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Effect of Nalidixic Acid on DNA Replication by Toluene-Treated *Escherichia coli*

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ABSTRACT **Nalidixic acid inhibits DNA synthesis in toluene-treated *E. coli*, strain B/r, as it is well known to do *in vivo*. Both semiconservative and repair syntheses are affected, though to different degrees. Density-transfer experiments indicate that chromosomal replication is reinitiated when nalidixic acid is removed from toluene-treated cells after exposure to the acid for one generation *in vivo*. For cells *in vivo* or after toluene-treatment, reinitiation is not seen in asynchronous cultures exposed briefly to nalidixic acid or in cells prevented from synthesizing proteins during their exposure to the acid. Reinitiation occurs at the chromosomal origin but, unlike the effect seen *in vivo*, replication at the old site persists.**

When the permeability of *Escherichia coli* is increased by treatment with toluene, the cells are able to synthesize DNA from exogenous deoxynucleoside triphosphates (1). This synthesis proceeds at 10–20% the rate *in vivo*, and resembles normal synthesis in most respects (2, 3). Such preparations may serve to assay soluble factors in DNA replication, which may not permeate intact cells (4). Experiments with toluene-treated cells may also indicate which processes are not normally limited by cell permeability.

Nalidixic acid (NAL) inhibits DNA synthesis in *E. coli* (5, 6) but its mode of action is unknown. The rate and site of renewed DNA synthesis *in vivo* after removal of NAL indicate that if the inhibitor is present at the time when the cell would otherwise have initiated a new cycle of DNA synthesis, the removal of NAL will trigger reinitiation (7). Should this initiation occur, the preexisting site of replication remains inactive (8).

Since the rate of DNA replication *in vivo* is normally regulated by internal controls on the interval between successive replication cycles (9, 10), experiments with agents that induce or interfere with initiation of DNA replication may give useful insights concerning normal regulation processes. Experiments with NAL on toluene-treated *E. coli* were undertaken to test the similarity of DNA replication *in vivo* and after toluene treatment, and to indicate, if possible, how NAL might disrupt the normal cycle of chromosome replication. It was found that cells reinitiate a cycle of DNA replication upon removal of NAL before or after toluene treatment. Toluene treatment does not eliminate their sensitivity to NAL either.

Abbreviation: NAL, nalidixic acid.

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These experiments were done by use of a density transfer protocol to demonstrate that semiconservative DNA replication in toluene-treated cells continues only in those chromosomal regions that were about to be replicated *in vivo*. Cells with ^{14}C-labeled chromosomal regions were treated with toluene and then allowed to incorporate nucleotides including [^{3}H]dCTP and substituting the heavy analogue BrdUTP for dTTP. Pycnographic analysis of their lysates permitted comparison of nucleotide uptake into DNA of various densities, the density transfer of chromosomal label, and the recovery of unaltered chromosome fragments. Such experiments permit the measurement of replication *in vivo* and *in vitro* and can serve to distinguish among possible sites of chromosomal replication.

MATERIALS AND METHODS

Reagents, the bacterial strain, conditions of culture, toluene treatment, deoxynucleotide incorporation, lysis, and DNA analysis have been described (3). The experiments reported below differ by the use of NAL and of different methods to label specific portions of the chromosome. Cells of *E. coli*, strain B/r, Thy$^-$, His$^-$, with appropriately labeled DNA were grown to a titer of 2 to 3 $\times$ 10^8/ml, then chilled, concentrated 20-fold, and shaken for 5 min at 37° with 1% toluene. To this was added 4.5 volumes of a mixture containing BrdUTP, dCTP, dGTP, dATP, ATP, NAD, $MgCl_2$–potassium phosphate buffer (pH 7.4), and [^{3}H]dCTP where appropriate. After a 20-min incubation at 37°, the cells were chilled and washed free of this mixture, then warmed and lysed with sodium dodecyl sulfate, digested with Pronase, and sheared to give DNA fragments of molecular weight 3 $\times$ 10^7. For pycnography the lysates were mixed with CsCl solution and centrifuged in a fixed-angle rotor, and the fractions were added directly to scintillation-counting fluid.

NAL, a gift of the Sterling–Winthrop Research Foundation, was dissolved in 0.1 N NaOH, stored at 4°, and used at concentrations of 25 μg/ml in growth media and reaction mixtures. It was removed from cells, after treatment with toluene, by dilution with 50 volumes of cold 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$, from which the cells were then sedimented (at 5000 $\times$ g for 5 min) and resuspended in the incubation mixture for DNA replication. In one experiment, when NAL was removed from viable cells, this was accomplished by Millipore (0.45 μm) filtration and washing with NAL-free medium at 37°.

Cells were treated with toluene after their chromosomes had been labeled in one or two of three different ways: (*1*) over

a large fraction of the newly replicated portion of the chromosome, (*2*) in a small region approximately straddling the growing forks, or (*3*) in a small region at the chromosomal origin, where the replication cycle commences. The first two modes of labeling do not require synchrony of replication. In order to label a large, recently replicated portion of the chromosome, cells were grown for a large fraction of their 50-min mass-doubling period after addition of [*methyl*-^{14}C]thymine. Small regions were labeled by addition of 0.2 μg/ml of labeled thymidine to cells growing in media containing 3 μg/ml of unlabeled thymine. The thymidine is then taken up preferentially, but its presence induces the enzyme thymidine phosphorylase (EC 2.4.2.4), which rapidly cleaves it to thymine (11), which is already present, unlabeled, in excess. 85% Of the incorporation of label into Cl_3CCOOH-precipitable material was complete after 5 min. After subsequent growth for one mass-doubling period it was possible to show that this procedure labeled only the small region of the chromosome being replicated during the pulse.

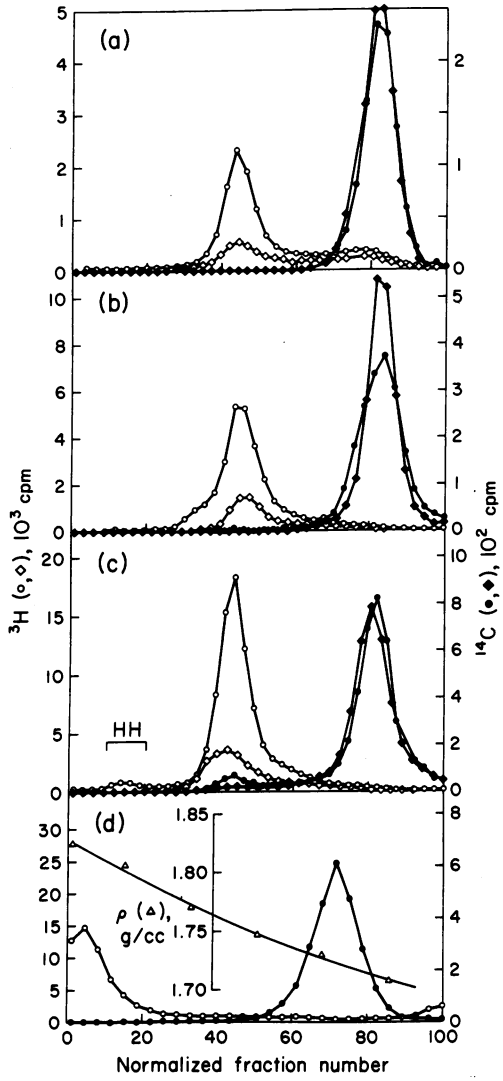

FIG. 1. Effect of NAL on the rate and site of DNA replication as revealed by DNA label density transfer experiments. Cells (10^8 to 4×10^8 cells per gradient tube) were prelabeled with [^{14}C]thymine (●, ◆, 0.1 μCi/ml, 6 μg/ml) for 23 min. Next the cells were treated with NAL (25 μg/ml) for (*a*) 0 min (no NAL added), (*b*) 8 min, or (*c*) and (*d*) 50 min, treated with toluene, washed free of NAL, and incubated for 20 min at 37° in a reaction mixture containing 70 mM potassium phosphate (pH 7.4), 13 mM $MgCl_2$, 0.13 mM NAD, 1.3 mM ATP, 33 μM dATP, 33 μM dGTP, 20 μM BrdUTP, and 20 μM [^{3}H]dCTP (○, ◇, 0.5 μCi/ml). Replicate reaction mixtures either contained (◇, ◆) or lacked (○, ●) NAL (25 μg/ml). Lysates were prepared and centrifuged to equilibrium in CsCl solution but lysate (*d*) was first heated at 98° for 5 min.

Chromosomal origins were labeled by the method of Bird and Lark (12), by use of amino-acid starvation to prevent the initiation of new replication cycles, while permitting those in progress to be completed. Thymine was then removed and amino acids were restored, so that synchronous initiation occurred when thymine was later restored. We adapted this procedure to our strain, starving cells (10^8/ml) of histidine for 200 min, then of thymine for 35 min, before addition of thymine (3 μg/ml) and [^{14}C]thymidine (0.2 μg/ml, 25 nCi/ml). Cells were transferred to new media after rapid filtration and washing on Millipore (0.45 μm) filters at 37°.

Some aliquots of the culture to be treated with toluene were assayed for chromosomal density transfer *in vivo*, during 20 min in media containing 10 μg/ml of bromodeoxyuridine (BrdU, from Cal Biochem) instead of thymine. Such cells were then harvested and treated with toluene as usual, but immediately washed, lysed, and digested for pycnography.

RESULTS

Several effects of exposure to NAL, either before or during *in vitro* DNA replication, are evident in Fig. 1. DNA synthesized *in vitro* is labeled with BrdUTP and [^{3}H]dCTP (*hollow symbols*), while the DNA made before toluene treatment was labeled with [^{14}C]thymine (*solid symbols*). The ^{14}C prelabel was used to reveal that the chromosomal site of replication was shifted after treatment with NAL.

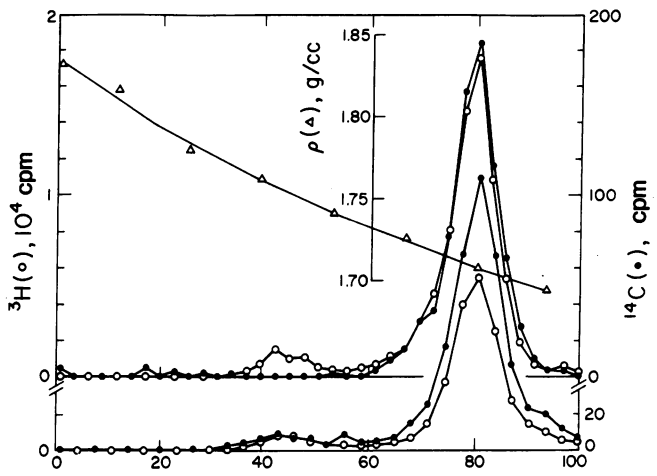

FIG. 2. Density transfer persists at the preexisting growing fork. At the time that NAL (25 μg/ml) was added to cells growing with a 51-min doubling time, they had been labeled *at* the growing fork for 6 min with [^{3}H]thymidine (○, 1 μCi/ml, 0.2 μg/ml), added 54 min earlier and labeled *behind* the growing fork with [^{14}C]thymine (●, 0.1 μCi/ml, 6 μg/ml, final concentration) added 35 min before the NAL. Cells were treated with toluene after exposure to NAL for 10 min (*upper curves*) or 60 min (*lower curves*), then washed and incubated in reaction mixtures like those of Fig. 1, but lacking NAL, and containing 33 μM dCTP in place of [^{3}H]dCTP. Lysis and pycnography followed as usual.

After growth for 23 min in [^{14}C]thymine, cells were either treated with toluene at once (Fig. 1*a*) or after exposure to NAL for 8 or 50 min (Fig. 1*b* and *c*, respectively). A similar prelabeling procedure has been used to demonstrate that only the DNA *ahead* of the growing forks was replicated by toluene-treated cells (3). In agreement with this, cells that had been unexposed or briefly (8 min) exposed to NAL before toluene treatment (Fig. 1*a* and *b*) failed to transfer ^{14}C to hybrid density upon incubation with nucleotides including BrdUTP. When the exposure to NAL before toluene treatment was prolonged to 50 min (Fig. 1*c*), density transfer occurred, indicating a new site of *in vitro* synthesis, in the chromosomal region labeled *behind* the growing forks for 23 min. Comparisons of the areas under the ^{3}H and ^{14}C curves indicate that the rate of *in vitro* synthesis per cell is doubled after prolonged exposure to NAL, but this result may reflect an increase in either the number of growing forks or the rate of DNA chain elongation. Essentially identical pycnographs were obtained when NAL was removed just before, instead of just after, toluene treatment (unpublished results). The label densities of a heat-treated lysate (Fig. 1*d*) are those of light- and heavy-density single strands, indicating that the hybrid densities of ^{3}H and ^{14}C labels are due to a normally dissociable product.

Regardless of prior treatment, exposure to NAL *in vitro* inhibits both semiconservative and repair replication as measured by uptake of [^{3}H]dCMP residues into hybrid and light species, respectively. In Fig. 1*a*, incubation with NAL inhibits semiconservative and repair synthesis by 70 and 40%, respectively. Exposure to NAL before toluene treatment (Fig. 1*b* and *c*) results in a 70% diminution of the low density ^{3}H "tail," but this uptake is still sensitive to NAL. In Fig. 1*c*, a minor amount of ^{3}H appears in the position characteristic of heavy duplex (HH).

To determine whether the preexisting growing fork could resume synthesis *in vitro* after the longer exposure to NAL *in vivo*, we did density transfer experiments after addition of NAL to cells with chromosomes labeled *at* the growing forks with [^{3}H]thymidine and *behind* the growing forks with [^{14}C]thymine. The cells were labeled as described in *Methods* and the legend of Fig. 2, so that when NAL was added the chromosomes had just been labeled with [^{14}C]thymidine for 35 min (51-min mass-doubling period) and previously labeled with [^{3}H]thymidine for a 6-min interval begun 54 min before toluene treatment in order to label a short region straddling the growing forks. After 10 or 60 min in NAL, cells were treated with toluene, removed from NAL, and allowed to incorporate nucleotides including BrdUTP. The regions replicated were then revealed by pycnography.

In Fig. 2, density transfer of ^{14}C from *behind* the growing forks requires the longer exposure to NAL (lower curves), as in Fig. 1. Since the ^{3}H label *at* the growing forks (○) is transferred to hybrid density equally well after 10- or 60-min prior treatment with NAL, some of these forks must remain active after prolonged NAL treatment, unlike the forks *in vivo* (7). The rate of DNA replication at the new forks is indicated by a ^{14}C density transfer of 7% in 20 min, which is 17% the rate of replication *in vivo*. This rate of replication, typical of toluene-treated cells, suggests that initiation occurs on only some of the partially replicated strands, if we assume that the rates of DNA chain elongation at new and old forks are equal. In Fig. 1*c* the rate of synthesis is only double that in *a* and *b*,

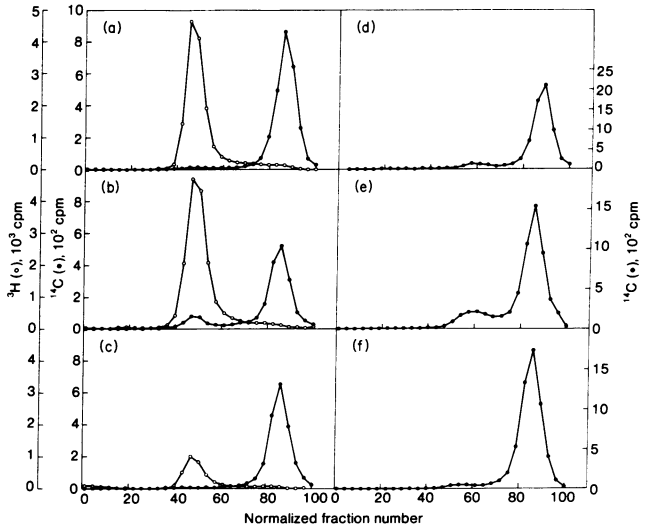

FIG. 3. NAL-induced reinitiation of chromosome replication *in vitro* and *in vivo*. Chromosome origins were labeled for 6 min with [^{14}C]thymidine (●, 25 μCi/ml, 0.2 μg/ml) upon initiation of replication, after amino-acid starvation, as described in *Methods*. Subsequent replication of labeled DNA was assayed by density transfer, either after toluene treatment (*a,b,c*) as in Fig. 1, (with [^{3}H]dCTP, ○) or *in vivo* (*d,e,f*), by transferring cells for 20 min to media substituting BrdU (10 μg/ml) for thymine. Between initiation of replication and assay of density transfer, cells were grown for 30 min (*a,d*); then NAL (25 μg/ml) was added for 55 min (*b,e*) or NAL was added and histidine was removed for 55 min (*c,f*).

so that if the old forks persist, one new pair of forks may account for the increase.

This experiment does not rule out the possibility of mutually exclusive activity at the old or new forks of each cell, as *in vivo* after prolonged NAL treatment. It is possible that some cells in the toluene-treated population may resume synthesis at the old forks while others reinitiate. This hypothesis seems unlikely: despite the considerable replication occurring at new forks, the density transfer of ^{3}H from the old forks is undiminished. Even if two new forks were initiated for each old fork inactivated, the doubling of [^{3}H]dCTP incorporation seen after such treatment (Fig. 1) would require that nearly all the activity at the old forks cease, but this activity is seen to remain undiminished, as measured by density transfer of ^{3}H in Fig. 2. Unfortunately, the activity of toluene-treated cells varies in different experiments (3), so such rate measurements cannot determine reliably the number of growing forks.

To determine whether the new sites of replication corresponded to the chromosomal origins, density transfer experiments were done with cells labeled at the origin(s) with [^{14}C]-thymidine (Fig. 3), as described in *Methods*. Little replication of chromosomal origins would be expected 30 min after the starvation-induced synchronous initiation, followed by growth in media supporting a 50-min doubling time. This is reflected in the slight amount of density transfer seen *in vitro* (Fig. 3*a*) or *in vivo* (Fig. 3*d*). If the 30-min period of growth after synchronous reinitiation were followed by exposure to NAL for 55 min, its removal *in vitro* or *in vivo* precipitated reinitiation, as evidenced by much-increased density transfer of the labeled origin (Fig. 3*b* and *e*). That this was not merely due to redistribution of the label is indicated by the absence of density-transfer when cells were prevented from synthesizing proteins

during their exposure to NAL, either by histidine starvation (Fig. 3c and f) or by 100 μg/ml of chloramphenicol (unpublished experiments). Again, the effect is similar *in vivo* and *in vitro*.

This experiment demonstrates the initiation of new replication at the origin after the removal of NAL *in vitro*—just as *in vivo*. It does not indicate whether the new replication commences only or even mainly at the origin, but analysis of the amount of label transferred suggests the latter, at least, is true. In Fig. 3b, 15% of the origin label has been transferred to hybrid density in 20 min. This high rate of ^{14}C density transfer taken together with [^{3}H]dCTP incorporation at 1/5 the *in vivo* replication rate indicates that the labeled portion of the chromosome (14%) is being replicated preferentially. Replication at the origin accounts for almost half of the DNA synthesis commencing at a new site when NAL is removed *in vitro*.

DISCUSSION

The rate of DNA synthesis in rapidly growing *E. coli* and *Bacillus subtilis* cells is controlled by adjustment of the interval between successive initiations of chromosome replication (9, 10). If toluenized cells can also control their DNA synthesis in this way, they might be used to assay for initiation-regulating factors (4). Burger (3) showed that toluene-treated cells continue DNA replication, but only in that chromosomal region that was about to be replicated *in vivo*. Matsushita, White, and Sueoka (13) confirmed this, but White, Matsushita, and Sueoka (manuscript in preparation) showed that toluene-treated *B. subtilis* cells soon preferentially diminish replication of the genetic markers nearer the chromosomal origin, which suggests that these cells cannot initiate new cycles of DNA replication when they are due.

The experiments reported here indicate that initiation at the origin may be induced *in vitro* as *in vivo* by removal of NAL after a suitable period of exposure. This *in vitro* "initiation" might well be trivial if the only effect of NAL were to block DNA chain elongation and the cells had completed all the necessary preliminaries to initiation while exposed to NAL *in vivo*. Nonetheless, these experiments indicate that, at the time of toluene treatment, the cells' osmotic integrity is not necessary for reinitiation to proceed. Moses and Richardson (1) have demonstrated that the wall of toluene-treated cells does not prevent the reaction of endogenous DNA polymerase with antibodies, of cellular DNA with exogenous DNase, which suggests that these cells are permeable to some macromolecules.

In many respects, the replicative potential of DNA in toluene-treated cells reflects the state of the cells before treatment. A notable difference is that replication is seen both at the new and old forks *in vitro*, after prolonged exposure to NAL. This observation indicates that the process that inactivates the old fork *in vivo* does not occur in toluene-treated cells even after prolonged exposure to NAL, or that toluene treatment can reverse this inactivation. No experiments were done with cells removed from NAL before toluene treatment to find out whether the old forks remain inactive *in vitro*. Uncoupling of NAL-induced reinitiation from the inactivation of the old growing forks may be considered a regulatory aberration. It may arise either through changes in cell permeability or through some other toluene-induced structural derangement.

The decreased sensitivity of toluene-treated cells to inhibition by NAL does not imply that toluene treatment has affected the reaction sensitive to NAL, or that the immediate effect of NAL has changed, quantitatively or qualitatively. It is not necessary to suggest, for example, that viable cells concentrate NAL—which has been disproved by H. Reiter (personal communication). If the 80–90% inhibition of DNA replication resulting from toluene treatment occurs at some other point in the pathway than that susceptible to NAL, there is no reason to expect that the residual activity of toluene-treated cells will be as sensitive as that of viable cells.

The decrease in nonconservative replication by toluene-treated cells that had been exposed to NAL *in vivo* has not been further investigated.

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