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

Printed in U.S.A.

Mechanism of Action of Nalidixic Acid on *Escherichia coli*

IV. Effects on the Stability of Cellular Constituents

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Received for publication 15 September 1965

ABSTRACT

COOK, THOMAS M. (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), WILLIAM H. DEITZ, AND WILLIAM A. GOSS. Mechanism of action of nalidixic acid on *Escherichia coli*. IV. Effects on the stability of cellular constituents. J. Bacteriol. 91:774–779. 1966.—Treatment of *Escherichia coli* 15TAU with nalidixic acid resulted in degradation of the nucleic acids of the cells, whereas protein was unaffected. Deoxyribonucleic acid (DNA) degradation appeared to be more extensive than ribonucleic acid degradation during periods of comparable bactericidal action. The onset of DNA degradation was evident prior to a measurable bactericidal effect. However, within the range of 2 to 20%, DNA degradation was accompanied by a decrease in viable cell numbers. Degradation of DNA to acid-soluble material occurred only under conditions permitting the bactericidal action of nalidixic acid. Arrest of the bactericidal action of nalidixic acid by the addition of dinitrophenol or chloramphenicol also inhibited DNA degradation. The acid-soluble products, which were excreted into the medium, have not been characterized completely, but probably were not phosphorylated.

Nalidixic acid exerts a bactericidal effect upon proliferating (but not upon nonproliferating) cultures of a susceptible organism, such as *Escherichia coli*. Associated with this lethal effect is a rather specific inhibition of deoxyribonucleic acid (DNA) synthesis (4). Nalidixic acid strongly inhibits the incorporation of radioactive precursors (adenine and uracil) into DNA, but has much less effect on the simultaneous incorporation of these precursors into ribonucleic acid (RNA; 5).

Other bactericidal agents, such as mitomycin C, phleomycin, and edeine, also are known to impose a relatively selective inhibition of DNA synthesis (7, 9, 11). The blockage of DNA synthesis by mitomycin C is accompanied by depolymerization of the DNA (but not RNA) of the cell and loss of the resulting degradation products into the surrounding medium (8). In contrast, no such degradation of DNA was observed in thymineless death (8) or with edeine treatment (7).

It was found previously (4) that the bactericidal action of nalidixic acid upon uniformly labeled cells preceded the release of radioactivity into the medium. This could be interpreted as ruling out rapid and extensive membrane damage similar to that produced by quaternary ammonium compounds. However, these data provided no information on the stability of individual cellular constituents during the early stages of drug action. This report presents the results of studies on the effects of nalidixic acid on the integrity of macromolecular cell components of *E. coli*.

MATERIALS AND METHODS

Bacterial cultures. The methods used for the cultivation of *E. coli* 15TAU and *E. coli* 198 and for changes of media have been described previously (4). In describing nutritional conditions, (+T, −AU) will refer to a medium containing thymine, but not arginine or uracil; similarly, (+T, +AU) will refer to complete growth medium containing thymine, arginine, and uracil.

Stability of macromolecules during bactericidal action of nalidixic acid. Cultures of *E. coli* 15TAU were labeled specifically in the protein, RNA, and DNA fractions by incubation for not less than three generations in the presence of 0.1 μc/ml of C^{14}-labeled L-arginine, uracil, and thymine, respectively. The cells were washed and transferred to fresh unlabeled medium for 15 min to permit utilization of residual radioactivity in the metabolic pool. The labeled cultures then were incubated in the presence and absence of nalidixic acid. To determine the total

amount of radioactivity which had been rendered acid-soluble, samples of whole cultures were extracted with cold perchloric acid (0.25 N, final concentration). Acid-insoluble material and perchlorate (after neutralization with KOH) were removed by membrane filtration, and radioactivity was determined with a liquid scintillation spectrometer (Packard Instrument Corp., Chicago, Ill.). To determine the amount of material which had been excreted into the medium, samples were filtered without acid treatment, and the radioactivity in the filtrate was measured.

Examination of the excreted material by paper electrophoresis was carried out as follows. After removal of cells by centrifugation, the spent medium was treated with acid-washed charcoal. The adsorbed material was eluted from the charcoal with ammoniacal ethyl alcohol (50% ethyl alcohol adjusted to *p*H 9.0 with ammonia), concentrated by lyophilization, and dissolved in a small volume of distilled water. A sample of this material was spotted on Whatman no. 1 filter paper moistened with 0.1 M citrate buffer (*p*H 5.0) and subjected to a potential of 700 v for 90 min. After drying, the location of radioactive spots was determined by use of the Vanguard 880 Autoscanner (Vanguard Instrument Co., LaGrange, Ill.).

The DNA of *E. coli* 198 was labeled by incubation for four generations in the presence of 5 μg/ml of 5-fluorodeoxyuridine (FUDR) and C^{14}-labeled thymidine (0.1 μc/ml) plus uracil (10 μg/ml) and thymine (13 μg/ml). Subsequent handling was as described above.

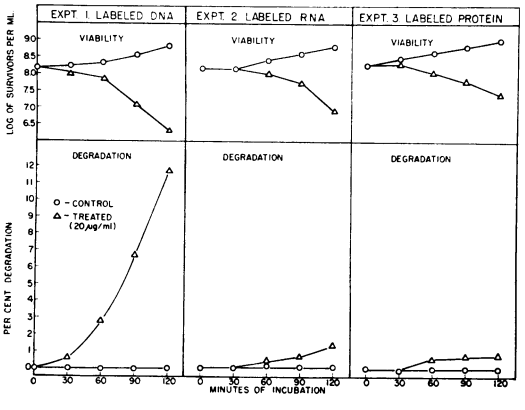

FIG. 1. *Stability of cellular constituents of Escherichia coli 15TAU exposed to nalidixic acid. In three separate experiments, the degradation of radioactive DNA, RNA, and protein, respectively, to acid-soluble materials was monitored during incubation of the prelabeled cultures in the presence and absence of nalidixic acid (20 μg/ml). The per cent degradation was calculated from the increase in acid-soluble radioactivity as a fraction of the initial acid-insoluble radioactivity. The initial level of acid-insoluble C^{14}-thymine, uracil, and arginine was found to be 4,900, 19,057, and 6,179 count/min per ml of culture, respectively.*

Chemicals. The following radioactive chemicals were obtained from New England Nuclear Corp., Boston, Mass.: thymine-2-C^{14} (45.4 mc/mmole), thymidine-2-C^{14} (30 mc/mmole), uracil-2-C^{14} (27.8 mc/mmole), and uniformly C^{14}-labeled L-arginine (222 mc/mmole). Chloramphenicol was obtained from Parke, Davis & Co., Detroit, Mich. FUDR was a gift from J. Berger, Hoffman-La Roche, Inc., Nutley, N.J.

RESULTS

Stability of DNA, RNA, and protein of nalidixic acid-treated cells. When cultures of *E. coli* 15TAU labeled specifically in the DNA, RNA, or protein fractions were exposed to a bactericidal concentration of nalidixic acid (20 μg/ml), it was found that there was a pronounced degradation of nucleic acids (Fig. 1). After an exposure of 120 min, during which time approximately 99% of the cells were rendered nonviable, approximately 13% of the initial acid-insoluble C^{14}-thymine (DNA) had been degraded into acid-soluble products. In the experiment with RNA-labeled cells, it appeared that RNA degradation (approximately 2%) was not as extensive as DNA degradation during a period of roughly equivalent loss of viability. Stability of protein was essentially unaffected by exposure to nalidixic acid.

TABLE 1. *Excretion of DNA degradation products by Escherichia coli 15TAU during bactericidal action of nalidixic acid**

Count/min per ml of C^{14}-thymine				
	Control		Treated (20 μg/ml of nalidixic acid)	
Time	Total acid-soluble material	Cell-free filtrate	Total acid-soluble material	Cell-free filtrate
min				
30	34	29	36	27
60	67	18	60	42
90	36	13	96	79
120	28	23	163	134
180	21	22	298	281
240	19	16	439	452
300	15	24	673	589
360	17	24	837	808

* A culture of *E. coli* 15TAU prelabeled in the DNA fraction by growth in the presence of C^{14}-thymine (0.1 μc/ml) was washed and transferred to nonradioactive growth medium. The initial acid-insoluble C^{14}-thymine in the resulting culture was 1,380 count/min per ml. Nalidixic acid was added to one portion of culture to a final concentration of 20 μg/ml. The total acid-soluble radioactivity and the amount excreted into the cell-free filtrate were determined.

TABLE 2. *Degradation of DNA in cultures of Escherichia coli 198 treated with nalidixic acid**

Nalidixic acid	Viable cells (per cent of original inoculum)	DNA degraded
μg/ml		%
0	355	0
5	49	9
25	5	20
100	6	25

* The DNA of a culture of *E. coli* 198 was labeled by growth in the presence of FUDR (5 μg/ml) and C^{14}-labeled thymidine (0.1 μc/ml). The culture was transferred to nonradioactive medium with and without nalidixic acid. After 120 min, the viable count and the amount of radioactivity rendered acid-soluble were determined.

The DNA degradation products did not build up within the cells, but rather were excreted into the surrounding medium (Table 1). After 360 min of exposure to 20 μg/ml of nalidixic acid, the number of viable cells was reduced to less than 1% of the original value. At this time, approximately 61% of the labeled DNA had been degraded to acid-soluble material. The bulk of this radioactivity could be accounted for by excreted material. Examination of the excreted material by paper electrophoresis (0.1 M citrate buffer, *p*H 5.0) indicated that it was not in the form of a deoxynucleotide. In contrast to the phosphorylated derivatives of thymidine, the radioactivity remained at the origin. This indicates that the excreted material was probably thymidine or thymine, but this material has not been characterized further.

The possibility that the observed instability of DNA in nalidixic acid-treated *E. coli* 15TAU could be due to some peculiarity of this strain was checked. The DNA in *E. coli* 198, a wild-type organism, was labeled specifically in the DNA fraction by growth in the presence of FUDR and C^{14}-labeled thymidine. When these cells were subsequently washed and transferred to fresh medium containing nalidixic acid (25 μg/ml), the bactericidal action of the drug was accompanied by degradation of the DNA (Table 2).

Correlation of bactericidal action and DNA degradation. Next, the effect of varying concentrations of nalidixic acid on DNA degradation in *E. coli* 15TAU was examined. Both the loss of viability and the degradation of DNA to acid-soluble material were correlated with the concentration of nalidixic acid employed (Fig. 2). To facilitate comparison of DNA degradation

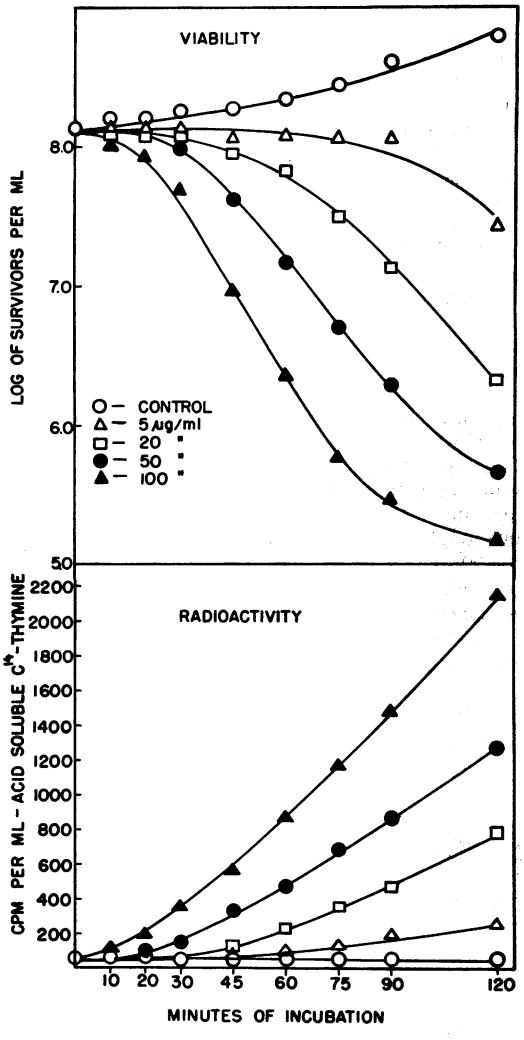

FIG. 2. *DNA degradation in Escherichia coli 15TAU as a function of nalidixic acid concentration.*

and loss of viability, the data of Fig. 2 have been redrawn in Fig. 3. It is apparent in Fig. 3 that loss of viability paralleled the degradation of DNA over a wide range.

Previously, we had shown that the inhibition of DNA synthesis by nalidixic acid in the nutritionally deficient medium (+T, −AU), did not lead to the death of the cells (5). Therefore, it was of considerable interest to compare the lethal action of the drug and DNA degradation in complete and deficient media.

The results of such a comparison are shown in Fig. 4. In the complete medium (+T, +AU), the untreated control culture proliferated and no DNA degradation occurred, whereas the cul-

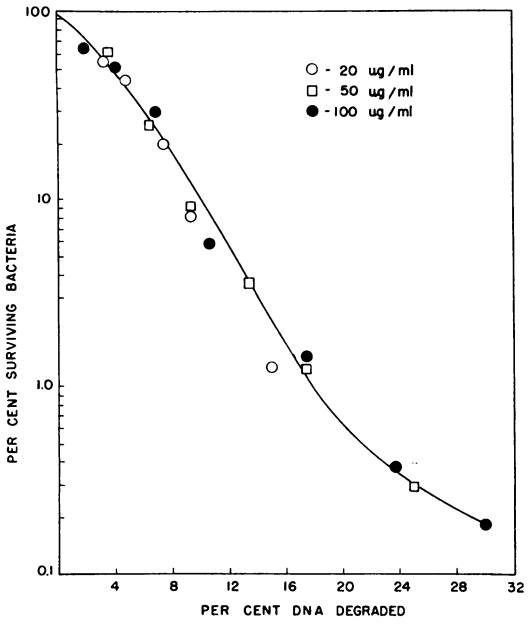

FIG. 3. *Correlation of bactericidal action and DNA degradation in Escherichia coli 15TAU. These data are taken from Fig. 2.*

ture treated with nalidixic acid (20 μg/ml) began to lose viability after a 60- to 75-min lag. In this case, degradation of the DNA in the treated culture was clearly evident before any detectable loss of viability.

In contrast, the culture treated with nalidixic acid in the deficient medium (+T, −AU) showed no loss of viability and only a negligible increase in the amount of acid-soluble radioactivity. Similar results were obtained also when either uracil or arginine was omitted singly from the medium.

It has been found that bacteriostatic compounds such as dinitrophenol or chloramphenicol will arrest the bactericidal action of nalidixic acid (5). It was of interest to determine whether these agents also had an effect on DNA degradation. As shown in Fig. 5, the addition of dinitrophenol (1 mg/ml) or chloramphenicol (20 μg/ml) to a culture of *E. coli* 15TAU did, in fact, decrease both the loss of viability and the degradation of DNA caused by nalidixic acid (20 μg/ml).

DISCUSSION

The bactericidal action of nalidixic acid now may be described in the following manner. Nalidixic acid rapidly inhibits DNA synthesis in growing cells of *E. coli*. This inhibition renders the DNA of such cells vulnerable to attack by

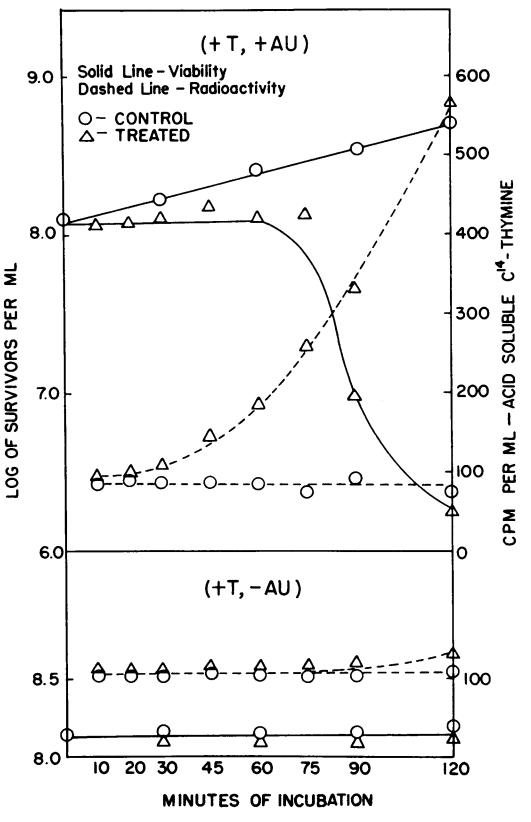

FIG. 4. *Requirement for RNA and protein synthesis for DNA degradation in cultures of Escherichia coli 15TAU treated with nalidixic acid. A culture prelabeled by growth in C^{14}-thymine was transferred to complete (+T, +AU) and nutritionally deficient (+T, −AU) media, with and without nalidixic acid (20 μg/ml).*

endogenous nucleases, and the genetic material of the cell is ultimately destroyed. Whether DNA degradation occurs in all strains of *E. coli* is uncertain. It has been found (Bouck and Adelberg, *personal communication*) that DNA degradation is not as extensive in some strains of *E. coli* as in those strains used for our studies. We confirmed this finding and will subsequently present a more extensive comparison of strain differences.

That DNA degradation is not an inevitable consequence of the action of nalidixic acid is evident from the results in nutritionally deficient medium. Under these conditions, it has been possible to separate the primary inhibition of DNA synthesis from the secondary bactericidal effect (5). Although DNA synthesis in *E. coli* 15TAU is blocked by nalidixic acid in the (+T, −AU) medium, there is no detectable loss of viability and essentially no degradation of DNA.

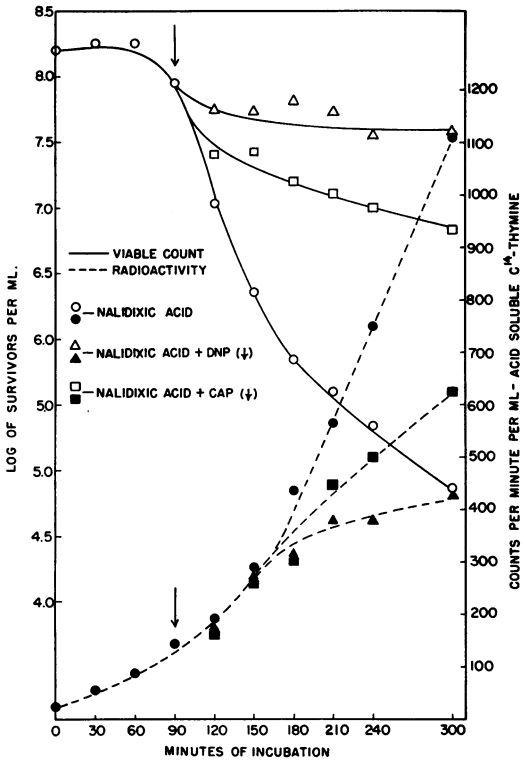

FIG. 5. *Inhibition by 2,4-dinitrophenol (DNP, 1 mg/ml) and chloramphenicol (CAP, 20 μg/ml) of DNA degradation in Escherichia coli 15TAU treated with nalidixic acid (20 μg/ml).*

It is interesting to compare the effects of nalidixic acid and mitomycin C. It has been reported that mitomycin C causes a degradation of DNA, but not RNA, of treated cells (8). However, a subsequent study has indicated that some degradation of RNA occurs in mitomycin C-treated cells, and indeed may be responsible for the release of RNA-bound deoxyribonuclease (6). We find some RNA degradation in nalidixic acid-treated cells of *E. coli* 15TAU. Mitomycin C, which has been shown to cause cross-linkage of the DNA strands, will rapidly kill *Bacillus subtilis* at a time when no depolymerization of DNA is observed (10). This is taken to indicate that the mitomycin-induced breakdown of DNA is an accessory phenomenon, only secondarily related to the lethal effect. DNA breakdown induced by mitomycin is enhanced when protein synthesis is blocked by chloramphenicol (2). In contrast, we find chloramphenicol to restrict DNA degradation and loss of viability in nalidixic acid-treated cells.

In *E. coli*, an interesting interrelationship has been demonstrated between sensitivity to ultraviolet (UV) irradiation and mitomycin C, that is, the ability to excise photoproducts from irradiated DNA, and DNA-degradation after treatment with either agent (1). Enhanced sensitivity to UV irradiation results from a mutation at any of three genetic loci (the *uvr* loci). Such sensitive mutants (*uvr*$^-$ mutants) show a collateral sensitivity to mitomycin C. These mutants are unable to excise thymine dimers from irradiated DNA, and show much less DNA degradation after treatment with either UV light or mitomycin C. From this it has been inferred that *uvr*$^-$ mutants lack the enzymes necessary to repair defective regions of DNA. The repair mechanism is thought to involve enzymatic excision of defective portions, with local single-strand degradation being initiated at the resulting gaps in the phosphodiester backbone. This is followed by resynthesis of the missing portions. It would be of interest to know whether or not the DNA degradation observed after nalidixic acid treatment is also genetically controlled.

We would like to point out explicitly that the present results caution against the uncritical use of nalidixic acid as a specific inhibitor of DNA synthesis in biochemical and genetic studies. Unless the degradation of DNA is controlled, treatment of cultures with nalidixic acid to prevent DNA synthesis may lead to anomalous results. It is possible to control DNA degradation in *E. coli* 15TAU by the use of the (+T, −AU) medium or by chloramphenicol. Of course, however, these conditions impose additional inhibitions of RNA and protein synthesis.

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