Inhibition of Ribonucleic Acid Synthesis by Nalidixic Acid in *Escherichia coli*

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The effect of low concentrations of nalidixic acid on ribonucleic acid (RNA) synthesis in *Escherichia coli* was examined. It was observed that RNA synthesis in exponentially growing cells was not significantly affected, in harmony with previous studies. However, RNA synthesis was markedly depressed by nalidixic acid during starvation for an amino acid or during chloramphenicol treatment. This effect was not caused by increased killing or inhibition of nucleoside triphosphate synthesis by nalidixic acid. The pattern of radioactive uracil incorporation into transfer RNA or ribosomes was not changed by the drug. The sensitivity of RNA synthesis to nalidixic acid in the absence of protein production may be useful in probing the amino acid control of RNA synthesis.

The antibacterial agent nalidixic acid (NAL), markedly inhibits the synthesis of deoxyribonucleic acid (DNA), whereas ribonucleic acid (RNA), protein, and lipid production are affected to a considerably lesser degree (8, 20). NAL treatment of *Escherichia coli* elicits responses similar to thymine deprivation, and therefore this drug is frequently used to arrest DNA synthesis specifically (4). The effect of NAL on macromolecular synthesis in nongrowing cells, however, has not been extensively investigated. Inhibition of DNA synthesis is reported to exist even in the presence of chloramphenicol (6). But the effect of NAL on the formation of RNA or lipid in the absence of protein synthesis has not been explored. The subject of this report is the effect of NAL on RNA synthesis when protein production is halted. We find that, in the absence of net protein synthesis, NAL exerts a considerably greater inhibitory effect on RNA synthesis than in logarithmically growing cells. This observation may be helpful in unraveling the mode of amino acid control of RNA synthesis.

MATERIALS AND METHODS

**Materials.** [2-14C]uracil and d-[(14C)glucose (uniformly labeled) were purchased from New England Nuclear Corp., Boston, Mass.; [5-3H]uracil was purchased from Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y. NAL grade B was obtained from Calbiochem, Los Angeles, Calif.; chloramphenicol and uridine 5′-triphosphate (UTP), Type VI, were from Sigma Chemical Co., St. Louis, Mo. Polyethyleneimine-cellulose plates were purchased from Brinkmann Instruments Inc., Westbury, N.Y. All other chemicals were reagent grade, commercially available products.

**Organisms.** The strains of *E. coli* used are shown in Table 1.

**Media.** The *E. coli* 15 THU cells were grown in a minimal salts medium (5) supplemented with: glucose, 0.3% (wt/vol); histidine, 20 mg/liter; thymine and uracil, 10 mg/liter each; thiamine, 0.1 mg/liter. Other strains were supplemented with their required amino acids at 50 μg/ml. A gyrotory water-bath shaker was employed (New Brunswick Scientific Co., New Brunswick, N.J.).

NAL stock solutions (1 mg/ml) were prepared using 0.01 N NaOH as the solvent. Chloramphenicol stock solutions (1 mg/ml) were prepared with distilled water as solvent.

**Uridine triphosphate determination.** One-milliliter portions of bacterial cultures were treated with 0.4 ml of 4 M sodium formate, pH 3.4. The cells were centrifuged, and 50 μl of clarified formate extract was chromatographed on polyethyleneimine plates as described by Rogerson and Ezekiel (14).

**Radioactive label uptake determination.** To monitor RNA synthesis, parallel samples of 0.5 ml were withdrawn into (i) 0.1 ml of ice-cold 50% trichloroacetic acid, and (ii) 0.1 ml of 5.5 N NaOH solutions. The trichloroacetic acid-treated samples were held at 0 to 4°C for a minimum of 30 min. These were then collected on glass fiber filter paper, washed with 0.05% trichloroacetic acid, and dried. Each filter paper was placed in a vial with toluene-based scintillation liquid, and the radioactivity was determined by using a model 2111 Packard liquid scintillation spectrometer. The NaOH-treated fractions were held at 37°C for 16 h, 0.1 mg of albumin carrier was added, and the samples were acidified with 0.1 ml of 6 N HCl and 1 ml of 10% trichloroacetic acid. These fractions were treated the same as was described above. Results from the NaOH-treated samples were subtracted.
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TABLE 1. Strains of E. coli tested for inhibition of RNA synthesis by NAL

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth requirement</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 THU NF 126</td>
<td>Thymine, histidine, uracil, arginine, lysine, thiamine</td>
<td>S. Cohen</td>
</tr>
<tr>
<td>NF 156</td>
<td>Histidine, tryptophan, arginine</td>
<td>N. Fiil</td>
</tr>
<tr>
<td>GR-1 K12W6</td>
<td>Methionine, biotin</td>
<td>E. Borek</td>
</tr>
<tr>
<td>GR-1 W122-33</td>
<td>Glycerol, isoleucine, leucine lysine, methionine, threonine valine, thiamine</td>
<td>C. F. Fox</td>
</tr>
</tbody>
</table>

from those of the trichloroacetic acid-treated samples. Colorimetric determinations, preparation of cell extracts, and sucrose density gradient centrifugations were performed as previously described (11).

RESULTS

The rate of radioactive uracil incorporation into RNA was followed in logarithmically growing cells as well as in cultures exposed to NAL and chloramphenicol. The results shown in Fig. 1 indicate that, whereas NAL did not initially inhibit the net rate of RNA synthesis in logarithmically growing cells, this drug almost immediately depressed the formation of RNA in the absence of protein synthesis.

When the net synthesis of protein was halted by histidine starvation, the rate of uracil uptake into RNA diminished by about 70%, because in E. coli THU RNA synthesis is stringently controlled (12). The residual synthesis of RNA during histidine deprivation, however, was also inhibited considerably by NAL (unpublished observations).

Determination of the RNA content of cells by the orcinol method (13) during NAL and chloramphenicol treatment gave results similar to studies of radioactive isotope incorporation. Therefore, the apparent interference with RNA synthesis is not caused by obstruction in the uptake of radioactive uracil into the cells.

Neither is the inhibitory effect of NAL on RNA synthesis due to an increased rate of killing. On the contrary, it has been reported that chloramphenicol counteracts the bactericidal action of the drug (6). We have confirmed this observation by viability determinations (unpublished data).

The synthesis of DNA of exponentially growing cells was markedly inhibited by NAL under the experimental conditions shown in Fig. 1 (1, 3, 6, 8, 9, 20). Whereas this depression of DNA synthesis did not retard the production of RNA in logarithmically growing cells, it was conceivable that in the absence of protein synthesis there would be a closer coupling of RNA and DNA production. Thus, interference with DNA manufacture could impede RNA synthesis as well. We have stopped DNA synthesis by thymine starvation and determined the effect of NAL on RNA production in the presence and absence of chloramphenicol. In Fig. 2 we show that blocking DNA and protein synthesis does not inhibit RNA synthesis, but the inhibitory effect of NAL on RNA still exists in the absence of DNA production.

Cells pretreated with NAL for 1 h showed the same capacity to synthesize RNA in the presence of chloramphenicol as the untreated organism, provided that NAL was removed prior to the addition of chloramphenicol (Fig. 3). In this experiment, the incorporation of glucose was used to monitor any differences between the NAL-pretreated and control cultures, due to possible differences in general metabolic activity. The results indicate that the NAL-induced changes in the cell are quickly reversed by protein synthesis, so that in separating NAL and chloramphenicol treatments inhibition to RNA synthesis cannot be shown.

Addition of NAL to chloramphenicol-pretreated cells resulted in an immediate inhibi-

FIG. 1. Rate of RNA synthesis during NAL treatment. An exponentially growing culture of E. coli THU was divided into four portions and treated as indicated in the figure. Chloramphenicol (CHL) was used at final concentration of 100 μg/ml, NAL at 20 μg/ml. (Whereas it was not done in this particular experiment, in subsequent repeats to all cultures not receiving NAL, equal volumes of 0.01 N NaOH were added. This variation did not change the result.) At times indicated, 2-ml portions were removed in duplicate, each receiving 0.1 μCi of [14C]uracil, and incubated at 37 C for 4 min. The reactions were stopped by either trichloroacetic acid or NaOH, and samples were worked up as described in Materials and Methods.
Materials and methods

An exponentially growing culture of E. coli THU was centrifuged, washed twice, and resuspended in thymineless medium, containing 0.05 μCi, 10 μg of [14C]uracil per ml. It was divided into six fractions and supplemented as shown in the figure. NAL was used at 10 μg/ml, chloramphenicol at 100 μg/ml. Two control fractions are not shown. These were a +thy control, for comparison with −thy and −thy + NAL, and +thy + CHL control for comparison with the −thy + CHL and −thy + CHL + NAL fractions.

Parallel cultures MCi, 1.5 × 10^8 organisms/ml, were grown. One was treated with 20 μg of NAL per ml for 1 h; the other remained untreated. Cultures were centrifuged, washed twice, and resuspended in media containing 100 μg of chloramphenicol per ml. The cultures were further subdivided, one-half of each receiving [14C]uracil (0.1 μCi; 10 μg/ml); the other half received 1.5 μCi, 3 μg of [14C]glucose per ml. From the [14C]uracil-containing cultures parallel samples were taken, one treated with trichloroacetic acid in the cold, the other treated with NaOH as described in Materials and Methods. The [14C]glucose-containing fractions were treated with trichloroacetic acid in the cold, before collecting them by filtration.

Fig. 2. Effect of chloramphenicol and NAL treatment on RNA synthesis during thyme starvation. An exponentially growing culture of E. coli THU was centrifuged, washed twice, and resuspended in thymineless medium, containing 0.05 μCi, 10 μg of [14C]uracil per ml. It was divided into six fractions and supplemented as shown in the figure. NAL was used at 10 μg/ml, chloramphenicol at 100 μg/ml. Two control fractions are not shown. These were a +thy control, for comparison with −thy and −thy + NAL, and +thy + CHL control for comparison with the −thy + CHL and −thy + CHL + NAL fractions.

Several laboratories reported changes in the cell envelope of E. coli after NAL treatment (10, 16). In the absence of protein synthesis (and likely cell envelope repair), exposure to NAL could lead to leakage of precursor molecules of macromolecular synthesis. This would result in a more or less general inhibition of all macromolecular synthesis. Therefore, we compared the effect of NAL on RNA synthesis with the impact of this drug on total macromolecular synthesis. Total macromolecular synthesis was followed by the uptake of radioactive glucose into the cold trichloroacetic acid-insoluble fraction. The results in Fig. 5 show that the ratio of uracil to glucose incorporation is measurably lowered in the presence of NAL.

We have also measured the capacity of cells to manufacture UTP during chloramphenicol and NAL treatments. In Table 2 we show the results; the amounts of UTP produced in the presence of chloramphenicol are comparable in magnitude to the quantity of UTP made in the presence of both chloramphenicol and NAL.

The results of Fig. 5 and Table 2 lead us to believe that the observed inhibition of RNA synthesis by NAL in the absence of protein production is caused neither by leakage of macromolecular precursors from the cells nor by a general inhibition of macromolecular production.

The pattern of ribosomal and transfer RNA synthesized during NAL treatment is not altered. In the presence of chloramphenicol incomplete ribosomal particles are produced (15) and NAL inhibits this process considerably (Fig. 6).

Fig. 3. Effect of pretreatment of E. coli THU with NAL on subsequent RNA synthesis in the presence of chloramphenicol. Parallel cultures were grown. One was treated with 20 μg of NAL per ml for 1 h; the other remained untreated. Cultures were centrifuged, washed twice, and resuspended in media containing 100 μg of chloramphenicol per ml. The cultures were further subdivided, one-half of each receiving [14C]uracil (0.1 μCi; 10 μg/ml); the other half received 1.5 μCi, 3 μg of [14C]glucose per ml. From the [14C]uracil-containing cultures parallel samples were taken, one treated with trichloroacetic acid in the cold, the other treated with NaOH as described in Materials and Methods. The [14C]glucose-containing fractions were treated with trichloroacetic acid in the cold, before collecting them by filtration.

Fig. 4. Effect of NAL on RNA synthesis during chloramphenicol treatment. To a logarithmically growing culture of E. coli THU 100 μg of chloramphenicol per ml was added. At times 0, 30, 60, and 90 min, samples were removed, each receiving 0.2 μCi, 0.02 μg of [14C]uracil per ml. These were then divided into equal halves and one received 20 μg of NAL per ml. Thirty minutes of incubation later, trichloroacetic acid was added to the cells, and the radioactive uracil uptake into RNA was determined as described in Materials and Methods.
FIG. 5. Comparison of the effect of NAL plus chloramphenicol on RNA synthesis with the rate of total macromolecular synthesis. An exponentially growing culture of E. coli THU received chloramphenicol (100 µg/ml) and was divided into two halves. One received [14C]uracil (0.1 µCi; 10 µg/ml), the other [14C]glucose (0.6 µCi; 3 µg/ml). Each half was further subdivided into two fractions, one of each receiving 20 µg of NAL per ml. Samples were taken at 0, 20, 40, and 60 min. The samples were treated as described in the legend to Fig. 3.

FIG. 6. Effect of NAL treatment on the pattern of RNA synthesis. A 200-ml culture of E. coli THU was grown to late logarithmic growth phase. During the last two generations of growth, 0.03 µCi, 10 µg of [14C]uracil per ml was present. The culture was centrifuged, washed twice, resuspended in 400 ml of fully supplemented non-radioactive growth medium, and incubated for 20 min at 37 C. It was then divided into four 100-ml fractions: A, B, C, and D. To B 20 µg of NAL per ml; to C 100 µg of chloramphenicol per ml; and to D 20 µg of NAL and 100 µg of chloramphenicol per ml were added. All four fractions were further incubated for 20 min. Then each fraction received 0.25 µCi, 0.02 µg of [3H]uracil per ml. Ten minutes later the cells were collected on crushed ice and treated as described in reference 11. Solid lines are [14C]uracil; dotted lines are [3H]uracil.

DISCUSSION

The experimental results reported in the previous section were obtained with E. coli 15 THU. Other strains of this organism may or may not yield the same results. In our hands, strains NF 126, NF 156, and K12W6 reacted similarly to 15 THU, whereas strains W122-33 and GR-1 did not show increased inhibition of RNA synthesis by NAL in the absence of protein synthesis. However, in the strains of E. coli that did not display increased inhibition of

TABLE 2. Uracil incorporation into UTPε

<table>
<thead>
<tr>
<th>Sample</th>
<th>Min</th>
<th>nmole of uracil incorporated into UTP (ml culture/min)</th>
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<tbody>
<tr>
<td>Log cells</td>
<td>0</td>
<td>0.378</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.483</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.438</td>
</tr>
<tr>
<td>Log cells</td>
<td>0</td>
<td>0.382</td>
</tr>
<tr>
<td>plus NAL</td>
<td>30</td>
<td>0.451</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.497</td>
</tr>
<tr>
<td>Log cells</td>
<td>0</td>
<td>0.350</td>
</tr>
<tr>
<td>plus CHL</td>
<td>30</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.292</td>
</tr>
<tr>
<td>Log cells</td>
<td>0</td>
<td>0.349</td>
</tr>
<tr>
<td>plus NAL</td>
<td>30</td>
<td>0.314</td>
</tr>
<tr>
<td>plus CHL</td>
<td>60</td>
<td>0.300</td>
</tr>
</tbody>
</table>

* Forty milliliters of a logarithmically growing culture of E. coli THU was divided into four fractions: A, B, C, D. Fraction A was the untreated control; it received 1 ml of distilled water and 0.2 ml of 0.01 N NaOH; B received 1 ml of distilled water and 0.2 ml of NAL (1 mg/ml); C received 1 ml of chloramphenicol solution (1 mg/ml) and 0.2 ml of 0.01 N NaOH; and D received 1 ml of chloramphenicol solution and 0.2 ml of NAL. At 0, 30, and 60 min, 0.9-ml portions were added to 0.05 ml of [14C]uracil (0.5 µCi, 16 µg) and incubated at 37 C for 5 min. The reaction was stopped by 0.5 ml of 4 M sodium formate, pH 3.4, and the samples were handled as described in Materials and Methods.
RNA synthesis by NAL in the absence of protein synthesis, RNA synthesis was more sensitive to inhibition by NAL during logarithmic growth than in the other strains mentioned. These strain-related differences in response to NAL are not yet understood.

Unfortunately, we cannot at this time identify the metabolic event during amino acid starvation or chloramphenicol treatment that is the target of NAL. We have shown that NAL inhibition of RNA synthesis in the presence of chloramphenicol is probably not the result of a lack of metabolic precursors available for RNA synthesis.

Since NAL does not act directly on the nucleic acid-producing enzymes (20), the effect of this drug on nucleic acid biosynthesis is most likely a secondary one. RNA synthesis has been shown to be sensitive to the amount of intracellular guanosine tetraphosphate (2). This nucleotide has a depressing effect on RNA synthesis either by inhibiting the biosynthesis of RNA precursors or by binding to the RNA polymerase initiation factor sigma (17). The manufacture of guanosine tetraphosphate is stimulated during amino acid starvation but inhibited during chloramphenicol treatment (7). Since NAL is inhibitory to RNA synthesis under both types of metabolic restrictions, it is unlikely that the drug's effect is connected with the presence or absence of guanosine tetraphosphate.

The mechanism that connects the control of RNA synthesis with protein production is not well understood (see ref. 18 for example). Thus, further exploration of the mode of action of NAL in *E. coli* may be of value in identifying the process that operates in the absence of protein production and that appreciably influences the rate of RNA synthesis.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


