recB*<sub>21</sub>) and oxolinic acid-resistant DNA gyrase (from strain N4156) were added where indicated in the amount of 3 units (0.3–0.4  $\mu$ g) per assay. The final concentration of oxolinic acid, where indicated, was 50  $\mu$ g/ml. After incubation for 60 min at 30°, acid-insoluble radioactivity was determined.

ColE1 DNA synthesis were early replicative intermediates (6) (T. Itoh and J. Tomizawa, unpublished). The inhibition by oxolinic acid was largely relieved by adding DNA gyrase purified from the oxolinic acid-resistant strain N4156, while enzyme from the sensitive strain N99*recB*<sub>21</sub> was relatively ineffective (Table 2).

To confirm the identification of DNA gyrase with the factor conferring oxolinic acid resistance on this system, we examined the chromatographic behavior of the two activities on phosphocellulose. As is shown in Fig. 2, there is rather accurate cochromatography of the two activities; the factor conferring oxolinic acid resistance is found only in fractions with DNA gyrase activity. These results suggest that a component of DNA gyrase is the target of oxolinic acid inhibition in this DNA-synthesizing system.

**Relaxation of Supercoiled DNA by DNA Gyrase.** In the absence of ATP, DNA gyrase preparations were capable of transforming supercoiled DNA to a relaxed form. This activity was not apparent under the assay conditions previously described (1), but could be demonstrated if the magnesium ion concentration was raised to 6 mM (Fig. 3*b*). In this modified assay condition, DNA gyrase still displayed its DNA-supercoiling activity in the presence of ATP.

The relaxing activity was inhibited by 50  $\mu$ g of oxolinic acid

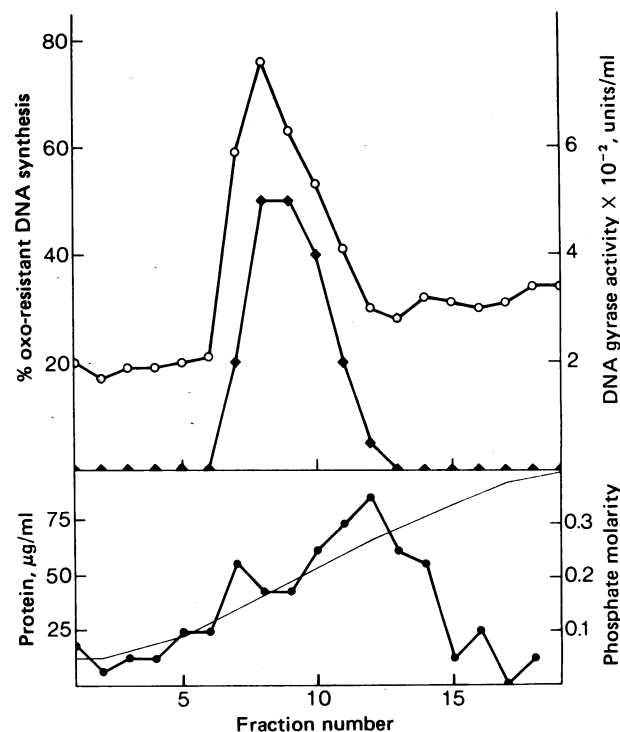


FIG. 2. Cochromatography of DNA gyrase activity with stimulatory activity for oxolinic acid-inhibited ColE1 DNA replication. The ratio of DNA synthesis in the presence of oxolinic acid (Oxo) to that in its absence is plotted. DNA gyrase was purified from strain N4156. Individual fractions from the phosphocellulose column were assayed for DNA gyrase activity (as described in ref. 1) and for their ability to stimulate oxolinic acid-inhibited ColE1 DNA replication. The replication assay was as described in the legend of Table 2 with the following exceptions: 2  $\mu$ l (26  $\mu$ g of protein) of a 40–70% ammonium sulfate fraction derived from strain NT525, 50  $\mu$ g of oxolinic acid per ml, and 2  $\mu$ l of each column fraction were added; the DNA concentration was reduced to 2.5  $\mu$ g/ml. Incorporation in the absence of drug varied from about 0.7–2.0 pmol of dTMP. The sloping baseline of DNA synthesis is due to the varying amounts of phosphate carried over with the DNA gyrase fractions. The pass-through fraction from the column, containing two-thirds of the total protein applied, had no detectable DNA gyrase activity or stimulatory activity in the DNA replication assay. O, % oxolinic acid-resistant DNA synthesis;  $\blacklozenge$ , DNA gyrase activity;  $\bullet$ , protein; —, phosphate molarity.

per ml (Fig. 3*c*), while the similar activity of DNA gyrase from the *nalA*<sup>R</sup> strain was not affected (Fig. 3*e* and *f*). The relaxing activity was totally insensitive to novobiocin at a level that blocks DNA supercoiling by DNA gyrase (Fig. 3*d*). Assay of fractions from a phosphocellulose column showed that the relaxing activity cochromatographed with DNA gyrase activity (data not shown).

This relaxing activity appears to be distinct from the nicking-closing enzyme  $\omega$  (14) by several criteria. (i) It was not inhibited by antibody directed against homogeneous  $\omega$  protein (Fig. 3*g*), at a level sufficient to block totally the activity of an equal weight of  $\omega$  protein. (ii) Relaxation by  $\omega$  protein was not inhibited by oxolinic acid at the concentration used here (data not shown).\* (iii) In addition, DNA gyrase is able to relax positively supercoiled DNA. When relaxed closed-circular DNA was incubated in the absence of ATP with DNA gyrase and

\* Much higher concentrations of oxolinic acid (above 500  $\mu$ g/ml) do inhibit  $\omega$  protein activity. However,  $\omega$  protein purified from a *nalA*<sup>R</sup> strain is inhibited to the same extent (our unpublished results). This inhibition thus appears to be unrelated to the function determined by the genetic locus for oxolinic acid resistance.

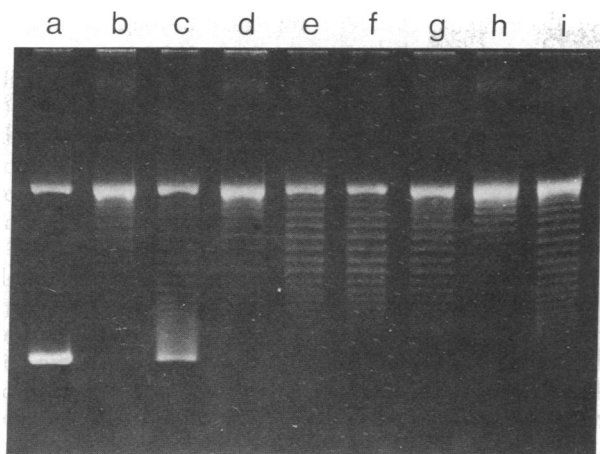


FIG. 3. Relaxation of supercoiled ColE1 DNA by DNA gyrase and its inhibition by oxolinic acid. (a) Intracellularly supercoiled ColE1 DNA containing some open circular DNA. (b, c, d, and g) The same DNA incubated with DNA gyrase from the sensitive strain N3048: (b) without drug; (c) with 50  $\mu\text{g}$  of oxolinic acid per ml; (d) with 10  $\mu\text{g}$  of novobiocin per ml; (g) with 2  $\mu\text{l}$  of antiserum against  $\omega$  protein. (e and f) The same DNA incubated with DNA gyrase from the oxolinic acid-resistant strain N4156: (e) without drug; (f) with 50  $\mu\text{g}$  of oxolinic acid per ml. (h and i) Relaxed closed circular ColE1 DNA incubated with DNA gyrase from strain N3048: (h) without ethidium bromide; (i) with 0.8  $\mu\text{g}$  of ethidium bromide per ml. Ethidium bromide was removed after the reaction by shaking with *n*-butanol. DNA gyrase was added at a level of 2 units per reaction. Reaction conditions were as described (1) except that ATP was omitted and the concentrations of several reagents were changed to the following values: 6 mM  $\text{MgCl}_2$ , 1.8 mM spermidine, 5 mM dithiothreitol, 9  $\mu\text{g}$  of *E. coli* tRNA per ml, and 0.36 mg of bovine plasma albumin per ml. Reactions were stopped by incubation at 80° for 5 min.

sufficient ethidium bromide to produce partial positive supercoiling, the DNA was reisolated, after removal of ethidium bromide, in a partly (negatively) supercoiled form (Fig. 3*f*). The DNA must thus have become relaxed in the reaction mixture. By contrast,  $\omega$  protein is known to have little or no activity on positively supercoiled DNA generated in this way (14) and was additionally shown not to carry out such a reaction under the reaction conditions used for relaxation by DNA gyrase.

The simplest interpretation of these results is that the nicking-closing activity of DNA gyrase is carried by the subunit associated with oxolinic acid sensitivity, and that this nicking-closing activity is distinct from the previously described  $\omega$  protein. A similar interpretation has been suggested in a parallel study by Sugino *et al.* (15).

**DNA Gyrase Induces Double-Strand Breakage of DNA in the Presence of Oxolinic Acid.** If ColE1 DNA is incubated with DNA gyrase in the presence of oxolinic acid and the product is then treated with sodium dodecyl sulfate and proteinase K, some of the DNA is converted to a full-length linear form, as judged by comigration with an endonuclease *Eco*R1 digest of ColE1 DNA (Fig. 4*b* and *c*). (*Eco*R1 is known to cleave ColE1 DNA at one site.) Some single-strand breakage could also be demonstrated. The cleavage reaction did not require ATP (Fig. 4*d*) nor was it specific for supercoiled DNA; relaxed ColE1 DNA was an equally good substrate (data not shown), as was linear ColE1 DNA (see below). Incubation with oxolinic acid was essential (Fig. 4*e*); both sodium dodecyl sulfate and proteinase K were required in the second incubation for DNA breakage to occur (Fig. 4*f* and *g*). Brief heating of assay mixtures to 80° before the addition of sodium dodecyl sulfate and proteinase K blocked the formation of linear DNA (Fig. 4*h*). When the effects of individual additions were monitored

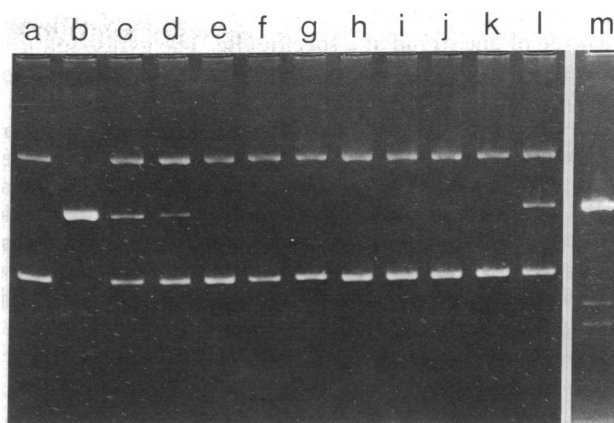


FIG. 4. Double-strand breakage of DNA induced by DNA gyrase in the presence of oxolinic acid. Intracellularly supercoiled ColE1 DNA (containing some open circular DNA) was treated under a variety of conditions. (a) DNA alone; (b) endonuclease *Eco*R1 digest of the DNA; (c) complete system for breakage (DNA was incubated for 60 min at 25° with DNA gyrase from the sensitive strain N3048, ATP, and oxolinic acid; then EDTA, sodium dodecyl sulfate, and proteinase K were added sequentially, and the incubation was continued for 30 min at 37°); (d) ATP omitted; (e) oxolinic acid omitted; (f) proteinase K omitted; (g) sodium dodecyl sulfate omitted; (h) reaction mixture heated to 80° for 5 min before addition of sodium dodecyl sulfate; (i-j) DNA incubated with DNA gyrase from the oxolinic acid-resistant strain N4156 as in (c), in the absence (i) and presence (j) of oxolinic acid; (k and l) DNA incubated with DNA gyrase from the coumermycin-resistant strain NI748 as in (c), in the absence (k) and presence (l) of oxolinic acid. The first incubations were carried out under the conditions of Fig. 3, but with 4 units of DNA gyrase, 1.6 mM  $\text{MgCl}_2$ , 200  $\mu\text{g}$  of oxolinic acid per ml, and ATP (where added) at 0.2 mM. Final concentrations of reagents added for the second incubation were: 11 mM EDTA, 2 mg of sodium dodecyl sulfate per ml, and 90  $\mu\text{g}$  of proteinase K per ml (added from a 1 mg/ml aqueous solution). After the second incubation, samples were shaken with chloroform/isoamyl alcohol and prepared for agarose gel electrophoresis as described (1). (m) Result of incubating an endonuclease *Eco*R1 digest of ColE1 DNA under the conditions described in (c).

by cesium chloride/ethidium bromide centrifugation, it was found that treatment with sodium dodecyl sulfate shifted some of the DNA to a lighter density, most probably as a protein-DNA complex. Subsequent incubation with proteinase K shifted this material to the density characteristic of linear and open-circular DNA (data not shown).

We infer that a rather stable gyrase-DNA complex is formed in the presence of oxolinic acid and that it can then be activated to cleave the DNA by adding sodium dodecyl sulfate and proteinase K. The first complex appears to be noncovalent, because it can be disrupted by heating. The second complex formed by the action of sodium dodecyl sulfate is stable to concentrated cesium chloride or to incubation at 80°, and thus may well be covalent. Formation of the first complex is slow. In kinetic experiments (not shown) maximal cleavage was obtained only if DNA gyrase was incubated with DNA and oxolinic acid for at least 1 hr.

As evidence that the cleavage reaction is a property of DNA gyrase itself rather than of a contaminant in the enzyme preparation, we note that the reaction has the expected genetic specificity. Enzyme from the *nalA*<sup>R</sup> strain produces very little cleavage (Fig. 4*i* and *j*); on the other hand, enzyme from the *cou*<sup>R</sup> strain behaves similarly to wild-type enzyme (Fig. 4*k* and *l*).

These results have some features in common with the known properties of relaxation complexes (16). Certain plasmid DNA species can be isolated with a bound protein. Treatment of the

complex with a protease or a protein denaturant results in breakage of one strand at a specific site. The gyrase-induced cleavage appears to differ from this system in the predominance of double-strand breaks.

The analogy of gyrase-induced breakage to that caused by a relaxation complex can be extended to the finding of a degree of site specificity. When an endonuclease *EcoRI* digest of ColE1 DNA is incubated with DNA gyrase and oxolinic acid and then treated with sodium dodecyl sulfate and proteinase K, fragments of defined sizes are seen upon gel electrophoresis (Fig. 4*m*). There appear to be several sites of breakage, of differing strength, in ColE1 DNA.

## DISCUSSION

These experiments show that oxolinic acid acts by inhibiting a component necessary for DNA gyrase activity. Growth of the cells, *E. coli* chromosome replication (4, 5) ColE1 DNA replication in extracts (ref. 7 and Table 2), DNA supercoiling *in vivo*, and DNA supercoiling catalyzed by purified DNA gyrase are all inhibited by oxolinic acid, and all these properties become resistant to the drug as the result of a mutation at the *nalA* locus. Furthermore, *in vitro* ColE1 DNA synthesis inhibited by oxolinic acid can be largely restored by the addition of purified DNA gyrase from the resistant strain. Thus a component of DNA gyrase is the target for the interaction with oxolinic acid that blocks DNA replication.

Together with our previous results showing that one component of DNA gyrase activity is associated with the genetic locus for coumermycin resistance (2), the present work implies that at least two distinct functional units are involved in DNA gyrase activity. In order to introduce negative (underwound) superhelical turns into DNA, DNA gyrase must perform two functions. It must impose a twisting stress tending to overwind a region of the DNA molecule, and it must carry out a nicking-closing reaction within that region to relieve the positive winding stress. Our experiments suggest that DNA gyrase does have a nicking-closing activity that is capable of relaxing supercoiled DNA, including positively supercoiled DNA. This activity can plausibly be associated with the oxolinic acid-sensitive component of the enzyme, because relaxation is inhibited by oxolinic acid but not by the other family of drugs, novobiocin and coumermycin, which also inhibit the supercoiling activity. In related experiments, Sugino *et al.* (15) have purified a protein with an oxolinic acid-sensitive nicking-closing activity but little or no supercoiling activity. This protein probably functions as the oxolinic acid-sensitive component of DNA gyrase.

The simplest model for DNA gyrase activity would then predict that the coumermycin-sensitive subunit carries out the energy transducing role of coupling ATP hydrolysis to production of a twisting stress on the DNA. For the present, how-

ever, the existence of other components beyond the two now defined is not excluded.

There remains one puzzling feature of oxolinic acid inhibition. While the drug concentration required to affect *in vivo*  $\lambda$  DNA supercoiling by an appreciable fraction is comparable to the concentrations needed to inhibit the cell-free reactions studied here, the growth of the cells is blocked by  $1/50$  the concentration. Nevertheless, the same mutation confers resistance to both classes of effects. Either the cells must be sensitive to very small changes in DNA supercoiling or, more plausibly, some more subtle interaction of DNA gyrase or of its oxolinic acid-specific component is involved. It is tempting to ask whether an intracellular analogue exists for the oxolinic acid-induced breakage of DNA that we have observed. In this connection, we note the observation (17) that chromosomal DNA isolated from *E. coli* treated with nalidixic acid has a markedly reduced single-strand molecular weight.

We thank Sue Wickner, Howard Nash, A. Richard Morgan, and Rolf Sternglanz for valuable discussions, and A. Sugino and N. Cozzarelli for generously exchanging unpublished information.

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