ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1973, p. 479-486

Vol. 4, No. 4
Printed in U.S.A.

Studies on the Mechanism of Action of Nalidixic Acid

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Received for publication 28 June 1973

With three independent techniques (absorption spectrophotometry, measurement of the deoxyribonucleic acid [DNA] melting temperature, and equilibrium dialysis), no evidence has been found for the binding of nalidixic acid to purified DNA. Also, no evidence has been found to support the hypothesis that nalidixic acid is permanently modified to a new, active compound by the bacterial cell. By using an in vitro DNA replication system developed by Bonhoeffer and colleagues, soluble extracts from nalidixic acid-sensitive cells have been shown to confer nalidixic acid sensitivity on the DNA synthesis of lysates from nalidixic acid-resistant cells. The activity in the extracts is only present in sensitive cells and is nondialyzable and heat sensitive. Finally, two known nalidixic acid-resistant mutants of *Escherichia coli*, mapping at *nal A* and *nal B*, respectively, have been tested to determine whether either of them is a transport mutant. It has been shown that *nal* B^r is a transport mutant whereas *nal* A^r is not.

Nalidixic acid (NAL; 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine - 3 - carboxylic acid) is a specific, rapid, and reversible inhibitor of bacterial deoxyribonucleic acid (DNA) replication (3, 9). At present the mechanism of action of NAL is totally unknown. Previous work by other investigators has ruled out several possibilities. For example, none of the many purified enzymes involved in DNA metabolism are inhibited by NAL in vitro. Those tested include: (i) DNA polymerase I from *Escherichia coli* and *Bacillus subtilis* (1, 16); (ii) *E. coli* DNA polymerases II and III (M. Gefter, personal communication); (iii) endonuclease I, exonucleases I, II, and III, and ligase from *E. coli* (16); (iv) ω protein (J. Wang, personal communication); and (v) ribonucleotide reductase (P. Reichard, personal communication).

Furthermore, two lines of evidence suggest that NAL does not block the synthesis of the precursors of DNA—the deoxynucleoside triphosphates. First, the intracellular levels of the deoxynucleoside triphosphates are unaffected by NAL (M. Atkinson, personal communication; ref. 14). Second, in vitro DNA replication in both the cellophane disk system developed by Bonhoeffer and colleagues and in toluenized cells is sensitive to NAL even though the deoxynucleoside triphosphates are supplied in both cases (13, 16, 18). Consequently, NAL must be blocking some aspect of the DNA polymerization reaction itself and not just synthesis of the DNA precursors.

Based on the work described above, three possibilities remain for the way in which NAL inhibits DNA replication: (i) NAL binds directly to the DNA template; (ii) NAL binds to and inactivates one of the components of the DNA replication complex (possibly an unknown replication protein); or (iii) NAL is chemically modified by metabolizing bacteria to an active form, which then functions by either (i) or (ii). In this paper we present experiments which test all three of these possibilities.

In addition, we have carried out experiments with the two known NAL-resistant mutants of *E. coli*, mapping at *nal A* and *nal B* (10), to determine whether either of them is a transport mutant.

MATERIALS AND METHODS

Bacterial strains. *E. coli* D 110 (*pol* I^-, *endo* I^-) used in the in vitro DNA replication studies was a gift of C. Richardson. NAL^r C, a spontaneous NAL-resistant mutant of D 110, was isolated by its ability to grow in the presence of 40 μg of NAL per ml. For the characterization of the *E. coli nal A* and *nal B* NAL-resistant mutants, three strains of Hane and Wood were used: MH 5 (*nal* A^r), MH 4 (*nal* B^r), and the parent NAL-sensitive strain KL 16 (10). All three were a gift of M. Inouye. *E. coli* B was used in the used in the NAL modification studies.

NAL. NAL, obtained from Sterling-Winthrop Laboratories, was stored as a solid at 5 C. Concentrated stock solutions of the drug (1 to 10 mg/ml in 0.1 M NaOH) were generally prepared just before use. However, these solutions could be stored at 5 C for at least 1 week without any loss of activity.

NAL was radioactively labeled by New England Nuclear Corp. by using tritiated H_2O in a catalytic ^1H-^3H exchange procedure. The highly radioactive NAL had to be purified before use. A complete purification of the ^3H-NAL was obtained in two steps by using preparative thin-layer chromatography (details are in the Ph.D. thesis of G. J. B., State University of New York at Stony Brook, 1972). The final purified product had a specific activity of 10^7 counts per min per μg of NAL.

NAL-DNA binding studies. Absorption spectra were measured in a Cary 15 spectrophotometer. A double compartment cuvette containing NAL and DNA, either mixed or unmixed, was placed in the sample beam, while DNA alone was in the reference beam. In order to examine specifically the spectrum of NAL, the concentration of DNA in the reference beam was adjusted to cancel the absorbance of DNA in the sample beam. Spectra were measured at a final concentration of 2.2×10^{-6} M NAL, dissolved in phosphate buffer, pH = 7, in the presence of 0.1 M monovalent cation. Molar ratios of NAL to DNA base pairs ranging from 0.04 to 1.0 with both calf thymus and λ DNA were examined.

Melting temperatures of DNA were measured in a Zeiss PMII spectrophotometer equipped with a temperature-controlled sample compartment. The solvent for the DNA solutions was sodium phosphate buffer, pH = 7. The final concentrations of Na^+, NAL, and DNA for the different experiments are given in Table 1.

Equilibrium dialysis experiments using ^3H-NAL were performed by placing a 1.0-ml mixture of calf thymus DNA (5.6×10^{-4} M in base pairs) and ^3H-NAL (5×10^{-6} M) inside a standard dialysis bag, and dialyzing versus 4 ml of SSC (0.15 M NaCl, 0.015 M Na_3 citrate) for 20 h at 5 C. Samples (5 μliters) were then removed from inside and outside the bag and assayed for ^3H by counting in 10 ml of Bray scintillation fluid (2).

NAL modification studies. An exponentially growing culture of *E. coli* B was incubated with ^3H-NAL (total concentration, 5 μg/ml) for 15 min at 37 C. The culture was rapidly chilled to 0 C, made acidic by the addition of concentrated HCl, and then extracted with $CHCl_3$. NAL is very soluble in $CHCl_3$ and insoluble in aqueous solutions when the pH is less than 7. After separation of the two phases, the $CHCl_3$ phase, containing about 98% of the input radioactivity, was concentrated to about 0.1 ml by evaporation and then chromatographed by thin-layer chromatography (TLC). The adsorbent for the TLC glass plates was cellulose (E. Merck, 20 by 20 cm, 0.25 mm thick), and the solvent for the experiment described in Fig. 1 was *n*-butanol, H_2O, 30% NH_4OH (79:14:7). Pure NAL, always used as a reference, has an R_f = 0.45 in this solvent. After chromatography, the plate was thoroughly dried and divided into 1-cm fractions, and each fraction was scraped into a counting vial. The vials were filled with a dioxane-H_2O-based scintillation fluid specially developed to extract compounds from TLC adsorbents (20). The fluid also contained

TABLE 1. *Effect of NAL on T_m of DNA*

DNA	Na^+ concn [M]	R[a]	T_m(C)	
			-NAL	+NAL
Lambda	0.035	1.0	79.0	79.2
Calf thymus	0.063	0.2	79.1[b]	78.0
Calf thymus	0.034	1.0	74.7[b]	73.9

[a] R = moles of NAL/moles of DNA base pairs. In the two experiments with R = 1, [NAL] = 1.7×10^{-5} M; in the single experiment with R = 0.2, [NAL] = 8.6×10^{-6} M.

[b] T_m values were calculated from the empirical equation: % guanine plus cytosine = 2.44 (T_m - 81.5 - 16.6 log [Na^+]); ref. 12.

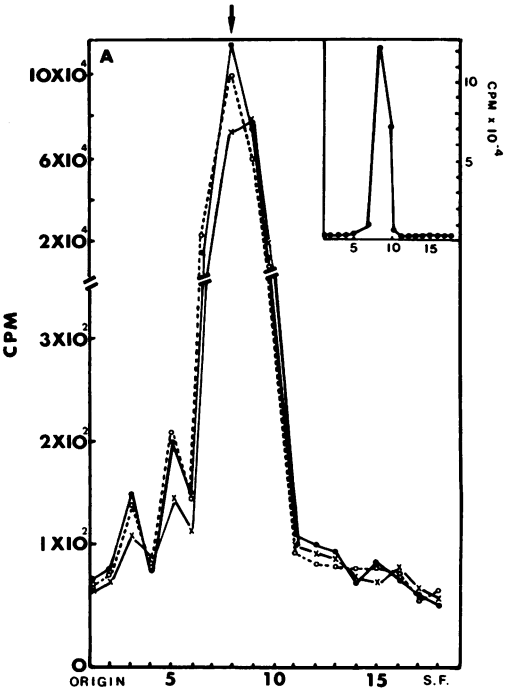

FIG. 1. *Thin-layer chromatograms of ^3H-NAL after incubation with bacteria. See detailed procedure in Materials and Methods. Three samples were chromatographed in parallel: ●, ^3H-NAL incubated with a culture for 15 min; ×, ^3H-NAL incubated with cell-free medium for 15 min; ○, ^3H-NAL added to a culture just before $CHCl_3$ extraction. The arrow represents the position of pure NAL. Note the change in scale in the main figure. The insert depicts the chromatogram of the experimental sample redrawn at full scale.*

4% (wt/wt) Cab-O-Sil to suspend particulate matter. Samples were counted in a Packard Tri-Carb liquid scintillation counter.

In vitro DNA replication. The basic procedure used was the same as that of Bonhoeffer and colleagues (18) with the following exceptions. Cellophane squares (11 mm^2), cut from Technicon standard dialyzing membrane (no. 105-0010), were used instead of the disks Schaller et al. used (18). Generally, 4 to 6 squares were placed on each agar plate, and all of the samples on a plate were lysed at approximately the same time. All incubations were carried out for 20 min at 35 C. In experiments in which extracts were added, the extract was first spread and dried on the cellophane square before spreading the lysozyme and cells. Drying of extracts and lysates was accomplished by blowing cold air over the samples with a hair dryer. Before precipitating the DNA with TCA, Schaller et al. (18) heated each sample to 80 C for 5 min. We found this step unnecessary, and omitted it. Nitrocellulose filters (Matheson-Higgins, 25 mm diameter, 0.45 μm pore size) were used to collect the precipitates. Samples were counted in toluene containing 4 g of Omnifluor (New England Nuclear) per liter in a Packard Tri-Carb liquid scintillation counter.

EDTA treatment of NAL-resistant mutants. Ethylenediaminetetraacetic acid (EDTA) treatment was essentially that of Leive (11). Cultures (10 ml) of MH 5 (*nal* A^r), MH 4 (*nal* B^r), or KL 16 were grown at 37 C in a minimal, tris(hydroxymethyl)aminomethane (Tris)-based medium (11) to a density of 2×10^8 cells/ml. The cells were washed once with 0.12 M Tris, pH = 8.0, and then resuspended in 1 ml of the same Tris buffer containing 2×10^{-4} M EDTA. After a 2-min incubation at 37 C, 10 ml of fresh medium was added. This terminated the EDTA treatment and allowed growth to begin. Control cultures were treated in the same way, except EDTA was omitted during the 2-min incubation. Control or EDTA-treated cultures were incubated for 4 min in fresh medium. Then ^{3}H-thymidine (0.1 ml of 100 μCi/ml) was added, and the cultures were split into two flasks with and without NAL. The concentration of NAL was 20 μg/ml for experiments with the *nal* A^r strain and 3 μg/ml for experiments with the *nal* B^r strain. Samples (1 ml) were removed periodically, precipitated with 15% trichloroacetic acid, and assayed for radioactivity as described in the previous section.

RESULTS

Nalidixic acid-DNA binding studies. Although several investigators have suggested that NAL inhibits DNA synthesis by binding directly to the DNA template (4, 5, 23), no experimental data have been published to either establish or eliminate this possibility. Therefore, in this study we have used three independent techniques to determine whether there is any interaction between NAL and purified DNA. In view of the rapid reversibility of the drug's effect on bacteria, one would expect that if NAL binds to DNA, the binding would be relatively weak.

The first method used was simply to measure the ultraviolet absorption spectrum of NAL in the presence and absence of DNA. It was found that the spectrum of NAL, including its absorption maxima at 258 and 330 nm, is essentially unchanged after mixing the drug with DNA. Both purified calf thymus DNA, native and denatured, and native bacteriophage lambda DNA were tested at several concentrations. The ratio of moles of NAL to moles of DNA base pairs was varied from 1.0 to 0.04. In all cases, no change in the NAL absorption spectrum could be detected. As a control, the spectrum of actinomycin D was measured under the same conditions, mixed and unmixed with DNA. Relatively large spectral changes were observed with this compound; they were identical to those found previously (17).

The second method used to detect binding was the measurement of the melting temperature (T_m) of DNA in the presence and absence of NAL. The T_m of calf thymus or lambda DNA was essentially unchanged by the presence of NAL, even with equimolar amounts of NAL and DNA base pairs (Table 1).

The final and most sensitive method used was that of equilibrium dialysis using tritium-labeled NAL. Typical results are outlined in Table 2. Again, there is no indication of binding of nalidixic acid to DNA. This is true whether the DNA is native, heat denatured, or in the presence of 2 mM Mg^{2+} ions. Association constants greater than 10 M^{-1} (in base pairs) are ruled out by the data in Table 2. However, significant binding was detected between NAL and bovine serum albumin, a protein known to bind a wide variety of compounds. As an additional control, the binding of ethidium bromide to DNA was clearly demonstrated by using the same equilibrium dialysis procedures (data not shown).

Nalidixic acid modification studies. One reason why all the studies which have tested the effect of NAL on purified or partially purified

TABLE 2. *Equilibrium dialysis of NAL and DNA*

Sample	Counts per min (in)	Counts per min (out)
Native DNA	18,920	19,150
Native DNA + Mg^{2+} [a]	13,850	14,010
Denatured DNA	13,715	13,770
BSA[b]	9,395	7,330

[a] In this experiment, 2×10^{-3} M Mg^{2+} was present.

[b] Concentration of BSA (bovine serum albumin) was 0.3 mg/ml.

cell components have given negative results may be that the drug is modified to an active form by the bacterial cell. In order to determine whether, in fact, NAL is metabolized to a new compound upon exposure to a bacterial culture, we have performed the following experiment.

^{3}H-labeled NAL at a final concentration of 5 μg/ml was incubated with an exponentially growing culture of *E. coli* B for 15 min at 37 C. The entire culture was then made strongly acidic and extracted with chloroform. The chloroform extract, containing 98% of the radioactivity in the culture, was concentrated and applied to a cellulose TLC plate. Two additional control samples were chromatographed at the same time: (i) ^{3}H-NAL added to the bacterial culture a fraction of a second before the $CHCl_3$ extraction, and (ii) ^{3}H-NAL incubated for 15 min at 37 C with growth medium containing no cells and then extracted with $CHCl_3$. The ^{3}H profiles of the chromatograms were then determined and compared with that of pure ^{3}H-NAL chromatographed on the same plate. Figure 1 shows the results of chromatography with a solvent containing *n*-butanol, H_2O, 30% NH_4OH (79:14:7). Only one major peak, containing 99.9% of the radioactivity and migrating at the position of pure NAL, is visible. There are two additional very minor peaks, but since the peaks are also present in both control samples, they must be insignificant. The entire experiment has been repeated using two other TLC solvent systems with essentially the same result—more than 99.9% of the ^{3}H migrates at the position of pure NAL (G. J. B., Ph.D. thesis). Thus the $CHCl_3$ extract of the culture clearly does not contain any modified NAL.

Nevertheless, a modified form of NAL could have remained behind in the aqueous phase, representing 2% of the input radioactivity in the culture. This possibility has been eliminated by the observation that 2% of the input radioactivity also remains in the aqueous phase in both of the control samples. Also, reextraction of the aqueous phase with $CHCl_3$ does not reveal any difference in the amount of radioactivity remaining in the aqueous phase for the experimental or control samples.

The experiment was also done by extracting the cell pellet instead of the whole culture with chloroform. Even though only 0.1% of the total input radioactivity remained in the pellet, the TLC profiles showed no peaks except that of pure NAL. Consequently, we believe it is very unlikely that NAL is permanently modified to a new active form.

In vitro DNA replication and the conferral of NAL sensitivity to NAL-resistant lysates. Bonhoeffer and co-workers have recently developed an in vitro DNA replication system which appears to have many of the characteristics of normal, in vivo DNA replication. Their method involves lysing cells gently and at high concentration on cellophane disks. The disks are then placed on top of a 50-μliter drop of incubation buffer containing adenosine triphosphate and the four deoxynucleoside triphosphates. The resultant DNA synthesis is semiconservative, proceeds at a rate comparable to that found in vivo, and is sensitive to NAL (18). Since the activity of the lysates can also be complemented by the addition of exogenous macromolecules (15), this system provides a powerful tool for investigating the DNA replication apparatus. We have used the cellophane disk in vitro replication system in this study with the ultimate goal of identifying the component(s) of the replication complex involved in the inhibition of DNA synthesis by NAL.

Initially, DNA synthesis was carried out in lysates of *E. coli* D 110 (*pol* I$^-$, *endo* I$^-$). The overall rate of synthesis was determined to be approximately 2.5 pmol of thymidine triphosphate incorporated per min per 10^8 lysed cells for at least the first 20 min of incubation at 35 C. It was found that the synthesis is almost completely dependent on the presence of adenosine triphosphate and is inhibited about 55% by 100 μg of NAL per ml. (Two hundred micrograms of NAL per ml does not increase the amount of inhibition.) These results are in good agreement with those of Bonhoeffer and coworkers who used a similar strain, *E. coli* H 560 (18).

Four spontaneous mutants of D 110, whose growth on agar plates is resistant to at least 40 μg of NAL per ml, were then isolated and tested in the replication system for their sensitivity to NAL. Since in vitro DNA synthesis in all four mutants was found to be resistant to NAL, we concluded that the mutants are internally resistant, and not resistant because of a block in the transport of NAL into the cell (see next section). One of the mutants, designated NAL^r C, displayed the most NAL resistance and was therefore chosen for all further work. Its DNA synthesis in vitro is inhibited only about 7.5% by 200 μg of NAL per ml as compared to 55% inhibition with D 110, the NAL-sensitive parent strain.

The following crucial question was then asked: could NAL sensitivity be conferred on lysates of NAL^r C by adding soluble extracts from the NAL-sensitive strain, D 110? If the answer were yes, then it would be possible, in principle, to purify the NAL-sensitive component by using the in vitro replication system as a functional assay.

Soluble extracts of both the NAL-sensitive strain (NAL^s = D 110) and the NAL-resistant strain (NAL^r C) were obtained by using the gentle lysis procedure of Godson and Sinsheimer (8). After rapidly sedimenting all of the membranes and DNA, the supernatant, containing most of the soluble proteins, was used directly in the in vitro DNA replication assays as described in Materials and Methods.

The results of experiments involving the addition of extracts to NAL^r C lysates are summarized in Table 3 and Fig. 2. NAL sensitivity is clearly conferred on NAL^r C lysates by the addition of NAL^s (D 110) extracts. The effect is specific for NAL^s extracts; Table 3 shows that extracts from NAL^r C cells do not confer sensitivity on the resistant lysates. Clearly, there must be some soluble component in the cell which determines whether DNA synthesis is sensitive or resistant to NAL. In addition, the amount of NAL sensitivity conferred is dependent on the amount of NAL^s extract added. (Ten microliters of extract corresponds to the amount of lysate obtained from approximately 4×10^7 cells, and there are about 4×10^7 to 5×10^7 lysed cells in each sample). Forty microliters of extract appears to confer close to the maximal amount of sensitivity in these experiments (Fig. 2). If more than 40 μliters of extract is used, the overall rate of DNA synthesis even in the absence of NAL decreases markedly. Because 55% inhibition is the maximal possible inhibition (that is the value obtained for the NAL^s strain D 110 itself), then about one-half of that maximal amount of inhibition is actually obtained in these experiments by the addition of 40 μliters of extract.

Preliminary characterization of NAL^s extracts has shown that they can be extensively dialyzed in the cold (20 h at 5 C) and still retain their activity. Therefore, the NAL-sensitive component(s) must be a fairly stable macromolecule with a molecular weight of about 10,000 daltons or greater. In addition, heating the extract to 80 C for 5 min results in the loss of almost all the activity. Further isolation and characterization of the NAL-sensitive component is in progress.

TABLE 3. *NAL inhibition of DNA synthesis in NAL^r C lysates as a function of the amount of NAL^s or NAL^r extract added*

Volume of extract added (μliters)	Extract added			
	NAL^s		NAL^r C	
	Inhibition ± σ (%)[a]	N[b]	Inhibition ± σ (%)	N
0	7.5 ± 8.6	20		
2	9.3 ± 4.9	2		
5	18.6 ± 4.2	2		
10	21.3 ± 4.5	8	3.9 ± 7.6	5
25	26.7 ± 5.1	8	5.8 ± 3.7	5
40	29.1 ± 4.6	4	9.0 ± 4.4	4

[a] Percentage of inhibition = 100(1 − incorporation with NAL/incorporation without NAL); σ = standard deviation.

[b] N = number of experiments conducted.

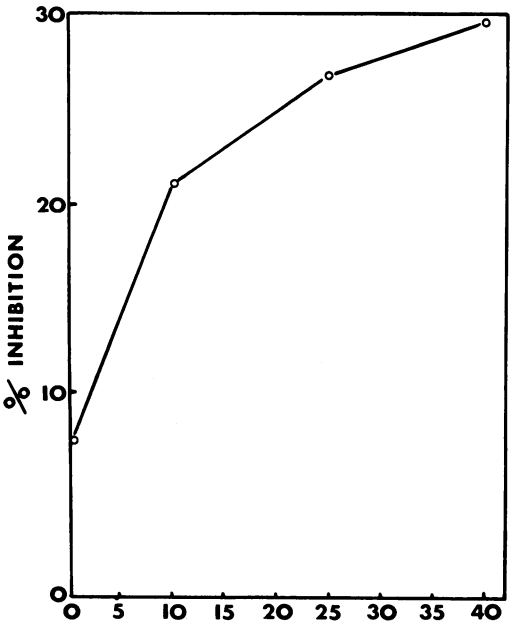

FIG. 2. *Conferral of NAL sensitivity on NAL-resistant lysates as a function of the amount of sensitive extract added. The data are taken directly from Table 3.*

Characterization of NAL-resistant mutants. *E. coli* NAL-resistant mutants are easily obtained by growing bacteria in the presence of NAL. Hane and Wood have characterized such mutants and found that they fall into two groups: (i) NAL A^r (growth at 40 μg/ml or higher), and (ii) NAL B^r (growth at 4 μg/ml, but no growth at 10 μg/ml). Mutants in the two groups map at two completely separate sites on the *E. coli* genome, *nal A* and *nal B* (10). We were interested to determine whether either of the two types of NAL-resistant mutants was a transport mutant. Our experiments involved temporarily increasing the permeability of the *E. coli* mutants by treating them with a low concentration of EDTA, according to the method developed by Leive (11), and then testing whether they became more sensitive to NAL.

Figure 3 shows the results of an experiment

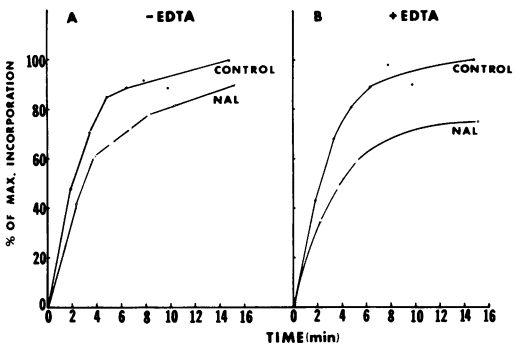

FIG. 3. *NAL sensitivity of DNA synthesis with and without EDTA treatment in E. coli MH5 (nal A^r). See Materials and Methods for details of the EDTA treatment. NAL at 20 μg/ml and ^{3}H-thymidine were both added at 0 min.*

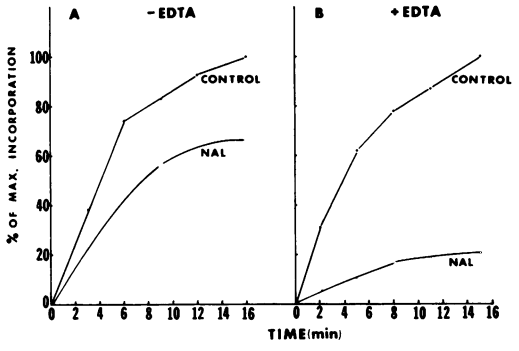

FIG. 4. *NAL sensitivity of DNA synthesis with and without EDTA treatment in E. coli MH 4 (nal B^r). See Materials and Methods for details of the EDTA treatment. NAL at 3 μg/ml was added at −7 min, and ^{3}H-thymidine was added at 0 min.*

with strain MH 5, resistant to more than 40 μg of NAL per ml and mapping at *nal A* (10). It can be seen that DNA synthesis, monitored by ^{3}H-thymidine incorporation, is quite resistant to 20 μg of NAL per ml, both before and after EDTA treatment. By contrast, DNA synthesis in a related NAL-sensitive strain, KL 16 (10), is about 80% inhibited by NAL under similar conditions (data not shown).

Figure 4 shows the results of a similar experiment with strain MH 4, resistant to 4 μg of NAL per ml and mapping at *nal B* (10). In this case, DNA synthesis is relatively resistant (66% of the control) without EDTA treatment, and sensitive (20% of the control) after EDTA treatment. On the basis of the data presented in Fig. 3 and 4, we conclude that mutants mapping at *nal A* are internally NAL resistant, whereas mutants mapping at *nal B* are transport mutants which can be made sensitive to NAL by EDTA treatment. As described in the previous section, the four spontaneous NAL-resistant mutants of *E. coli* strain D 110 were found to be resistant to 40 μg of NAL per ml both in vivo and in vitro. Thus they are not transport mutants and are presumably of the *nal A* type.

DISCUSSION

With three independent techniques (absorption spectrophotometry, measurement of the DNA melting temperature, and equilibrium dialysis), no evidence has been found for the binding of nalidixic acid to purified DNA. Association constants greater than 10 M^{-1} (in base pairs) are ruled out by the equilibrium dialysis data in Table 2. Most drugs which bind to DNA have binding constants greater than 10^5.

In addition, no evidence has been found to support the possibility that NAL is permanently modified to a new, active compound by the bacterial cell. With ^{3}H-NAL and three different thin-layer chromatographic systems, no new tritium-labeled compounds were detected in growing cultures of *E. coli* which had been incubated with the drug for 15 min. However, negative results such as this are always difficult to prove conclusively. It is conceivable that either the amount of NAL which is converted to the active form is so small that it was undetectable in these experiments, or NAL is changed in the cell to an unstable active form which then rapidly converts back to NAL during the extraction and analysis.

By using the in vitro DNA replication system of Bonhoeffer and colleagues (18), we have developed a promising approach for isolating the NAL-sensitive cellular component. We have demonstrated that a soluble extract from NAL-sensitive cells is able to confer sensitivity on a lysate from NAL-resistant cells. The effect is specific for extracts from NAL-sensitive cells; extracts from NAL-resistant cells do not confer sensitivity on the resistant lysates (see Table 3 and Fig. 2). The activity is nondialyzable and heat sensitive. Experiments are underway in our laboratory to purify and characterize the NAL-sensitive component.

At present we are unable to confer complete sensitivity on NAL-resistant lysates. The effect is about half of the maximum possible; specifically, we find 29% NAL inhibition of DNA synthesis with 40 μliters of sensitive extract, to be compared with 55% for the sensitive strain, D 110, itself. Adding more than 40 μliters of extract decreases the amount of DNA synthesis even in the absence of NAL. Possibly nucleases

in the extract destroy the replicating chromosome. Since 40 μliters of sensitive extract is made from 1.6×10^8 cells and the resistant lysate is made from 4×10^7 to 5×10^7 cells, there should be an excess of the sensitive component. We have not been able to determine why the amount of sensitivity conferred is not greater. Possibly there is heterogeneity in the ability of the lysed cells to take up the sensitive extract.

Complementary experiments have also been conducted in which extracts of NAL-resistant cells were added to lysates of the NAL-sensitive strain, D 110. We were unable to confer resistance on sensitive lysates. This is consistent with the genetic data of Hane and Wood showing that sensitivity is completely dominant to resistance in partial diploids for the *nal A* marker (10). One explanation of the dominance of sensitivity to resistance in both our experiments and in living cells would be that NAL is chemically modified to an active inhibitor by sensitive cells and it cannot be modified by resistant cells. However, as documented above, we have seen no evidence for a permanent chemical modification of NAL by growing bacteria. Therefore, the dominance of sensitivity to resistance remains unexplained.

We have examined two NAL-resistant mutants, mapping at *nal A* and *nal B*, respectively, to test whether either of them becomes more sensitive to NAL after EDTA treatment known to increase the permeability of the cell (11). The data of Fig. 3 and 4 show that the *nal A* mutant remains resistant whereas the *nal B* mutant becomes much more sensitive to NAL after EDTA treatment. Thus, we conclude that the *nal B* mutant is a transport mutant whereas the *nal A* mutant is not.

Finally, we offer some speculation about the nature of the NAL-sensitive target in *E. coli*. Is it a DNA replication protein? As described in the introduction, no known purified enzyme involved in DNA metabolism is inhibited by NAL in vitro. Furthermore, the six proteins defined by the *E. coli* temperature-sensitive DNA replication mutants, *dna A, B, C-D, E, F,* and *G* (21), are probably not inhibited by NAL. The evidence follows. First, *dna A* and *dna C-D* are initiation mutants, and NAL inhibits DNA replication itself and not just initiation. Second, *dna E* is the structural gene for DNA polymerase III (7), an enzyme not inhibited by NAL (M. Gefter, personal communication). Third, *dna F* is the structural gene for the B1 subunit of ribonucleotide reductase (6), and, as argued in the introduction, NAL must inhibit DNA replication at a stage beyond the deoxynucleoside triphosphates. Finally, the *dna B* and *dna G* proteins are required for the in vitro conversion of single-stranded ϕx DNA to the double-strained RF (19, 22), and NAL does not inhibit that reaction (K. Geider, personal communication; R. Wickner, personal communication). Also, *dna B* and *dna G* functions are needed for the in vivo synthesis of *oop* ribonucleic acid, a ribonucleic acid probably involved in λ DNA replication, and NAL does not inhibit the synthesis of *oop* ribonucleic acid (S. Hayes and W. Szybalski, Fed. Proc. 32:529, 1973.).

Thus, if the *E. coli* NAL-sensitive target is indeed a protein, and if NAL rather than a modified form is the active inhibitor (as our data suggest), then the protein may be an undiscovered component of the DNA replication apparatus.

ACKNOWLEDGMENTS

We are indebted to Judith P. Baird for advice throughout the course of this work. We thank Baldomero Olivera for suggestions about the in vitro replication system.

This study was supported by grant no. NP-45A from the American Cancer Society.

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