ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1985, p. 200-206
0066-4804/85/080200-07$02.00/0
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Vol. 28, No. 2

Membrane Permeability Changes Associated with DNA Gyrase Inhibitors in *Escherichia coli*

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Received 13 February 1985/Accepted 7 May 1985

Inhibition of DNA synthesis in *Escherichia coli* B/r by a DNA gyrase inhibitor results in cell death after a 50-min lag period. Examination of the cells under phase-contrast and electron microscopes revealed that they appeared to undergo plasmolysis coincident with the onset of cell death. The inhibited cells were also found to become susceptible to low levels of detergent at this time. With a fluorescent membrane probe, the level of membrane permeability was assessed and found to increase concurrently with the decrease in culture viability. Analysis of the cell envelope constituents revealed that, other than a shift in the protein/lipid ratio, the compositions of the cell membranes were unperturbed.

There has been a renewed interest in DNA gyrase inhibitors with the recent introduction of new quinolone derivatives with increased in vitro activity and improved pharmacokinetics (3). Treatment with these DNA gyrase inhibitors ultimately leads to loss of bacterial viability. A number of studies have focused on the effects that these antibiotics have on the topology of DNA and gene expression (for review, see reference 6). Cessation of DNA synthesis by gyrase inhibitors activates a cascade of events within the cell involving a wide variety of biochemical pathways, including cell filamentation (12). Only a few studies have addressed the physiological consequences of DNA replication inhibition on the bacterial cell envelope (21, 22).

During a series of studies on DNA replication inhibitors, we noted morphological changes consistent with a loss of membrane integrity and leakage of cytoplasmic constituents. In this report, the effect of a DNA replication inhibitor on the structure and function of the bacterial envelope is examined. Substantial alterations in the integrity of the membranes are documented, and their possible relationship to loss of viability is discussed.

MATERIALS AND METHODS

Bacterial strain. *Escherichia coli* B/r T^-, a thymine-requiring auxotroph, was derived from a prototrophic *E. coli* B/r strain (obtained from George Khachatourian, University of Saskatchewan, Saskatchewan, Saskatoon, Canada) by an amethopterin selection procedure (4). The strain was a low-level thymine requirer (*thyA deoB*) that grew fully on 2 μg of thymine per ml. Cells were treated with 20 μg of nalidixic acid (Nal; Sigma Chemical Co., St. Louis, Mo.; prepared in 0.5 N NaOH) per ml, which is 10 times the MIC. Chloramphenicol (Sigma) was also used at 10 times the MIC (25 μg/ml).

Medium. The organisms were grown in M9 medium (16) containing 0.4% glucose and supplemented with 5 μg of thymine per ml. (M9 buffer refers to the above composition, without glucose or thymine). The strain was maintained on M9 medium solidified with 1.5% agar.

Growth conditions. A sample (0.5 ml) of an overnight culture was inoculated into 100 ml of prewarmed M9 medium and grown with aeration at 35°C for at least five doublings before the start of an experiment. Viable counts were obtained by making serial 10- or 100-fold dilutions in M9 buffer and spreading 0.1-ml samples in triplicate on M9 agar plates with a sterile glass rod. After incubation for 48 h, the plates were counted. Culture absorbance was read at 540 nm in a Perkin-Elmer model 124 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) with M9 buffer as a reference.

Microscopy. Phase-contrast microscopy was carried out with a Zeiss microscope. For photography, cells were spread (either immediately or after fixation with 0.85% formaldehyde) on slides with thin agar layers.

Cells were prepared for electron microscopy by adding an equal volume of 3% (wt/vol) glutaraldehyde in 0.067 M cacodylate buffer (pH 7.3) to cells in M9. After 5 min of fixation, the cells were washed twice in cacodylate buffer (0.067 M, pH 7.3) and postfixed in 1% OsO_4 in cacodylate buffer for 5 min. The cells were washed, dehydrated, and embedded in Epon 812.

The sections were cut on a Sorvall Porter-Blum MT-2 ultramicrotome equipped with a diamond knife. Sections were stained with uranyl acetate (5 min) and Reynolds lead citrate (3 min). The stained sections were viewed in a Hitachi model HS-8 electron microscope. All electron microscopy reagents were from Polyscience, Warminster, Pa.

Detergent susceptibility assay. Sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Rockville Center, N.Y.; 10% stock solution in distilled water) was added with vigorous stirring to the culture to make the final concentration of SDS 0.1%. The cultures were incubated with shaking at 35°C, and the turbidity was followed at 540 nm.

Isotope incorporation and loss studies. One-milliliter of 1-ml cultures were incubated in the presence of 5 μCi of [^{3}H]thymine (New England Nuclear Corp., Boston, Mass.; diluted to 1 μCi/μg). At timed intervals, 50-μl samples were placed on paper disks, immersed in ice-cold 5% trichloroacetic acid with 1% sodium pyrophosphate, washed twice in cold 95% ethanol and once in cold acetone, and air dried on

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a wire screen. The disks were placed in scintillation vials, and 10 ml of toluene-based scintillation fluid was added. Leakage of cytoplasmic constituents was measured by prelabeling of a 10-ml culture with [^{3}H]uracil (24.9 Ci/mmol; New England Nuclear) for 30 min, washing twice by centrifugation (7,700 × *g*, 5 min) and suspending the sample in M9 buffer, with a final suspension in M9 medium. Loss of incorporated radiolabel was assessed by removing 500-μl portions into microfuge tubes, centrifugating (Eppendorf 3200 centrifuge; Brinkman Instruments, Westbury, N.Y.), and counting 250 μl of supernatant fluid in Biofluor (New England Nuclear). A portion (100 μl) was also tested for trichloroacetic acid-precipitable counts on paper disks as described above. Peptidoglycan breakdown was measured by prelabeling cells with *N*-acetyl-D-[^{3}H]glucosamine (1.7 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 1 h, washing as above, and removing 1-ml samples into tubes with 5% trichloroacetic acid on ice. After centrifugation, 1 ml of 4% SDS was added, and the tubes were placed in boiling water for 20 min. The SDS-insoluble material was collected on membrane filters (Millipore Corp., Bedford, Mass.), washed twice with ice-cold water, dried, and counted in a toluene-based scintillation system.

Fluorescent membrane probe technique. The fluorescent membrane probe technique used was that of Nieva-Gomez et al. (17). Samples (5 ml) of the cultures were chilled and centrifuged at 4°C at 7,000 × *g* for 5 min. The cells were washed, centrifuged, and suspended in M9 buffer. The optical density of all samples was adjusted to 0.6 (approximately 1.5 × 10^8 cells per ml). Three milliliters of each sample was removed, and *n*-phenyl-1-napthylamine (NPN; Eastman Organic Chemicals, Rochester, N.Y.) dissolved in methanol was added to the suspension to a concentration of 3 μM. The culture samples containing NPN were pipetted into quartz cuvettes (1-cm light path) and placed in a Bowman-Aminco spectrofluorometer. Excitation was at 340 nm, and emission was read at 420 nm at a 90° angle to the excitation beam. For the determination of the emission spectrum, the emission wavelength scale was manually changed in 10-nm increments, and measurements were recorded as arbitrary units by using the instrument markings. To determine the proportion of cell-bound fluorescent probe, the samples were centrifuged at 7,700 × *g* for 10 min, and the fluorescence in the supernatant fluid was recorded.

Analysis of membrane components. Pulse-labeling of membrane proteins was carried out in 20-ml samples of cells (either control or Nal treated) by washing and suspending in prewarmed low-sulfate (2 μg/ml) M9 medium with 5 μCi of Na[^{35}S]O_4 (43 Ci/mg; New England Nuclear) per ml for 6 min and terminating with excess $MgSO_4$. Cells were concentrated to 5 ml and broken by sonication (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) with glass beads in a safety hood, and membranes were collected at 100,000 × *g*. After two additional washes with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.4), this material was used as the cell envelope fraction. For analysis of cytoplasmic and outer membranes, the cytoplasmic membrane was solubilized with Triton X-100 (Sigma Chemical Co.) by the method of Schnaitman (20). The outer membrane was collected by centrifugation, and the cytoplasmic membrane was precipitated with ice-cold 10% trichloroacetic acid and washed twice with cold acetone. Membrane proteins were analyzed in the polyacrylamide gel electrophoresis system of Ames (1). Detection of radiolabeled proteins was by autoradiography on Kodak AR-2 film (Eastman Kodak Co., Rochester, N.Y.).

Pulse-labeling of membrane protein with [^{14}C]leucine (47 mCi/mmol; New England Nuclear) and lipid with [^{3}H]acetate (120 mCi/mmol; New England Nuclear) was carried out as described previously (8). After cell envelope preparation, the material was counted in Biofluor and corrected for ^{14}C overlap in the ^{3}H channel; 98% of the acetate label in the membrane was extractable into the chloroform-methanol phase.

Protein was determined in the cell envelope by the Lowry et al. method (14) with crystalline serum albumin as the standard. Phospholipids were extracted from dried cells into chloroform-methanol, and phosphorus was determined by the method of Ames and Dubin (2). Lipopolysaccharide was quantitated by determination of 2-keto-3-deoxyoctonate in the cell envelope as described by Osborn (19).

RESULTS

Effect of gyrase inhibitors on viability and morphology. Treatment of a logarithmically growing culture of *E. coli* B/r with 20 μg of Nal per ml led to an immediate halt in [^{3}H]thymine incorporation into the cold acid-insoluble fraction of the cells (Fig. 1). The onset of cell death, as measured by colony-forming criteria, ensued 50 min after the addition of Nal. The rate of cell death was reduced markedly when the cells were simultaneously treated with Nal and chloramphenicol (data not shown), as has been previously reported (7). Cultures of growing *E. coli* B/r that were treated with Nal contained a number of elongated rods. A striking feature was the appearance of areas of reduced cytoplasmic contrast (vacuoles) visible under both the phase-contrast and electron microscopes. Figure 2 shows these areas, which began to appear after 50 min of Nal treatment, when the viability of the culture started to decline. Very few vacuoles were present at the 60-min point, whereas the majority of cells contained them after 120 min of exposure to Nal. The areas

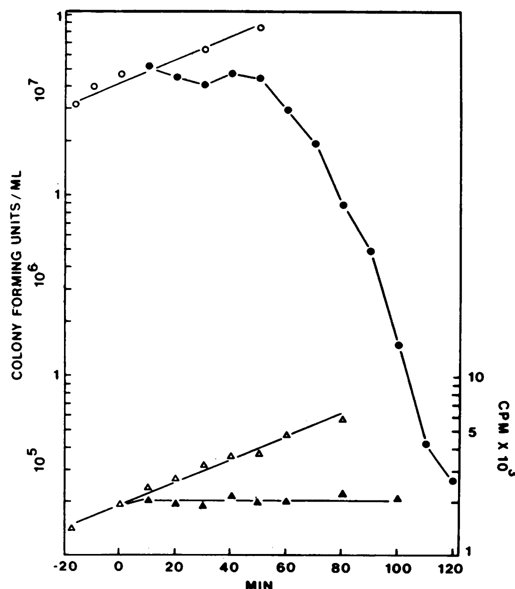

FIG. 1. Viability and incorporation of [^{3}H]thymine into the trichloroacetic acid-insoluble fraction. CFU of control cells (○) and Nal (20 μg/ml)-treated cells (●) are shown. Nal was added at time 0. Incorporation of labeled thymine into cells is shown for control (△) and treated (▲) cultures.

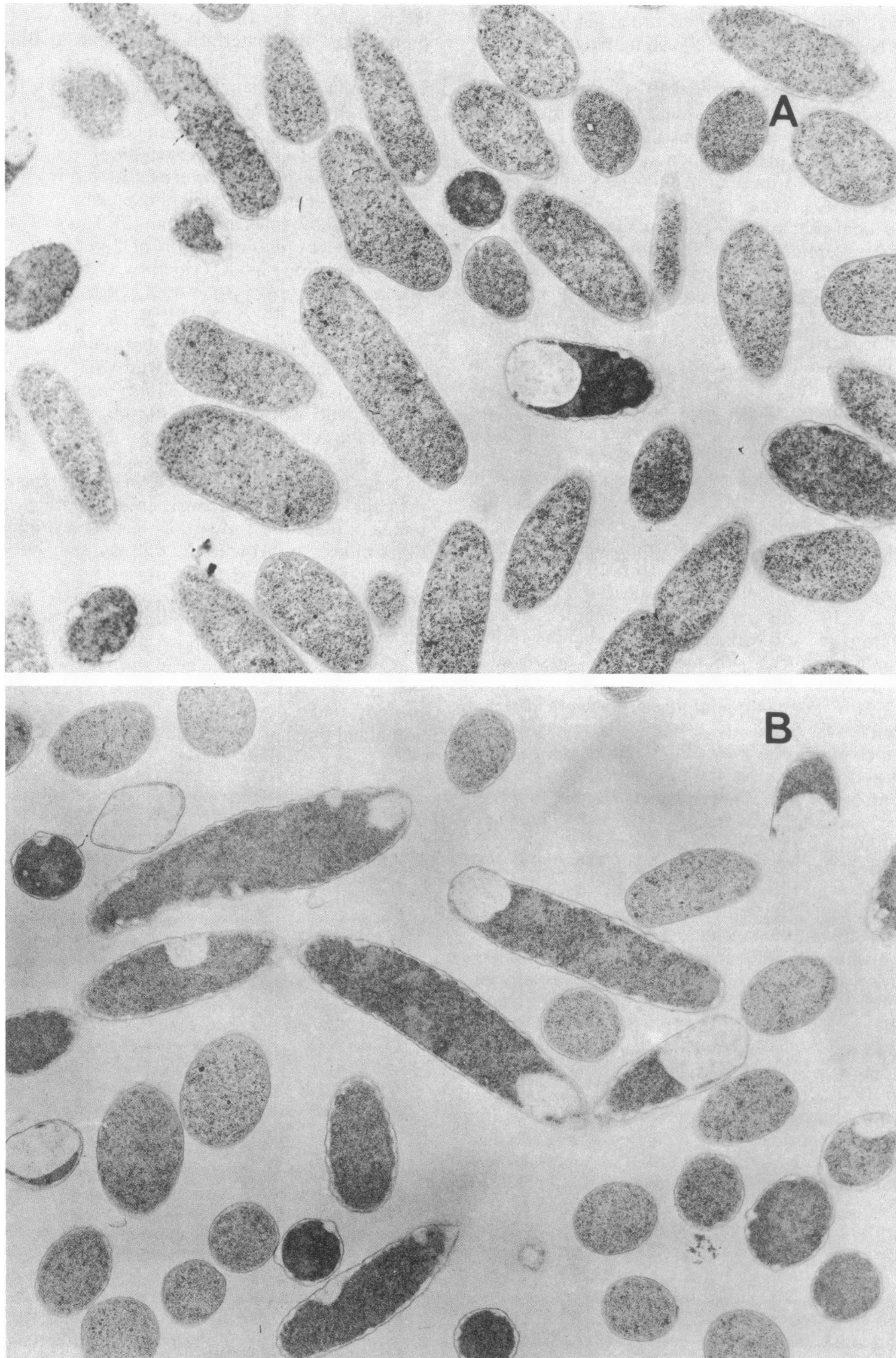

FIG. 2. Electron micrograph of a typical *E. coli* B/r culture after 60 min (A) and 120 min (B) of Nal treatment. The cytoplasm has retracted from the rigid outer cell membrane, and substantial areas that appear to be devoid of cytoplasmic material are visible.

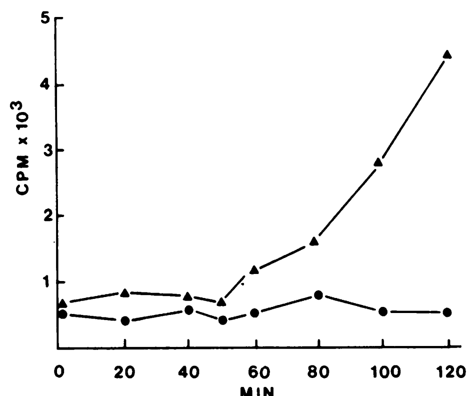

FIG. 3. Leakage of material prelabeled with [^{3}H]uracil. A culture was labeled, washed, and divided into two subcultures. The loss of label into the supernatant fluid of control (●) and Nal-treated (added at time 0) (▲) cultures is illustrated.

were not observed when the cells were treated simultaneously with Nal and chloramphenicol. Examination of these areas revealed that they were devoid of cytoplasm, as if substantial amounts of cell constituents had been lost, leading to cytoplasmic condensation. Experiments in which leakage of prelabeled material ([^{3}H]uracil) was followed confirmed that large amounts of trichloroacetic acid-soluble, uracil-containing substituents were lost, beginning 50 min after Nal addition (Fig. 3).

Effect of detergent on Nal-treated cells. Growing cells of *E. coli* B/r were not affected by a low concentration of the surfactant SDS. Treatment with Nal alone resulted in an increase in turbidity, due primarily to an increase in cell length. Cells were resistant to 0.1% SDS for 50 min after Nal addition (Fig. 4). Beyond this period, the culture became susceptible to detergent-mediated lysis, resulting in a decline in the turbidity. A Nal-treated culture that did not receive detergent exhibited no decrease in absorbance, although the turbidity did level off. The addition of 0.1% SDS to a culture that had been preexposed to Nal for 120 min led to a very rapid (<3-min) decline in turbidity. The addition of chloramphenicol to Nal-treated cultures inhibited the detergent-mediated lysis (data not shown).

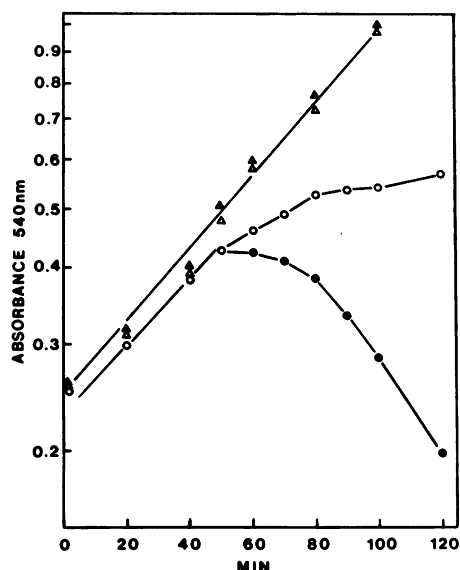

FIG. 4. Effect of detergent on Nal-treated cells. A large culture was divided into four subcultures. Two of the cultures were controls; one received no treatment (△), and one received 0.1% SDS at time 0 (▲). The two other cultures were treated with Nal at time 0, one with (●) and one without (○) 0.1% SDS, also added at time 0.

Peptidoglycan degradation triggered by Nal would be one explanation for the lysis observed. Loss of incorporated *N*-acetylglucosamine label from the hot SDS insoluble fraction was measured in control cells, Nal-treated cells, SDS-treated cells, and cells treated with both SDS and Nal. No differences were seen at either 60 or 120 min in the hot SDS-insoluble fraction from any of the treatments (data not shown).

Fluorescent membrane probe. The interaction of Nal-treated cells with the fluorescent probe NPN was studied. Cell samples that were exposed to Nal for the indicated periods received 3 μM NPN. Figure 5 demonstrates that the fluorescence in Nal-treated cells remained at the control level for 50 min, after which there was a sharp rise in fluorescent emission. Chloramphenicol was found to substantially suppress the increased fluorescence observed in Nal-treated cells.

It was necessary to establish that the increased fluorescence was due to cell-bound NPN and not to possible leakage of intracellular material, which may fluoresce, into the medium. Low-speed centrifugation (7,700 × *g*, 10 min) was found to remove the increased fluorescence from the sample, indicating that it was cell bound. The probe was also found to have undergone a shift in the emission maximum, from 440 to 410 nm, indicative of the probe being in a lipid environment (9, 17).

Comparison of cell envelope compositions. Membranes of control cells and cells treated with Nal for 120 min were prepared from large (2-liter) cultures, and washed three times with distilled water. The preparations were dried in vacuo and weighed, and protein and 2-keto-3-deoxyoctonate determinations were performed. Lipid phosphorus was determined after extraction of lipid from dried, weighed cells (Table 1). Protein content increased slightly during Nal

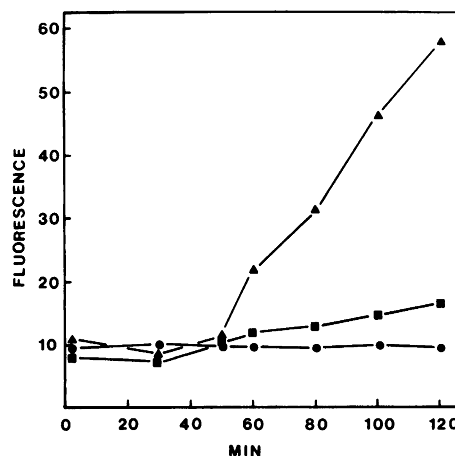

FIG. 5. NPN fluorescent probe interaction with *E. coli* B/r. The fluorescent response of the NPN probe was measured at intervals in samples from cultures that were either control cells (●), cells treated with Nal at time 0 (▲), or cells treated with Nal and chloramphenicol at time 0 (■). Fluorescence is in arbitrary units.

TABLE 1. Analysis of cell envelope components[a]

Cells[b]	Membrane protein (μg/mg of cell envelope)	KDO (μmol/mg of cell envelope protein)	Phospholipid phosphorus (μg/mg of cell dry wt)
Controls	560 (12)	0.095 (0.006)	51.4 (1.8)
Nal treated	578 (9)	0.084 (0.004)	44.7 (2.1)

[a] Values are averages of quadruplicate determinations, with the number in parentheses representing 1 standard deviation.

[b] Control cells and cells treated with 20 μg of Nal per ml for 120 min were assayed as described in the text for cell wall constituents.

treatment, whereas lipid and lipopolysaccharide synthesis declined slightly. Pulse-labeling of the membrane proteins synthesized during the 60 min after Nal addition showed only slight changes in synthetic rates of a few outer membrane proteins (Fig. 6), except for synthesis of the RecA protein (40,000 molecular weight), known to be induced under these conditions, and decreased levels of two higher-molecular-weight, minor outer membrane proteins (5, 12). There were no protein changes observed in the inner membrane fraction.

The difference in protein and phospholipid synthetic rates was investigated further by pulse-labeling control and Nal-treated cells with [^{3}H]acetate and [^{14}C]leucine. Envelopes were isolated, and the amount of each label was determined. Whereas [^{14}C]leucine incorporation paralleled cell mass closely in Nal-treated cells, [^{3}H]acetate incorporation into the chloroform-methanol–extractable phase occurred at a considerably lower rate. This is expressed as the relative

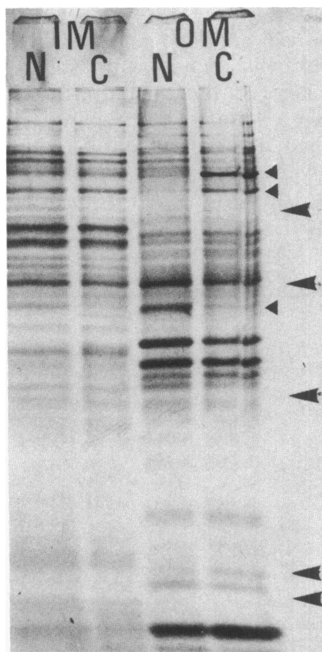

FIG. 6. Autoradiograph of proteins synthesized at 60 min after the addition of Nal. Cells were pulse-labeled for 6 min, and then the membrane fractions were isolated as described in the text. Abbreviations IM and OM, inner and outer membranes, respectively; N, Nal-treated cells; C, controls. Molecular weight markers (large arrows) at the right represent, from the top, 66,000-, 43,500-, 30,000-, 17,000-, and 12,400-molecular weight standards. The outer membrane changes are indicated by small arrows.

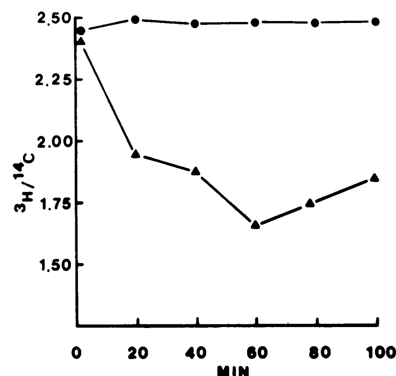

FIG. 7. Synthesis of protein and lipid in the cell envelope fraction. Shown are the ratios of [^{3}H]acetate incorporation to [^{14}C]leucine incorporation into cell envelopes isolated from control cells (●) and cells that received Nal at time 0 (▲).

ratios of incorporation in Fig. 7. Of particular importance was the finding that this imbalance occurred relatively early in Nal treatment, well before the onset of the membrane effects and viability loss.

DISCUSSION

Strains of *E. coli* B/r are noted for the rather lengthy delay between inhibition of DNA synthesis and the onset of rapid cell death (10). This property makes it possible to study physiological events after the addition of DNA replication inhibitors that may be associated with the lethal consequences.

The quinolone antibiotics have as their immediate target in the bacterial cell the DNA gyrase. Inhibition of this enzyme interferes with DNA supercoiling and, among other consequences, leads to differential expression of genes (26) and activation of the SOS response (6, 12, 23). The SOS response is a cascade of events involving a number of gene products. It leads to, among other things, cessation of respiration and blocked cell division. When the antibiotic is removed before the 50-min point in *E. coli* B/r or when protein synthesis is blocked by chloramphenicol, the lethal effect of Nal is not expressed.

In *E. coli* B/r strains, there are mutations at two loci, *lon* and *sulA*, which play roles in cell division. The *sulA* (*sfiA*) gene is induced as part of the SOS response to treatments such as Nal (6, 23). Thus, the response of *E. coli* B/r may not reflect that seen in wild-type strains. An additional complication is the recently reported effect of Nal on the *htpR* locus, which controls the heat shock response (11). The effect of Nal on *E. coli* therefore involves the activation of multiple genes, and the interplay among these is undoubtedly complex.

In the present study, the physiological response of *E. coli* B/r to Nal was examined, focusing on the properties of the cell envelope. Morphological abnormalities indicated that the cells were undergoing a process that superficially resembled plasmolysis. Indeed, the cells were found to be losing radiolabeled marker, beginning at the same time that the morphology change was first visible. These data suggested that the functional integrity of the *E. coli* B/r envelope was lost, beginning at the same time that ability to form colonies declined.

E. coli B/r is normally unaffected by concentations of detergents ranging up to 5%, due to the ability of the outer membrane to exclude these agents (15). Nal-treated cells lost

this capability and began to lyse 50 min after antibiotic addition. The optical clearing seen under these conditions was not due to large-scale activation of peptidoglycan hydrolases, since there was no substantial loss of peptidoglycan label even during prolonged treatment with Nal plus SDS.

The fluorescent probe NPN has been used to assess the penetrability of the outer membrane (9, 13). The use of NPN, which is an uncharged molecule, allows the monitoring of structural changes that occur in the *E. coli* cell envelope as a result of loss of membrane potential (17, 18). The response of the fluorescent probe was identical in control and Nal-treated cells up to the 50-min time point. Again, after 50 min of Nal treatment, a response indicative of membrane perturbation was obtained, consistent with the results in the other experiments.

Compositional data of cell envelopes of Nal-treated *E. coli* indicated that the only substantive changes were a decrease in the rate of lipid synthesis and minor changes in outer membrane proteins. The change in the rate of lipid incorporation occurred shortly after Nal treatment, well before the other manifestations documented. This imbalance in the protein to lipid ratio was sustained for the duration of Nal treatment. As expected, the most notable consequence of DNA inhibition was the increased synthesis of the 40,000-molecular-weight RecA protein (12). With regard to the RecA protein, it is unclear whether this protein is actually associated with the outer membrane or whether it preferentially partitions with the outer membrane when this particular preparative procedure is used (27). The two high-molecular-weight proteins whose synthesis ceased upon Nal addition deserve further attention. Several changes were reported in total cell protein synthesis upon Nal treatment (5, 26).

It is known that treatment of *E. coli* with azide, cyanide, and other agents that dissipate membrane potential causes morphological changes, leakage of intracellular components, detergent susceptibility, and fluorescent probe response identical to those observed in the present study (17, 24). Furthermore, inhibition of DNA synthesis by UV irradiation also results in detergent susceptibility, vacuoles in the cytoplasm, and inhibition of respiration (21, 22).

This suggests that in *E. coli* B/r, a consequence of DNA synthesis inhibition by Nal is a disruption of normal membrane integrity. This membrane permeability may be triggered by the physiological responses of the cell as a result of DNA inhibition (22). An interpretation consistent with the data is that the cells lose their capacity to maintain the proton motive force, possibly as a result of either a *recA*-induced process or the imbalance in membrane composition. Loss of the potential across the membrane is known to have profound effects on outer membrane permeability (9, 25).

The most interesting aspect of the current work is the temporal relationship of the membrane-related phenomena and loss of viability. It is intriguing to speculate that inhibition of DNA replication leads to a cascade of physiological events terminating in a loss of membrane integrity, which is ultimately the lethal event in these cells.

ACKNOWLEDGMENTS

We thank Alexander Tomasz for his enthusiastic support and Margaret Geller for assistance in preparing the manuscript.

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