JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 1112-1118 October, 1964
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MECHANISM OF ACTION OF NALIDIXIC ACID ON *ESCHERICHIA COLI*

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Received for publication 8 May 1964

ABSTRACT

GOSS, WILLIAM A. (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), WILLIAM H. DIETZ, AND THOMAS M. COOK. Mechanism of action of nalidixic acid on *Escherichia coli*. J. Bacteriol. **88**:1112-1118. 1964.—Nalidixic acid was lethal for proliferating cultures of *Escherichia coli*. Associated with this lethal effect was the formation of elongated, serpentine forms. Cultures treated with nalidixic acid were osmotically stable; lethality was observed in the presence of stabilizers. Although it was possible to demonstrate leakage of intracellular components from treated cells, this effect occurred only after 99% of the cells were nonviable. Nalidixic acid had little or no effect on respiration with glucose as substrate. If cellular growth was restricted by suboptimal temperature or nutritional deficiencies, the drug was not lethal. Chemical analysis of cellular constituents revealed that lipid, protein, and ribonucleic acid levels were of the same order of magnitude in control and drug-treated cells. Only deoxyribonucleic acid (DNA) levels were markedly lowered in drug-treated cells. These facts are consistent with the view that nalidixic acid interferes with the synthesis of *E. coli* DNA.

Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) is a new antibacterial agent, the synthesis of which was reported by Lesher et al. (1962). This naphthyridine has a broad spectrum of antibacterial activity; is especially active against gram-negative species, including those associated with urinary-tract infections (Deitz, Bailey, and Froelich, 1964); and is very well tolerated by laboratory animals and man (Lishman and Swinney, 1963). This report presents the results of preliminary studies to determine the mechanism of action of nalidixic acid.

We examined the effect of this compound on the growth, motility, respiration, integrity of the cell membrane and cell wall, and chemical composition of *Escherichia coli*.

MATERIALS AND METHODS

Cultures. E. coli strain 198 (ATCC 11229) and *E. coli* strain 15 ($T^-A^-U^-$; obtained from Department of Bacteriology, Rutgers University, New Brunswick, N.J.; hereafter referred to as *E. coli* 15 TAU) were maintained by periodic transfer on nutrient agar slants.

Growth studies. Except as noted below, experiments were carried out at 37 C with growing cultures in glucose (1%)-salts medium (medium C of Roberts et al., 1957). *E. coli* 15 TAU will not grow in this medium unless supplemented with thymine, L-arginine, and uracil (Kanazir et al., 1959), which we used at 2.0, 100.0, and 10.0 μg/ml, respectively. Flasks were inoculated with an overnight culture in the same medium to give an optical density (650 mμ) of 0.040 to 0.050, and were incubated with shaking in a water bath until the turbidity had approximately doubled [optical density (OD) = 0.090 to 0.110]. An OD of 0.100 was found to represent 10^8 to 1.2×10^8 viable cells per ml.

Bacterial viability was estimated by plating dilutions (in 1% peptone water) of bacterial cultures in Tryptone Glucose Extract Agar (Difco).

To determine the sensitivities of the cultures used in this study, a twofold tube dilution test was employed. Various amounts of nalidixic acid (in a volume of 0.1 ml) were added to 2 ml of glucose (1%)-salts medium inoculated with an overnight culture (1:1,000). After 16 to 18 hr at 37 C, the lowest concentration preventing visible turbidity was recorded as the minimal inhibitory concentration (MIC).

Photomicroscopy. Smears, prepared from 3-hr cultures of *E. coli* 15 TAU, were stained with gentian violet and photographed with a Zeiss Ultraphot II microscope on Panatomic-X film (Eastman Kodak Co., Rochester, N.Y.).

Spheroplast studies. Cells of *E. coli* 198 were exposed to nalidixic acid (25 μg/ml) in Tryptose Phosphate Broth with 20% sucrose and 0.008 M Mg^{++} as spheroplast stabilizers (Lederberg,

1956). Control cultures and cultures treated with penicillin G (1,000 units per ml) were also studied. After 4 hr, samples were diluted in 1% peptone water and in 1% peptone water plus stabilizers, and viability was determined in Tryptone Glucose Extract Agar containing stabilizers. Cell suspensions also were examined microscopically.

"Leakage" of radioactive materials. Cells from an overnight culture were centrifuged, washed, and resuspended in fresh medium containing 0.1% uniformly labeled C^{14}-glucose (0.27 μc/mg; New England Nuclear Corp., Boston, Mass.), and were incubated for 3.5 hr. The cells were centrifuged, washed with nonlabeled medium, and resuspended in fresh nonlabeled glucose-salts medium with and without nalidixic acid; incubation was then continued. At intervals thereafter, viable counts were made of each suspension. Concurrently, portions were filtered through membrane filters of 0.45-μ porosity (Millipore Corp., Bedford, Mass.), and the radioactivity in the filtrates was determined with a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Corp., Chicago, Ill.). Counting efficiency in this system was 42.2%.

Respiration studies. Standard manometric techniques (Umbreit, Burris, and Stauffer, 1957) were used to determine respiration rates of washed-cell suspensions prepared from young cultures (4 hr) of *E. coli* 15 TAU. Each Warburg vessel received 1.5 mg of bacterial protein, 100 μmoles of phosphate buffer (pH 7.0), 20 μmoles of glucose (tipped in after 5 min), and 0.2 ml of 20% KOH (in center well), in a total volume of 3.0 ml. Oxygen consumption was recorded at 37 C before and after the addition of inhibitors. Respiration rates were calculated as $Q_{O_2}(N)$.

Chemical analysis of cells. A culture of *E. coli* 198 grown to an OD (650 mμ) of 0.200 was divided into two portions, and nalidixic acid (2.5 μg/ml) was added to one. Immediately after addition of drug, and at 30-min intervals thereafter, 100-ml samples were withdrawn, chilled with ice, and centrifuged. The washed cells were fractionated by the method of Schneider (1957), except that total lipids were extracted by the method of Kaneshiro and Marr (1962). Lipid, nucleic acid, and protein determinations were made on the solvent-soluble, hot trichloroacetic acid-soluble, and hot trichloroacetic acid-insoluble fractions, respectively. Lipid phosphorus was estimated by the Fiske and SubbaRow technique described by Leloir and Cardini (1957). Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined by pentose analysis, with the use of the diphenylamine and orcinol methods described by Schneider (1957). Protein was measured by the method of Lowry et al. (1951).

RESULTS

Growth inhibition of E. coli by nalidixic acid. As reported previously (Deitz et al., 1964), nalidixic acid inhibits the growth of *E. coli*. With the tube dilution test, the MIC values for the two cultures employed were found to be 1.2 μg/ml for *E. coli* 198 and 2.3 μg/ml for *E. coli* 15 TAU.

Next, the effect of nalidixic acid on growth of *E. coli* was examined in more detail by use of shake-flask cultures. Under the experimental conditions, control cultures showed a linear increase in the logarithm of the viable cell count during the first 180 min of incubation. Generation times under these conditions were estimated to be approximately 39 min for *E. coli* 198 and 47 min for *E. coli* 15 TAU. When such cultures were treated with low levels of nalidixic acid (0.5 to 1.0 MIC), the growth rates were markedly reduced. At higher levels, there were drastic reductions in the viable populations. Loss of viability began earlier with higher concentrations (Fig. 1). At the highest levels used, the lethal effect of nalidixic acid was evident within one generation time. Despite the rapid loss of viability, there were no corresponding decreases in the turbidities of the treated cultures. It was noted that, at some levels of nalidixic acid which caused a profound decrease in viability, there was actually an increase in turbidity.

Morphological changes in nalidixic acid-treated cells. Microscopic examination of cultures exposed to a lethal concentration of nalidixic acid revealed extremely elongated, serpentine forms. The development of elongated cells of *E. coli* 15 TAU as a function of concentration is shown in Fig. 2.

After 3 hr of incubation in the presence of 0.3 μg/ml (Fig. 2B), most of the cells were similar to those of the control culture (Fig. 2A). At 1.0 and 3.0 μg/ml, respectively, the majority of the cells were elongated many times their normal length (Fig. 2C and 2D). At the highest concentration used in this particular study (10 μg/ml), relatively few cells could be located per field, and these were greatly elongated (Fig. 2E).

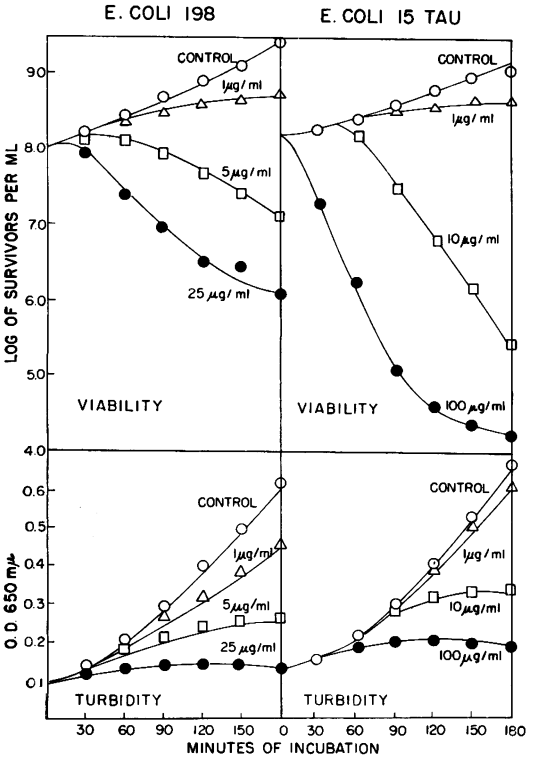

FIG. 1. *Growth inhibition of Escherichia coli by nalidixic acid: comparison of viability and turbidity.*

The elongated cells, although motile, were nonviable when diluted and plated on agar. Attempts then were made to determine whether these cells were osmotically fragile.

Osmotic fragility. Plate counts of control cultures grown in the presence of osmotic stabilizers increased threefold during the 4-hr experimental period, whereas a fourfold reduction was observed in cultures exposed to nalidixic acid (25 μg/ml). However, no changes in the viable counts of these cultures were observed when they were subjected to osmotic shock.

The viability of osmotically stabilized cultures treated with penicillin G (1,000 units per ml) decreased 40-fold. However, in contrast to the nalidixic acid-treated cultures, subjection of these cultures to osmotic shock resulted in a 4,000-fold decrease in viability (Table 1).

Microscopic examination during the course of the experiment revealed only elongated cells in the nalidixic acid-treated culture and no spheroplasts. Conversely, spheroplasts but no elongated cells were observed in the penicillin-treated culture.

Membrane damage. Cultures of *E. coli* 15 TAU labeled by growth in the presence of radioactive glucose were treated with nalidixic acid (100 μg/ml). After 90 min of exposure, the viable count of the control culture had increased 2.5-fold whereas the viable count of the drug-treated culture had decreased tenfold. During this same period, there were no differences in the radioactivity of cell-free filtrates from treated and untreated cultures. Not until the viable count had decreased 100-fold (120 min post-treatment) was there an increase in the rate of release of radioactivity from the treated cells. Thus, there was no apparent effect on membrane integrity during the period of rapid loss of viability (Fig. 3).

Effect of nalidixic acid on respiration. The respiration of washed-cell suspensions of *E. coli* 15 TAU was only slightly affected by nalidixic acid. With glucose as substrate, concentrations as high as 7.5 mg per vessel (33 μmoles) inhibited respiration by only 25%. At 1.5 mg per vessel (6.5 μmoles), there was no inhibition. In contrast, the respiration of such suspensions was inhibited some 60% by iodoacetate (10 μmoles; Table 2).

Conditions required for the action of nalidixic acid. The time at which cells exposed to nalidixic acid began to die coincided with the onset of vigorous growth of control cultures. This suggested that active growth is a prerequisite for drug action. To explore this possibility, the effect of nalidixic acid was examined in a nitrogen-free medium and at suboptimal growth temperatures. *E. coli* 198 was depleted of endogenous nitrogen pool by incubation at 37 C in the glucose-salts medium without NH_4Cl until no change in turbidity was observed. At this time, NH_4Cl (to 0.2%) was added to control cultures placed at 37 C. Nalidixic acid (10 μg/ml) was added to two similar portions, one of which was incubated at 2 to 4 C rather than at 37 C. A fourth portion received nalidixic acid but not NH_4Cl, and was incubated at 37 C. The numbers of viable cells in treated and control cultures were determined after 0, 1, 2, and 4 hr of incubation. The results showed that nongrowing cells are unaffected by nalidixic acid (Table 3).

To extend these observations, the action of nalidixic acid on *E. coli* 15 TAU was examined under various nutritional conditions. Young growing cultures were harvested, washed, and resuspended in the fully supplemented glucose-salts medium (added thymine, L-arginine, and uracil) and in modified medium with one or more of the

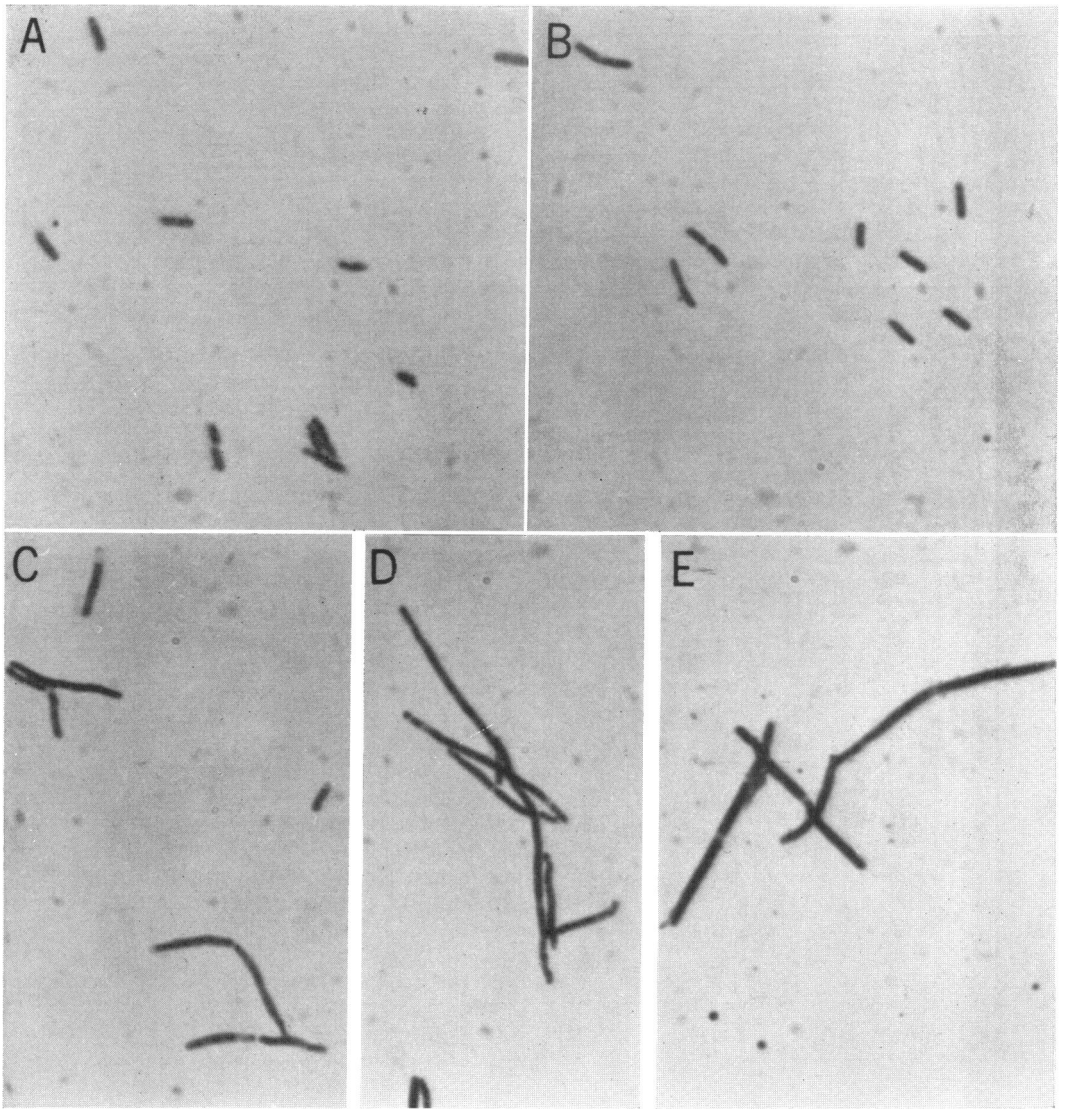

FIG. 2. *Morphological changes induced in cells of Escherichia coli 15 TAU by nalidixic acid. Smears were prepared from 3-hr cultures, stained with gentian violet, and photographed with a Zeiss Ultraphot II microscope. Magnification 1,800 ×. (A) control, (B) 0.3 μg/ml, (C) 1.0 μg/ml, (D) 3.0 μg/ml, (E) 10.0 μg/ml.*

supplements omitted. Samples were withdrawn at intervals from treated (10 μg/ml of nalidixic acid) and control cultures for viable-cell counts.

In complete medium without nalidixic acid, the number of viable cells increased from 10^8 to 1.2×10^9 per ml during the 3.5 hr of the experiment. In this case, the average generation time was approximately 60 min. However, when thymine was omitted from the culture, the cells died rapidly. After the onset of death. approximately 90% of the cells became nonviable within one generation time (Fig. 4).

When cells were incubated in the complete medium in the presence of nalidixic acid, there was a rapid loss of viability (Fig. 4). After a 30-min delay, death of the cells began at an accelerated rate which, by 90 min, was exponential. Approximately 90% of the cells were killed in one

TABLE 1. *Comparison of the viable counts of stabilized and osmotically shocked cultures**

Exposure	Controls		Treated			
	Stabilized	Shocked	Nalidixic acid		Penicillin G	
			Stabilized	Shocked	Stabilized	Shocked
hr						
0	4.0×10^8	4.1×10^8	4.0×10^8	4.1×10^8	4.0×10^8	4.0×10^8
4	12.0×10^8	12.0×10^8	1.1×10^8	0.9×10^8	1.3×10^7	1.0×10^5

* Cells of *Escherichia coli* 198 were exposed to nalidixic acid (25 μg/ml) or penicillin G (1,000 units per ml) in Tryptose Phosphate Broth containing 20% sucrose and 0.008 M Mg^{++} as spheroplast stabilizers. After 4 hr, cultures were diluted in 1% peptone water plus stabilizers or 1% peptone water without stabilizers (shocked), and were finally plated in Tryptone Glucose Extract Agar plus stabilizers.

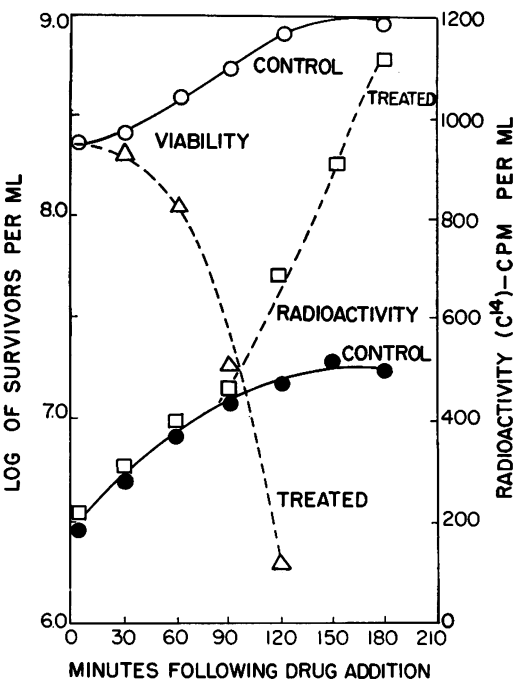

FIG. 3. *Loss of viability of Escherichia coli 15 TAU treated with nalidixic acid (100 μg/ml), and subsequent release of radioactive intracellular material.*

generation time. In the thymine-deficient medium, the death of the cells was almost identical in the presence or absence of nalidixic acid; that is, nalidixic acid exerted no lethal effect beyond that imposed by thymine deficiency itself.

The lethal effect of thymine deficiency is prevented by omission of arginine or uracil, or both (Kanazir et al., 1959; Maaløe, 1961). The pro-

TABLE 2. *Effect of nalidixic acid on respiration of Escherichia coli 15 TAU**

Inhibitor	Amt	$Q_{O_2}(N)$	
		Control	Inhibited
Nalidixic acid	1.5 mg (6.5 μmoles)	1,420	1,420
Nalidixic acid	7.5 mg (33 μmoles)	1,600	1,235
Iodoacetate	10 μmoles	1,420	467

* Each Warburg vessel received 1.5 mg of bacterial protein, 100 μmoles of phosphate buffer (pH 7.0), 20 μmoles of glucose (tipped in after 5 min), and 0.2 ml of 20% KOH (in center well), in a total volume of 3.0 ml. Oxygen consumption was recorded at 37 C before and after the addition of inhibitors.

TABLE 3. *Failure of nalidixic acid to affect the viability on nongrowing cultures of Escherichia coli**

Time of exposure	Control	Treated (10 μg/ml of nalidixic acid)		
	Complete (37 C)	Complete (37 C)	Complete (2 to 4 C)	Deficient (37 C)
hr				
0	2.2×10^8	—	—	—
1	2.3×10^8	2.2×10^8	2.0×10^8	2.2×10^8
2	5.4×10^8	8.9×10^7	2.5×10^8	2.3×10^8
4	2.4×10^9	9.8×10^6	2.1×10^8	1.9×10^8

* Nitrogen (as NH_4Cl to 0.2%) was added at time 0 to three portions of the depleted culture one of which was placed at 2 to 4 C (complete). A fourth portion received no nitrogen (deficient).

tective effect of multiple nutritional deficiencies on thymineless death is also shown in Fig. 4. Under these conditions of nutritional deficiency, nalidixic acid was not bactericidal.

From the lack of effect on cell wall, cell membrane, and respiration, the development of elongated forms, and the requirement for concomitant growth conditions, we were led to suspect an alteration of nuclear function. For this reason, we undertook a chemical analysis of nalidixic acid-treated cells.

Cellular composition. Cells of *E. coli* 198 grown in the presence and absence of nalidixic acid were fractionated and chemically analyzed for several major cellular constituents.

In the control culture, lipid, protein, RNA, and DNA increased about threefold during the 90-min incubation period. The ratio of DNA to protein remained relatively constant throughout the experiment. In the culture treated with nalidixic acid, only DNA failed to increase at the same rate as protein.

This specific inhibition of DNA synthesis is shown in Fig. 5. The increase (expressed as percentage of increase from time "0") in each cellular component is shown for the control and treated cultures. It is clear that the increase in lipid, protein, and RNA was of the same order of magnitude in both treated and control cultures. The increase in DNA, on the other hand, was much greater in the control cells than in those treated with nalidixic acid.

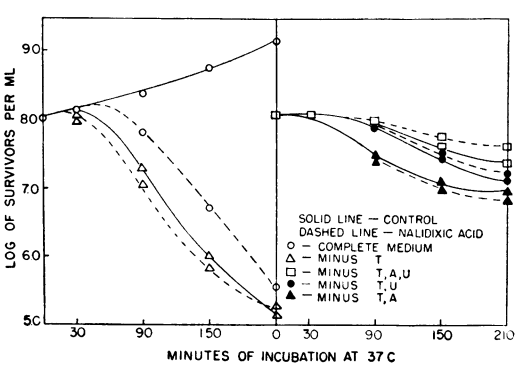

FIG. 4. *Survival of Escherichia coli 15 TAU exposed to nalidixic acid (10 μg/ml) in complete and deficient media. The complete medium was glucose (1%)-salts medium supplemented with 2 μg/ml of thymine (T), 100 μg/ml of L-arginine (A), and 10 μg/ml of uracil (U).*

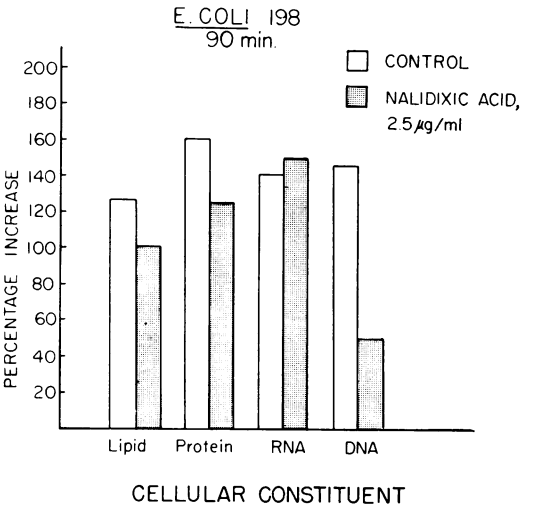

FIG. 5. *Chemical composition of control and nalidixic acid-treated (2.5 μg/ml) cultures of Escherichia coli 198, after incubation for 90 min.*

DISCUSSION

Nalidixic acid, even at low levels, exerts a bactericidal effect on *E. coli*. The lethal effect is manifested by an arrest of proliferation, accompanied by a distinctive morphological alteration of the susceptible cells. The drug is not lethal as long as growth is restricted by suboptimal temperature or nutritional deficiencies. The synchrony between the initiation of growth in control cultures and of death in treated cultures is clearly not coincidental. Chemical analysis of cellular constituents provides direct evidence of deficient nuclear (DNA) synthesis which, in the presence of competent cytoplasmic (RNA and protein) synthesis, results in unbalanced bacterial metabolism and death.

The development of extremely long, filamentous cells with concomitant loss of viability suggests that nalidixic acid acts in a manner similar to that induced by thymine deficiency (Cohen and Barner, 1954), by 5-fluorouracil deoxyriboside (Cohen et al., 1958), and, in certain cases, by vitamin B_{12} deficiency (Beck, Hook, and Barnett, 1962). These effects are observed under conditions otherwise permitting cellular proliferation, and are the consequence of intracellular desynchronization leading to unbalanced bacterial metabolism. The fact that there is little or no inhibition of respiration or synthesis of RNA or protein, and no direct effect on integrity of cell

wall or membrane, is consistent with the view that nalidixic acid blocks the synthesis of DNA.

Exposure of bacteria to low doses of ultraviolet light or to X rays, or to such agents as nitrogen mustards or mitomycin C, also causes the development of filamentous bacteria with concomitant loss of viability (Hughes, 1956; Billen, 1963; Reich, Shatken, and Tatum, 1961; Iyer and Szybalski, 1964). These agents act on and modify the integrity and nature of DNA; consequently, they affect both proliferating and nonproliferating cells. They are, for the most part, nonselective in their action, and are toxic to a wide variety of cells, including bacteria, fungi, and mammalian cells.

It becomes quite apparent that nalidixic acid is distinctly different from the above-mentioned agents that act on DNA. The fact that nalidixic acid is well tolerated by experimental laboratory animals and by man emphasizes the selectivity of this chemotherapeutic agent for certain bacterial cells.

The data presented here provide strong presumptive evidence that nalidixic acid exerts its primary action on the synthesis of DNA. Investigations are being continued to define the action of this drug in greater detail.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Hans P. Drobeck of this institute for the photomicroscopic studies. The excellent technical assistance of M. A. Walas is gratefully acknowledged.

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