JOURNAL OF BACTERIOLOGY, Feb., 1966
Vol. 91, No. 2

Mechanism of Action of Nalidixic Acid on *Escherichia coli*

III. Conditions Required for Lethality

WILLIAM H. DEITZ, THOMAS M. COOK, AND WILLIAM A. GOSS
Sterling-Winthrop Research Institute, Rensselaer, New York

Received for publication 15 September 1965

ABSTRACT

DEITZ, WILLIAM H. (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), THOMAS M. COOK, AND WILLIAM A. GOSS. Mechanism of action of nalidixic acid on *Escherichia coli*. III. Conditions required for lethality. J. Bacteriol. **91**:768–773. 1966.—Nalidixic acid selectively inhibited deoxyribonucleic acid (DNA) synthesis in cultures of *Escherichia coli* 15TAU. Protein and ribonucleic acid synthesis were shown to be a prerequisite for the bactericidal action of the drug. This action can be prevented by means of inhibitors at bacteriostatic concentrations. Both chloramphenicol, which inhibits protein synthesis, and dinitrophenol, which uncouples oxidative phosphorylation, effectively prevented the bactericidal action of nalidixic acid on *E. coli*. The lethal action of nalidixic acid also was controlled by transfer of treated cells to drug-free medium. DNA synthesis resumed immediately upon removal of the drug and was halted immediately by retreatment. These studies indicate that nalidixic acid acts directly on the replication of DNA rather than on the "initiator" of DNA synthesis. The entry of nalidixic acid into cells of *E. coli* was not dependent upon protein synthesis. Even in the presence of an inhibiting concentration of chloramphenicol, nalidixic acid prevented DNA synthesis by *E. coli* 15TAU.

Nalidixic acid, a specific inhibitor of deoxyribonucleic acid (DNA) synthesis, is bactericidal for cultures of *Escherichia coli* under conditions permitting ribonucleic acid (RNA) and protein synthesis. In contrast, no loss of viability is observed under conditions of restricted protein and RNA synthesis (3).

This communication presents the results of further studies on the mechanism of action of nalidixic acid on *E. coli*. We examined in some detail the conditions required for bacterial lethality and the effect of this chemotherapeutic agent on DNA synthesis of *E. coli*.

MATERIALS AND METHODS

The methods used for the cultivation and study of *E. coli* 15TAU have been described previously (2, 3). To indicate nutritional conditions, (+T, −AU) will refer to a medium containing thymine, but not L-arginine or uracil; similarly, (+T, +AU) will refer to complete growth medium containing thymine, L-arginine, and uracil. Unless otherwise stated, bacterial viability was estimated by plating dilutions (in 1% peptone water) of bacterial cultures in Tryptone Glucose Extract Agar (Difco). DNA synthesis was monitored by determining the amount of radioactive precursor (C^{14}-labeled thymine or thymidine) incorporated into trichloroacetic acid-insoluble material (2). To change media, cells were collected by membrane filtration (Millipore, 0.45 μ), washed, and resuspended in warm basal medium. Supplements then were added to give the desired medium.

Radioactive precursors. Thymidine-2-C^{14} (specific activity, 30 mc/mmole) and thymine-2-C^{14} (specific activity, 45.4 mc/mmole) were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Initiation of bactericidal action. Nalidixic acid inhibits DNA synthesis in *E. coli* without subsequent loss of viability in nutritionally deficient media (2, 3). The present study shows that the bactericidal action of the drug can be initiated by the addition of required nutrients to treated cells in deficient medium.

An exponentially proliferating culture of *E. coli* 15TAU was transferred from (+T, +AU) medium to (+T, −AU) medium containing C^{14}-labeled thymidine (0.05 μc/ml) with and without 20 μg/ml of nalidixic acid. After 110 min of incubation, both cultures were divided,

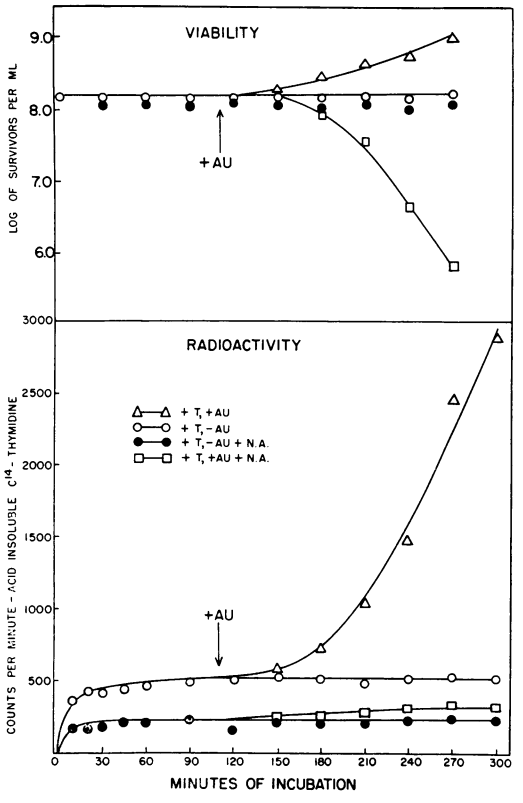

FIG. 1. *Initiation of the bactericidal action of nalidixic acid on Escherichia coli 15TAU by restoration of protein and RNA synthesis. Exponentially growing cultures were transferred from (+T, +AU) to (+T, −AU) medium containing C14-labeled thymidine (0.05 μc/ml) in the presence and absence of nalidixic acid (20 μg/ml). L-Arginine (A) and uracil (U) were added after 110 min. Samples were removed to ice-cold 10% trichloroacetic acid for determinations of radioactivity in acid-insolubles.*

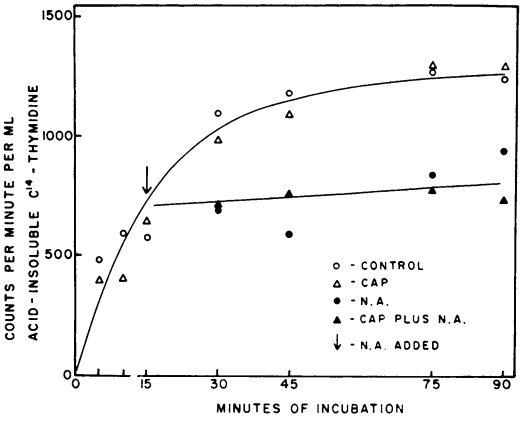

FIG. 2. *Failure of chloramphenicol (CAP) to prevent inhibition of DNA synthesis by nalidixic acid. An exponentially growing culture was transferred from (+T, +AU) to (+T, −AU) medium containing CAP (200 μg/ml) and C14-labeled thymidine (0.05 μc/ml). After 15 min, nalidixic acid (20 μg/ml) was added. Samples were removed to ice-cold 10% trichloroacetic acid for determinations of radioactivity in acid-insolubles.*

and L-arginine and uracil were added to a portion of each.

During incubation in the deficient medium (+T, −AU), DNA synthesis was promptly inhibited by nalidixic acid without any decrease in the number of viable cells (Fig. 1). About 60 min after addition of arginine and uracil to the nalidixic acid-treated culture, the cells began to lose viability, which coincided with the onset of proliferation and incorporation of isotope by the control culture. In the drug-treated culture, a slight incorporation of radioactive thymidine accompanied the loss of viability. In the absence of arginine and uracil, there was no further DNA synthesis and no change in viable-cell count in either the control or treated cultures.

In the nutritionally deficient medium, (+T, −AU), the results suggested that protein and RNA synthesis were not required for the uptake of nalidixic acid. Under these conditions, it was conceivable that a small amount of residual protein synthesis could occur, permitting elaboration of a nalidixic acid permease analogous to that suggested for the uptake of streptomycin (6). To test this possibility, a culture of *E. coli* 15TAU was transferred to (+T, −AU) medium containing chloramphenicol at a final concentration of 200 μg/ml. After 15 min, nalidixic acid at a final concentration of 20 μg/ml was added to one portion of the culture.

The results indicate that chloramphenicol does not interfere with the normal completion of the DNA replication cycle (Fig. 2). Nalidixic acid, even in the presence of chloramphenicol, inhibited DNA synthesis immediately. From these results, we concluded that uptake of nalidixic acid is not dependent upon the induction of permease.

Conditions controlling bactericidal action. The requirement for RNA and protein synthesis suggested that, once initiated, the bactericidal action of nalidixic acid could be arrested by subsequent treatment with appropriate bacteriostatic agents. Chloramphenicol, which inhibits protein synthesis selectively, and dinitrophenol, which uncouples oxidative phosphorylation, were used for this purpose.

A culture of *E. coli* 15TAU was treated with nalidixic acid (20 μg/ml). After reduction of the

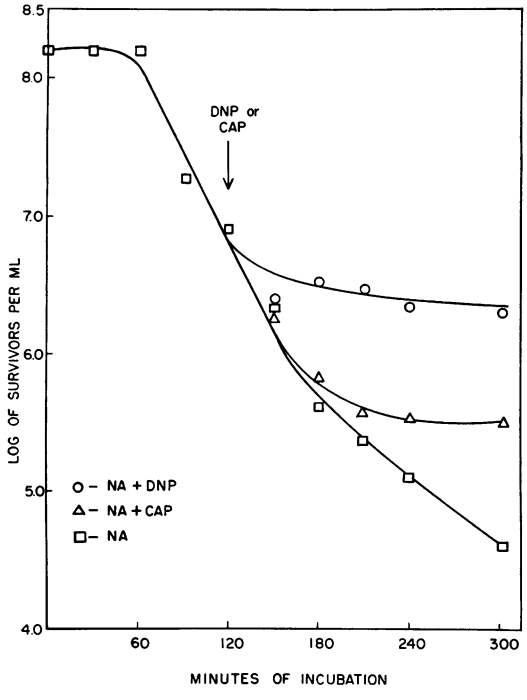

FIG. 3. *Arrest of the bactericidal action of nalidixic acid by chloramphenicol (CAP) and dinitrophenol (DNP). An exponentially growing culture was treated with nalidixic acid (20 μg/ml). After 120 min, separate portions of the culture were treated with CAP (20 μg/ml) and DNP (1.0 mg/ml).*

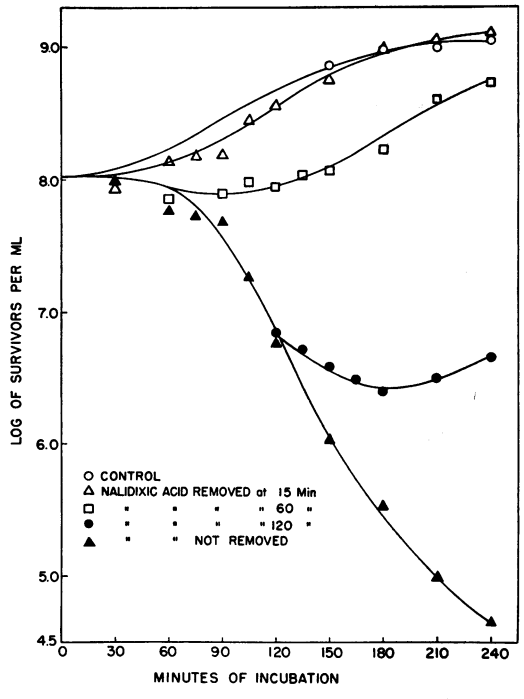

FIG. 4. *Resumption of proliferation in nalidixic acid-treated cultures after removal of drug. An exponentially proliferating culture was treated with nalidixic acid (20 μg/ml). After 15, 60, and 120 min, portions were removed and the optical density was recorded. The cells were collected on membrane filters (0.45 μ porosity), washed twice with warm basal medium, and immediately resuspended in drug-free medium at the optical density recorded prior to filtration.*

viable population by 90%, chloramphenicol and dinitrophenol (20 μg/ml and 1.0 mg/ml, respectively) were added to separate portions of the culture. Both chloramphenicol and dinitrophenol arrested the bactericidal action of nalidixic acid, but the action of dinitrophenol was far more rapid (Fig. 3).

We showed earlier that the inhibition of DNA synthesis by nalidixic acid could be reversed by filtration and transfer to drug-free medium (3). It has been suggested (Konetzka, *personal communication*) that our observations on DNA synthesis after removal of nalidixic acid could be explained by effects on the "initiator" (7, 8) of DNA synthesis similar to those proposed for the action of phenethyl alcohol (10). We examined this possibility in the following experiments.

We attempted to halt the bactericidal action of nalidixic acid by transferring treated cultures to drug-free medium. After various periods of incubation in the presence of nalidixic acid (20 μg/ml), portions of the treated culture were filtered, washed, and resuspended in warm drug-free medium. Transfer of the cells after 15 or 60 min permitted resumption of proliferation (Fig. 4). After 120 min of treatment, more than 90% of the initial population had been rendered nonviable. Transfer at this time resulted in continued loss of viability, but at a progressively decreasing rate. About 60 min after the transfer, proliferation resumed.

If the action of nalidixic acid were similar to that of phenethyl alcohol, one would expect resumption of DNA synthesis immediately after drug removal. Furthermore, a second treatment of this culture with nalidixic acid should not stop DNA synthesis immediately, but should permit completion of the replication cycle representing a 40% increase in the amount of DNA synthesized (10).

The DNA of a culture of *E. coli* 15TAU was labeled by incubation for several generations in the presence of C^{14}-labeled thymidine (0.01 μc/ml), and a portion of the culture then was treated with 20 μg/ml of nalidixic acid. After 30 min, a portion of this culture was transferred

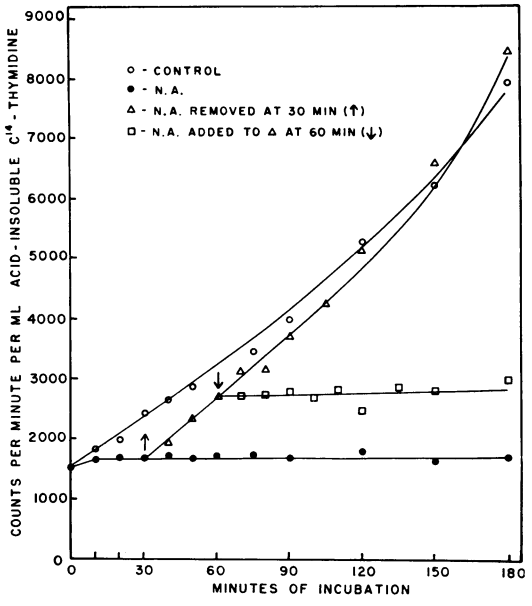

FIG. 5. *Failure of nalidixic acid to affect the "initiator" of DNA synthesis. Thirty minutes after treatment (20 μg/ml of nalidixic acid) of an exponentially growing culture, drug was removed from a portion of the culture by filtration and washing, and the cells were immediately resuspended in warm, drug-free medium. Thirty minutes after the transfer, a portion of this culture was again treated with a similar concentration of nalidixic acid. Samples were removed to ice-cold 10% trichloroacetic acid for determination of radioactivity in acid-insolubles.*

to drug-free medium. After an additional 30 min of incubation, the culture was again treated with the same dose of nalidixic acid.

DNA synthesis resumed immediately upon transfer of the treated cells to drug-free medium, but was immediately inhibited when again treated with nalidixic acid (Fig. 5). After the second treatment, the net increase in the amount of DNA was only 5% during a 2-hr period.

Again, it was conceivable that with a lower concentration of nalidixic acid one could demonstrate an effect on initiator similar to phenethyl alcohol.

The DNA of a culture of *E. coli* 15TAU was labeled by growth for several generations in the presence of C^{14}-labeled thymine (0.01 μc/ml). Portions of the culture then were treated with various concentrations of nalidixic acid ranging from 1.0 to 20.0 μg/ml.

Treatment with low levels of drug (1.0 and 3.0 μg/ml) resulted in only limited increases in the number of viable cells (Table 1). At 5.0 and 20.0 μg/ml, the drug was bactericidal. The

TABLE 1. *Relationship of nalidixic acid concentration to bacterial viability and DNA synthesis**

Nalidixic acid	Bacterial viability, per cent survivors at			DNA synthesis, per cent increase at	
	0 min	60 min	120 min	60 min	120 min
μg/ml					
0	100	215	450	140	346
1.0	100	199	300	111	252
3.0	100	200	190	53	146
5.0	100	199	39.5	36	72
20.0	100	44.5	3.0	7.8	6.0

* A culture of *Escherichia coli* 15TAU was labeled by growth in the presence of C^{14}-thymine (0.01 μc/ml) and portions were treated with various levels of nalidixic acid. Samples were diluted and plated in Tryptone Glucose Extract Agar. Additional samples were pipetted to ice-cold 10% trichloroacetic acid for determination of radioactivity in acid-insolubles.

amount of DNA synthesized was inversely proportional to the drug concentration. Most important, at none of the concentrations was the net increase in DNA limited to the value of approximately 40%, which would be expected if only "initiator" were inhibited. In fact, DNA synthesis was continuous during the experiment at all concentrations less than 20 μg/ml. At this concentration, the inhibition of DNA synthesis was virtually complete.

Relationship of bactericidal action to growth rate. Our studies to date have indicated that the bactericidal action of nalidixic acid requires conditions which permit proliferation of control cultures. It seemed reasonable that the rate of bactericidal action and growth rate should be correlative. A convenient means of regulating the generation times of microorganisms is alteration of the temperature of incubation.

To determine the relationship of the bactericidal action of nalidixic acid to growth rate, cultures of *E. coli* 15TAU were treated with 100 μg/ml of drug and were incubated at three different temperatures.

The growth rates of control cultures were dependent on incubation temperature, with the respective generation times estimated to be 135, 72, and 48 min at 20, 28, and 37 C (Fig. 6).

Loss of viability in the treated cultures was similarly temperature-dependent. At 37 C, the onset of bactericidal action was preceded by virtually no lag, whereas pronounced lag periods were observed at 20 and 28 C. At all temperatures, loss of viability progressed more rapidly

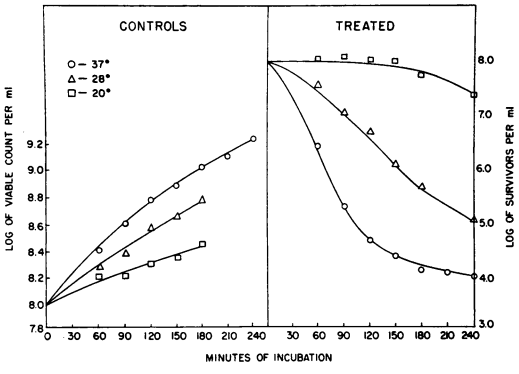

FIG. 6. *Relationship of the rate of bactericidal action of nalidixic acid to the rate of growth. An exponentially growing culture was treated with nalidixic acid (100 μg/ml) and portions were incubated at 37, 28, and 20 C.*

than proliferation in the corresponding control cultures.

Thus, we have shown that the bactericidal action of nalidixic acid can be stimulated or arrested by controlling the state of growth of the culture, i.e., by manipulation of incubation temperature or nutritional conditions, or by means of bacteriostatic agents such as chloramphenicol or dinitrophenol.

Considerations of the kinetics of bactericidal action. Examination of the kinetics of the bactericidal action of nalidixic acid shows that survival curves are characteristically biphasic (Fig. 6). Several possible explanations can be offered for the marked deceleration in the rate of bactericidal action. The surviving population could be composed of drug-resistant mutants or, alternatively, nutritionally deficient mutants unable to grow in this medium (+T, +AU). On the other hand, the surviving fraction might simply represent more slowly growing cells in the population.

The above possibilities were examined as follows. To test for the presence of drug-resistant mutants, samples of a culture of *E. coli* 15TAU treated with nalidixic acid (20 μg/ml) were plated in complex medium in the absence and presence of a similar concentration of drug. The presence of nutritionally deficient auxotrophs was examined by additional platings in synthetic agar (without drug).

Loss of viability, similar to that observed in previous experiments, occurred in the treated culture as shown by the viable counts in complex agar without drug (Table 2). Though the rate of bactericidal action declined, the treated cells continued to lose viability over the 6-hr experimental period. No colonies developed when as much as 1 ml of undiluted culture was plated on agar containing nalidixic acid. After 1 hr of treatment, the viable counts obtained in synthetic agar were slightly but consistently lower than those obtained in complex agar.

TABLE 2. *Viability of nalidixic acid-treated cultures as determined in the absence and presence of drug and in complex and synthetic media**

Treatment period	Viable count per ml		
	Complex agar		Synthetic agar, without drug
	Without drug	With drug	
hr			
0	1.1×10^8	None	1.0×10^8
1	7.5×10^7	None	7.8×10^7
2	3.1×10^7	None	2.1×10^7
3	9.3×10^5	None	8.8×10^5
4	8.9×10^4	None	7.1×10^4
6	7.4×10^3	None	5.7×10^3

* A culture of *Escherichia coli* 15TAU was treated with 20 μg/ml of nalidixic acid. Appropriate dilutions were plated in complex (Tryptone Glucose Extract Agar) and in synthetic (basal glucose-salts medium with added thymine, L-arginine, and uracil containing 1.5% agar) media. Undiluted 1.0-ml portions of the culture were plated in the complex agar containing 20 μg/ml of nalidixic acid.

DISCUSSION

Several pertinent points are evident from the data presented here. The bactericidal action of nalidixic acid on *E. coli* can be controlled by the regulation of protein and RNA synthesis. This was achieved by the use of bacteriostatic inhibitors, selected nutritional conditions, or temperature-regulated growth rates. It clearly is evident that the primary biochemical effect, inhibition of DNA synthesis, can be distinguished from the bactericidal action of the drug. Under conditions restricting protein and RNA synthesis, (+T, −AU), DNA synthesis is blocked, but there is no detectable loss of viability. In nutritionally complete medium, (+T, +AU), both chloramphenicol, which blocks protein synthesis, and dinitrophenol, which uncouples oxidative phosphorylation, effectively arrest the bactericidal action of nalidixic acid. It is evident also that chloramphenicol does not prevent entry of nalidixic acid into the cell. DNA synthesis is blocked by nalidixic acid in cultures pretreated with chloramphenicol. This fact suggests that uptake of nalidixic acid by *E.*

coli is not dependent on the synthesis of a permease.

The data presented here, furthermore, systematically rule out any primary action of nalidixic acid on the "initiator" of DNA synthesis. Inhibition of DNA synthesis is proportional to the concentration of nalidixic acid, even at sublethal doses. Removal of the drug from treated cultures results in immediate restoration of DNA synthesis. Retreatment of the same culture results in immediate inhibition of DNA synthesis. These results clearly demonstrate that the action of nalidixic acid on *E. coli* differs from that of phenethyl alcohol.

The deceleration of the rate of viability loss (Fig. 6) is not a result of selection of a resistant population. Furthermore, the cells are still vulnerable to the action of the drug, since they continue to die during prolonged incubation (Fig. 3 and Table 1). Several explanations can be offered for the presence of the surviving fraction. We have shown that treatment of a culture growing at a decreased rate results in a decreased rate of viability loss. If, among the originally treated population, there were cells with very slow growth rates, such cells might not be killed during the experimental period. It is also possible that some cells were growing very slowly as a consequence of drug treatment. As an alternative hypothesis, a portion of the treated cells may be better able to repair drug-imposed injuries and eventually form colonies on agar plates.

The presence of nutritionally deficient auxotrophs is suggested by the lower plate counts obtained in synthetic, as compared with complex, agar. If not present initially in the treated population, such cells may represent mutants arising as a consequence of drug action. Alternatively, nalidixic acid-damaged cells may be able to repair the lesions more efficiently on the complex than on the synthetic agar. Such a situation, though not inconceivable, would contrast with reports showing more efficient recoveries of *E. coli* in minimal media after treatment with ultraviolet (1, 5, 9, 11) or nitrogen mustard (4). Further investigations are being carried out to examine these possibilities.

The results of the drug-removal studies suggest that nalidixic acid is not firmly bound to sensitive cellular sites. Washing the cells may, therefore, dilute the drug to ineffective intracellular levels permitting resumption of proliferation. Additionally, if a repair mechanism is involved in recovery, the cells appear capable, once the drug is removed, of rapidly repairing the damaged site.

ACKNOWLEDGMENT

We acknowledge the advice and encouragement of E. W. Dennis and the technical assistance of Mary Walas.

LITERATURE CITED

1. ALPER, T., AND N. E. GILLIES. 1960. The relationship between growth and survival after irradiation of *Escherichia coli* strain B and two resistant mutants. J. Gen. Microbiol. **22**:113–128.
2. GOSS, W. A., W. H. DEITZ, AND T. M. COOK. 1964. Mechanism of action of nalidixic acid on *Escherichia coli*. J. Bacteriol. **88**:1112–1118.
3. GOSS, W. A., T. M. COOK, AND W. H. DEITZ. 1965. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. **89**:1068–1074.
4. HAROLD, F. M., AND Z. Z. ZIPORIN. 1958. Effect of nitrogen and sulfur mustard on nucleic acid synthesis in *Escherichia coli*. Biochim. Biophys. Acta **28**:482–491.
5. HILL, R. F., AND E. SIMSON. 1961. A study of radiosensitive and radioresistant mutants of *Escherichia coli* strain B. J. Gen. Microbiol. **24**:1–14.
6. HURWITZ, C., AND C. L. ROSANO. 1962. Chloramphenicol-sensitive and -insensitive phases of the lethal action of streptomycin. J. Bacteriol. **83**:1202–1209.
7. MAALØE, O., AND P. C. HANAWALT. 1961. Thymine deficiency and the normal DNA replication cycle. I. J. Mol. Biol. **3**:144–155.
8. NAKADA, D. 1960. Involvement of newly-formed protein in the synthesis of deoxyribonucleic acid. Biochim. Biophys. Acta **44**:241–244.
9. ROBERTS, R. B., AND E. ALDOUS. 1949. Recovery from ultraviolet irradiation in Escherichia coli. J. Bacteriol. **57**:363–375.
10. TREICK, R. W., AND W. A. KONETZKA. 1964. Physiological state of *Escherichia coli* and the inhibition of deoxyribonucleic acid synthesis by phenethyl alcohol. J. Bacteriol. **88**:1580–1584.
11. WOODY, P. L., J. D. MANDEL, AND J. GREENBERG. 1961. Resistance and cross-resistance of *Escherichia coli* mutants to anticancer agents. Radiation Res. **15**:290–297.