Mode of Action of Quinodixin and Substituted Quinoxaline-di-N-Oxides on *Escherichia coli*

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The effect of quinodixin on the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid, and protein in *Escherichia coli* KL 399 was examined under aerobic and anaerobic conditions. In the absence of oxygen the synthesis of DNA was completely inhibited by 10 ppm of quinodixin, whereas the synthesis of ribonucleic acid and protein were not affected. Quinoxaline-di-N-oxides (QdNO) induce degradation of DNA in both proliferating and non-proliferating cells. polA, recA, recB, recC, exrA, and uvrA mutants were more susceptible than the corresponding repair-proficient strains. All strains were more resistant in the presence of oxygen. Quinodixin was reduced to quinoxalin-N-oxide by intact *E. coli* cells or by a cell-free *E. coli* extract. Electron spin resonance measurements demonstrated the generation of free radicals during the reduction of quinodixin. Oxygen or deficiency of energy sources impaired the antibiotic activity and the reduction of QdNO. The QdNO reductase activity was demonstrated to be lower in QdNO-resistant mutants than in the susceptible parent strain. Based on these results it is concluded that an intermediate of reduction, probably a free radical, is responsible for the lethal effect of quinodixin. With three independent techniques no evidence has been found for binding of quinodixin to DNA.

The potent in vivo activity of various quinoxaline-1,4-di-N-oxides (QdNO) against diverse bacteria, *Entamoeba histolytica*, and *Chlamydiae* of the psittacosis-lymphogranuloma venereum group has been known for more than two decades (9, 24, 29, 35, 50). Like many antibiotics (e.g., penicillin or tetracyclines) and chemotherapeutics (e.g., nitrofuran or sulphonamide), QdNO increase the live weight gain in young chickens, pigs, and calves when added to the diet (5, 12, 16, 37). At present two QdNO, carbadox and olaquindox, are on the market as growth promoters in various countries; a third, quinodixin, has been withdrawn.

QdNO were first prepared as potential antagonists of vitamin K activity, but such antagonism has never been demonstrated (24, 35). Only a few further reports on the mode of action have been published. Studies on the effect of 2,3-dihydroxymethyl-quinoxaline-1,4-di-N-oxide on the incorporation of radioactive precursors into deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein in *Escherichia coli* revealed a specific inhibition of DNA synthesis (15). Morphological changes in cells of *E. coli* and *Staphylococcus aureus* treated with 2,3-dihydroxy-

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methyl-quinoxaline-1,4-di-N-oxides studied by using electron microscopy have been interpreted to result from impaired synthesis of DNA (41). The mode of action of myxin (1-hydroxi-6-methoxiphenazine-5,10-di-N-oxide), an antibiotic structurally related to the QdNO, has been investigated in more detail. Myxin inhibits DNA synthesis and induces single-strand breaks which lead to DNA breakdown (31, 32). In vitro studies showed that myxin binds to DNA by intercalation and inhibits RNA synthesis (22, 23).

Apparently contradictory to these results was a report showing that quinodixin is up to 100 times more effective under anaerobic conditions than in the presence of oxygen (21). To our knowledge no other inhibitor of DNA synthesis acts preferentially under anaerobic conditions. This led us to study the effect of QdNO on the synthesis of biopolymers under anaerobic conditions.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* W 8 was obtained from the Institut für Genetik, Universität Köln; *E. coli* QR 9, QR 26, and QR 29 were isolated from *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG)-treated *E. coli* W 8 by selection for increased quinodixin resistance. *E. coli* AB 1157, AB 1886, AB 2463, and AB 2500 (1) were
kindly supplied by W. Arber, Biozentrum, Basel, Switzerland. E. coli JC 3272, JC 3272 recA13, JC 3272 recB21, JC 3272 recC23, JC 3272 recF21, and JC 3272 recH21, selected from NTG-treated JC 3272 recB21, JC 2926 (1), PAM 5717 (18), JC 138, JC 139 (14), KL 399 (33), and UB 1139 (3) were all gifts from A. Moiolo-Batt and S. T. Jenkins, University of Bristol, England.

Bacteria were grown either in brain heart infusion (BHI) broth (Difco) or in M9 synthetic medium (30) supplemented with the amino acids required (0.5 mM), thiamine (10 μg/ml) and thymine (1 μg/ml).

Anaerobic incubation. Plates and activated iron wool (42) were placed in a desiccator. Before being sealed, the jar was flushed with CO₂.

Titration vessels (EA 875, Metrohm, Herisau, Switzerland) were used to perform experiments with cells growing in liquid media or with soluble cell extracts. The vessels were placed in an icebath, filled with reaction mixture, and sealed. To establish anaerobic conditions they were sparged with oxygen-free N₂ containing 5% (vol/vol) CO₂ (15 liters/h, Carba AG, Basel). The gas was sterilized by means of a cotton filter, purified, and humidified by passage through three flasks of alkaline pyrogallol, the last one warmed to the reaction temperature. After 15 min, the experiment was started by mounting the vessels in a water bath at 30, 37, or 42°C. Samples were withdrawn with sterilized needles.

Chemicals. Quinoxin (Grosfas, Imperial Chemical Industries), Carbadox (Mecadox, Pfizer), and Olaquinnox (Bay-O-Nox, Bayer) were obtained from commercial sources. QdNO solutions are sensitive to light and must be protected from daylight. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, calf thymus DNA, and bovine serum albumin were purchased from Serva Feinbiochemica, Heidelberg, German Republic of Germany. Trinitrated compounds were obtained from the Radiochemical Centre, Amersham, England.

Synthesis of macromolecules. Log-phase cells (2.5 × 10⁸ cells/ml) were grown in M9 medium supplemented with amino acids (final concentrations, 0.5 mM, except leucine at 0.05 mM), thiamine (10 μg/ml), and thymine and uracil (1 μg/ml). Either [methyl-³H] thymidine, [⁵⁷H] thymidine, [⁵⁻H] thymidine, or 1-[³-H] leucine (2.2 μCi/ml, final concentration) was added. Samples (0.4 ml) were withdrawn after 3 ml of ice-cold 10% trichloroacetic acid, and the acid-insoluble precipitates were collected by filtration. The filters were washed with 10 ml of ice-cold 1% acetic acid, dried at 100°C for 1 h, and used for radioactivity determination.

Degradation of DNA. Cells were grown overnight in supplemented M9 medium containing 1 μCi of [methyl-³H] thymidine per ml, harvested, washed, suspended in supplemented M9 medium without radioactive thymidine, and preincubated for 1 to 2 h. They were then transferred into reaction vessels, and the degradation of DNA was followed by measuring the increase of soluble radioactivity in the cold trichloroacetic acid fraction by using the method of Hall and Howard-Flanders (19).

Measurement of radioactivity. The filters or 0.1 ml portions of radioactive solution were placed into scintillation vials containing 8 ml of scintillation fluid [scintillation fluid: 3 liters of toluene, 1 kg of Triton X-100, 300 ml of water, 12 g of 2-(tert.-butyl-phenyl)-5-(4-biphenyl-1,3,5-triazidol)]. The radioactivity was determined in a liquid scintillation spectrometer (Packard TriCarb, B 2450).

Viable cell determination. Immediately after withdrawal, the samples were appropriately diluted with 0.9% NaCl and 0.1 ml was plated on BHI agar (Difco). The plates were aerobically incubated at 37°C for 24 h.

Determination of MIC. Late-log-phase cultures were diluted in saline to 10⁶ cells/ml, and 0.05 ml was distributed with a loop on BHI agar plates containing QdNO in serial twofold dilutions. Plates were incubated under aerobic or anaerobic conditions at 37°C for 24 h. The minimum inhibitory concentration (MIC) is defined as the arithmetic mean of lowest drug concentration which completely inhibited growth and the next, twofold-lower concentration.

Preparation of cell-free extracts. Frozen late-log-phase cells were ground with aluminum oxide (Carnag 507 C) in 0.067 M phosphate buffer (pH 7.2). After removing the aluminum oxide by centrifugation (5 min, 1,000 × g), the extract was centrifuged for 15 min at 48,000 × g. The supernatant cell-free extract was stored at 0°C and subsequently used for experiments during 4 days without loss of activity.

Determination of protein. Protein was determined by using the biuret reaction of Stickland (10). The absorbancies were converted to milligrams of protein by using bovine serum albumin as a standard.

Measurement of reduction of QdNO by intact E. coli. Late-log-phase cells were harvested, washed, and suspended in M9 medium, and the turbidity (optical density at 550 nm [OD₅₅₀]) was adjusted to 4.0 (2.5 × 10⁸ cells/ml). An appropriate amount of suspension was mixed with QdNO and placed in a incubation vessel as described. The samples withdrawn were mixed with a threefold excess of ice-cold ethanol, the cells were removed by centrifugation, and the absorbance of the supernatant was determined at 380 nm.

Measurement of reduction of QdNO by cell-free extracts of E. coli. Cell-free extract (2 mg of protein/ml, final concentration) was mixed with cysteine (pH 7, 1% final concentration), QdNO, and a reducing system at 0°C. Two reducing systems were used: (i) 386 nmol of reduced nicotinamide adenine dinucleotide (NADH), 172 μmol of glucose, and 18 enzyme units of alcohol dehydrogenase; or (ii) 386 nmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH), 60 μmol of glucose 6-phosphate, and 5.6 enzyme units of glucose 6-phosphate dehydrogenase. Phosphate buffer (0.067 M, pH 7.2) was added to give a final volume of 4 ml. Samples (0.5 ml) were mixed with 2 ml of ice-cold ethanol. The precipitate was removed by centrifugation, and the absorbance of the supernatant was determined at 380 nm.

Determination of the redox potential. Two combined platinum/calomel (3.5 M KCl) electrodes (Ea 217, Metrohm, Herisau, Switzerland) were inserted in each culture vessel. They were sterilized by immersion in 5% H₂O₂ for 30 min. The monitoring apparatus was an E510 pH meter (Metrohm).

ESR measurement. Electron spin resonance (ESR) observations were made with a Varian E 9
spectrophotometer employing 100-kc magnetic field modulation. The samples were placed in flat cells under anaerobic conditions.

Quinodixin/DNA binding studies. Calf thymus DNA and quinodixin were dissolved in 0.0015 M NaCl/0.00015 M trisodium citrate and mixed at different DNA-phosphate/drug ratios. The spectrophotometer cuvettes containing the appropriate mixtures were incubated for 24 h at room temperature under anaerobic conditions. Absorption spectra were measured in a Beckman DB G spectrophotometer at room temperature. Measurements on solutions containing DNA were made with reference to a blank containing the same concentration of DNA in buffer.

Equilibrium dialysis experiments using 3H-labeled DNA were performed by placing either 1.0 ml of calf thymus DNA (3 mg/ml), a mixture of calf thymus DNA (3 mg/ml) and reducing cell extract (final concentrations as described), or intact cells inside a dialysis bag (visking cellulose tubing). This material was dialyzed versus 25 ml of 0.0067 M phosphate buffer (pH 7.2) containing 6.2 \times 10^{-4} M 3H-labeled quinodixin (specific activity, 0.42 \muCi/\mumol) at 35°C for 15 h. Anaerobic conditions were established as described. Samples (0.1 ml) from inside and outside the bag were assayed for 3 h.

Separation of the DNA/3H-labeled quinodixin complex from free 3H-labeled quinodixin. Calf thymus DNA (1 mg/ml) was incubated anaerobically at 37°C for 1 h with 6.2 \times 10^{-4} M 3H-labeled quinodixin (specific activity, 1.66 \muCi/\mumol). The reduction of quinodixin was performed either by addition of a reducing cell extract or by addition of a deaerated solution of dithionite (Na_2S_2O_4) in five portions at 10-min intervals to give a final concentration of 31 \times 10^{-4} M. The reaction mixture (volume 0.75 ml) was then placed on a Sephadex G-25 column (2 by 30 cm) and eluted with 0.0067 M phosphate buffer (pH 7.2) containing 0.02% sodium azide. Fractions (3 ml) were collected, concentrated at 160°C to 0.1 ml, and, after addition of 8 ml of scintillation fluid, used for radioactivity determination.

RESULTS

Influence of quinodixin on the synthesis of macromolecules. We first examined the effect of quinodixin on biopolymer synthesis under aerobic and anaerobic conditions. Quinodixin inhibited the synthesis of DNA at the concentrations tested only in the absence of oxygen (Fig. 1). The syntheses of RNA and protein were not affected by 10 \mug of quinodixin per ml, whereas 100 \mug of quinodixin per ml impaired the synthesis of both macromolecules under anaerobic conditions. Higher drug concentrations were needed to block the synthesis of DNA also under aerobic conditions (unpublished data). Based on these results it was concluded that quinodixin is an inhibitor of DNA synthesis with the peculiarity to be much more efficient under anaerobic conditions.

Quinodixin-induced DNA degradation. Since the inhibition of DNA synthesis by various drugs results in extensive degradation of DNA (2, 8, 48), we determined the effect of quinodixin on the stability of uniformly labeled DNA in E. coli KL 399 by measuring the liberation of acid-soluble radioactivity. DNA was degraded to an acid-soluble form in cells exposed to quinodixin (Fig. 2). The rate of breakdown was a function of the drug concentration and was decreased by the presence of oxygen. Therefore, the decreased susceptibility of bacteria to quinodixin under aerobic conditions (21) could result from the decreased effect of quinodixin on DNA synthesis and DNA degradation in presence of oxygen.

Susceptibility of various repair-deficient strains to QdNO. DNA repair-deficient strains of E. coli are known to be more susceptible to many agents which impair the synthesis of DNA (28, 45, 48). Table 1 shows that repair mutants are also more susceptible to QdNO. The degree of susceptibility was dependent on the type of mutation present in the genome.

Under aerobic conditions the acquisition of a recA13, polA1, or recB21 mutation rendered the cell 20 to 80 times more susceptible to quinodixin. On the other hand the introduction of a recC22, uvrA6, or exrA mutation resulted in a less than 10-fold decrease in resistance to quinodixin in the presence of oxygen. These genes rendered the cells more susceptible to olaquindox and to quinodixin under anaerobic conditions too, but the differences between the various repair-deficient and -proficient strains were smaller than with quinodixin under aerobic conditions.

Repair deficiencies stimulate or block the degradation of DNA, depending upon the mutation present and the agent used to treat the cells. The rate of DNA breakdown is not always coupled to loss of viability (40, 51). Therefore, we investigated the stability of DNA in different repair-deficient strains exposed to quinodixin in correlation to the viable cell count.

<table>
<thead>
<tr>
<th>Strain and relevant genotype</th>
<th>MIC (\muG/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Quinodixin</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>JC 3272</td>
<td>70.3</td>
</tr>
<tr>
<td>JC 3272 recA13</td>
<td>1.8</td>
</tr>
<tr>
<td>JC 3272 recB21</td>
<td>3.1</td>
</tr>
<tr>
<td>JC 3272 recC22</td>
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</tr>
<tr>
<td>AB 1157</td>
<td>44</td>
</tr>
<tr>
<td>AB 1886 uvrA6</td>
<td>37.5</td>
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<td>AB 2463 recA13</td>
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</tr>
<tr>
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<td>12</td>
</tr>
<tr>
<td>JG 139</td>
<td>100</td>
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<tr>
<td>JG 138 polA1</td>
<td>1.2</td>
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In the presence of either a recA or a polA gene, the addition of quinoxin resulted in a rapid loss of viability coupled with extensive DNA breakdown (Fig. 3). Despite the strong lethal effect of quinoxin on recB strains, almost no degradation of DNA was observed. Therefore, DNA breakdown itself cannot be responsible for the lethal effect of quinoxin. This was stressed by the results where the uvrA strain was clearly more susceptible than the repair-proficient strains at rates of DNA degradation similar in both types of strains.

**Effect of chloramphenicol on quinoxin-induced DNA degradation.** By blocking the protein synthesis, the degradation of DNA induced by different agents is either stimulated (myxin) (2), decreased (ultraviolet light) (36), or

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**FIG. 1.** Effect of quinoxin on the synthesis of macromolecules under aerobic and anaerobic conditions. Log-phase cells of *E. coli* KL 399 (10⁸ cells/ml) were incubated at 30°C in supplemented M9 medium, and the incorporation of [methyl-³H]thymidine into DNA, of [³H]uridine into RNA, and of L-[G-³H]leucine into protein was determined by measuring the radioactivity in the trichloroacetic acid-insoluble fraction. The cells were preincubated for 15 min and at zero time were exposed to quinoxin. (A) Control without quinoxin; (B) 10 µg of quinoxin per ml; (C) 100 µg of quinoxin per ml. Aerobic conditions: vessels aerated with 10 liters of compressed air per h. Anaerobic conditions: vessels sparged with 10 liters of nitrogen per h containing 5% (vol/vol) CO₂.

**FIG. 2.** Degradation of DNA in *E. coli* KL 399 under aerobic and anaerobic conditions as a function of quinoxin concentration. Cells were incubated at 42°C in supplemented M9 medium for 30 min, and at zero time quinoxin was added. (A) Without quinoxin, aerobic; (B) without quinoxin, anaerobic; (C) 1 µg of quinoxin per ml, aerobic; (D) 1 µg of quinoxin per ml, anaerobic; (E) 10 µg of quinoxin per ml, aerobic; (F) 10 µg of quinoxin per ml, anaerobic.
completely blocked (nalidixic acid) (10). To examine the effect of protein synthesis on DNA breakdown induced by quindoxin, cells uniformly labeled with [3H]thymidine were preincubated anaerobically at 30°C in the presence or absence of chloramphenicol (50 μg/ml). After 30 min, different amounts of quindoxin were added, and the degradation of DNA was determined as described (19). Chloramphenicol decreased the rate of quindoxin-induced degradation of DNA (Fig. 4). A similar impairment of DNA breakdown after ultraviolet irradiation was observed in cells prestarved for essential amino acids (36). This was explained by a different type of DNA repair in prestarved cells. Since E. coli KL 399 carries a temperature-sensitive recA function, we could examine the influence of the recA gene on the effect of chloramphenicol on quindoxin-induced degradation of DNA. The experiment described in Fig. 4 was performed at 42°C using 10-times-lower quindoxin concentrations. The results obtained (unpublished data) were similar to those at 30°C so that the chloramphenicol effect is not dependent on an intact recA gene product. These experiments have shown that quindoxin affects the stability of DNA both in proliferating and non-proliferating cells.

Metabolism of quindoxin in E. coli. Metabolic activation is a well-known characteristic of many DNA attacking agents (6, 25, 26, 31, 34, 43). Preliminary experiments were done to detect changes in the absorption spectrum of quindoxin, and related QdNO, during the incubation with cells of E. coli W 8.

The peak at 398 nm in the absorption spectrum of chloroform-extracted quindoxin disappeared completely if 0.5 liters of M9 medium containing 62 × 10^-4 M (100 ppm) quindoxin was inoculated with 20 ml of a late-log-phase culture of E. coli W 8 and incubated overnight aerobically (with slight stirring) at 37°C. After removing the cells, the supernatant medium was extracted with chloroform. The absorption spectrum was then measured and compared with the spectrum of quindoxin, quinoxalin-N-oxide (QNO), and quinoxaline. The absorption spectra

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**Fig. 3. Effect of quindoxin on DNA degradation and viability of various DNA repair-proficient and DNA repair-deficient strains of E. coli K-12.** Cells were labeled overnight with [3H]thymidine, harvested, washed, suspended in supplemented M9 medium without radioactive precursors, and preincubated for 1 h at 37°C under anaerobic conditions. At zero time the experiment was started by adding 6 μg of quindoxin per ml. The viable cell count at zero time (about 4 × 10^9 cells/ml) was taken as 100%. (A) UB 1139 (repair proficient); (B) JG 139 (repair proficient); (C) AB 2500 wcrA6; (D) JG 3272 recB21; (E) JG 139 polA1; (F) JG 2926 recA13.
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Figure 4. Effect of chloramphenicol on the quindoxin-induced degradation of DNA in E. coli KL 399. Uniformly labeled cells were preincubated anaerobically at 30°C for 30 min. Chloramphenicol was present where indicated. At zero time quindoxin was added. (A) Control without quindoxin, the presence or absence of chloramphenicol did not affect the stability of DNA; (B) quindoxin (2 μg/ml) + chloramphenicol (50 μg/ml); (C) quindoxin (2 μg/ml); (D) quindoxin (20 μg/ml) + chloramphenicol (50 μg/ml); (E) quindoxin (20 μg/ml).

Figure 5. Absorption spectrum of quindoxin and some reduced derivatives in chloroform. Aqueous solutions (62 × 10⁻⁶ M) were prepared and extracted with an equal volume of chloroform. (a) Quindoxin reduced by E. coli W 8; (b) QNO; (c) quinoxaline; (d) quindoxin.

Figure 6. Effect of oxygen on the lethal effect and the reduction of quindoxin. E. coli W 8 cells were incubated in M9 medium at 37°C. Aerobic conditions (O₂) were established by sparging the vessel with compressed air (1,000 liters/h). During the anaerobic incubation, nitrogen (N₂) containing 5% (vol/vol) CO₂ (45 liters/h) was flushed instead of air. The redox potential (E cal) represents the arithmetic mean of the two values obtained with the electrodes inserted. Symbols: ▼, Changes of the incubation conditions by changing the sparging gas; ▼Q, addition of quindoxin (10 ppm final concentration).

of biologically reduced quindoxin (Fig. 5a) and QNO (Fig. 5b) were almost identical. A substance with a spectrum identical to QNO was also obtained by reducing quindoxin with an
equimolar amount of dithionite (Na₂S₂O₄) under anaerobic conditions. If QNO, biologically reduced quinoxin, or quinoxin was treated with a fivefold molar excess of dithionite, all three quinoxaline derivatives were reduced to quinoxaline.

Finally, the antibiotic activities of QNO and biologically reduced quinoxin were compared. A supernatant, containing 62 × 10⁻⁴ M biologically reduced quinoxin, was supplemented with glucose (45 mM final concentration) and potassium hydroxide (to restore pH 7.2) and sterilized by filtration. This medium was inoculated with *E. coli* W 8 and incubated overnight at 37°C under anaerobic conditions. Neither growth inhibition nor changes in the absorption spectrum of the biologically reduced quinoxin was observed. From this it was concluded that biologically reduced quinoxin had no antibiotic activity and was not further metabolized. No stable antimicrobial agent was produced by quinoxin reduction. Likewise QNO displayed no antibiotic activity and was not degraded by *E. coli* W 8.

Based on the absorption spectrum and the chemical and antibiotic properties, biologically reduced quinoxin seems to be identical to QNO.

**Effect of oxygen on the reduction of quinoxin.** A 500-ml volume of M9 medium was inoculated with *E. coli* W 8, incubated at 37°C, and sparged with 1,000 liters of compressed air per h. The oxygen concentration was measured indirectly by recording the redox potential. In bacterial cultures without special redox buffers, the measurement of the redox potential is essentially a measurement of the oxygen concentration and has the advantage of being more sensitive than polarographic oxygen determination (27). At the same time the turbidity (OD₅₅₀) and the colony-forming ability (viable cell number) were determined (Fig. 6). After 2 h, quinoxin (10 ppm) was added but it had no influence on any parameter recorded. Three hours later the aeration was stopped, and the vessel was sparged with nitrogen containing 5% (vol/vol) CO₂ (45 liters/h). Immediately the redox potential dropped because of the physical displacement (rapid phase) and the consumption of oxygen by respiration (slow phase). Under anaerobic conditions a rapid loss of viability was observed and at the same time the quinoxin concentration (OD₃₇₁) started to decrease. After 1 h the anaerobic incubation was stopped and the culture was aerated as before. Oxygen blocked the reduction of quinoxin immediately. The loss of viability slowed down, and after 1 h viable cell count began to increase. All the effects described were observed again when the cells were incubated a second time under anaerobic conditions (Fig. 6).

Despite the rapid loss of viability after the shift from aerobic to anaerobic conditions, the increase in turbidity continued. Microscopic examination of cells exposed to quinoxin during several hours revealed extremely elongated, filamentous forms (Fig. 7).

**Quinoxin reduction and loss of viability in *E. coli* cells starved for glucose.** The reduction of quinoxin was dependent on the presence of an energy source (Fig. 8). Glucose stimulated the reduction of quinoxin much more than succinate, and in correspondence with this result the loss of viability was more rapid and more extensive in the presence of glucose. In the absence of an exogenous electron donor or in the presence of succinate, cell death began after a lag phase. This lag might result from the need of a certain amount of damaged sites in DNA prior to the onset of cell death.

**Activity of QdNO reductase in QdNO-resistant mutants.** From *E. coli* W 8, treated with NTG, three QdNO-resistant mutants (QR 9, QR 26, QR 29) were obtained by selection for increased quinoxin resistance. The mutants were about 30 times more resistant than the parent strain to all QdNO tested, but no change in susceptibility to unrelated agents, including nitrofurantoin, was observed.

Carbadox was reduced at a five to six times higher rate by the susceptible *E. coli* W 8 cells than by the resistant mutants (Fig. 9). Similar results were obtained with either quinoxin or olaquindox. The experiments shown in Fig. 9 and 10 were performed with carbadox instead of quinoxin because carbadox was reduced more rapidly and the reduction rates could therefore be determined more accurately.

The decreased QdNO reduction rates in resistant strains could have been due to impaired uptake of QdNO or could have resulted from a mutated reductase enzyme. To discriminate between these two possibilities cell-free extracts were prepared and the reductase activity was determined (Fig. 10). When supplemented with an appropriate regenerating system, NADH or NADPH were used as electron donors. Extracts from QdNO-resistant strains reduced carbadox much slower than the extract from *E. coli* W 8.

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**Fig. 7.** Morphological changes induced in cells of *E. coli* W 8 by quinoxin (10 ppm). A 20-ml volume of BHI broth was inoculated with 2 ml of an overnight culture and incubated aerobically without shaking at 37°C. (a) Beginning of the incubation; (b) after 3 h; (c) after 10 h. Photographed with a Wild M 20 microscope; magnification, ×400.
This difference was found with both electron-donating systems although they were more pronounced with NADH. These observations strongly suggest that, in these strains, resistance to QdNO is a direct consequence of a decreased reductase activity.

**ESR measurements.** It has been shown by three different approaches that the lethal effect of quindoxin is tightly coupled with the reduction of quindoxin. On the other hand, QNO, the final product of quindoxin reduction, displayed no antibiotic activity. It was therefore assumed that the DNA-damaging compound is an unsta-
possible side or intermediate product of the reduction of quindoxin.

Studying the reduction of quindoxin with an ESR spectrometer, signals were obtained when dithionite, intact *E. coli* W 8 cells, or a soluble bacterial extract was given to quindoxin under anaerobic conditions. The existence of an ESR signal provides direct evidence for the presence of free radicals in the samples. The reduction of a 0.1 M quindoxin solution chemically with an equimolar amount of dithionite gave an ESR spectrum with a *g* value of 2.0049 ± 0.0001, and discernible hyperfine structure was found (Fig. 11). The free radical was identified to be QdNOH (Fig. 12). The maximal intensity (2.5 × 10⁻⁷ molespins/liter) did not change during the first 15 min. Thereafter the signal decayed with a half-life of about 40 min.

When intact bacterial cells or a cell-free bacterial extract was used to reduce quindoxin, an ESR signal without hyperfine structure was observed. A much smaller and clearly different signal was obtained if the biological material was incubated without quindoxin, so that the signal developed in the presence of quindoxin was due to a free radical produced during the reduction of quindoxin. The absence of hyperfine structure prevented the identification of this radical. It cannot be decided whether the lack of hyperfine structure resulted from a specific association between the free radical and a macromolecule as assumed in the case of rubiflavin (48) or if the rotational relaxation time of the free radical was unspecifically increased by the viscosity of the surrounding medium.

**Quindoxin/DNA binding studies.** For most of the directly DNA attacking compounds, covalent or noncovalent binding to the target has been described (38, 39, 46). In this study we have used three independent techniques to de-

![Fig. 10. Reduction of carbadox by cell-free extracts of *E. coli* W 8 and QdNO-resistant strains. Cell-free extracts were mixed with carbadox and either NADH, alcohol dehydrogenase, and ethanol (NADH system) or NADPH, glucose 6-phosphate dehydrogenase, and glucose 6-phosphate (NADPH system). The vessels were then preincubated, and the experiment was started as described in Fig. 10. Symbols: ○, *E. coli* W 8; □, *E. coli* QR 9; ▲, *E. coli* QR 26; △, *E. coli* QR 29.](image)

![Fig. 11. ESR spectrum of quindoxin free radical (QdNOH). A 1-ml volume of a 0.1 M solution of quindoxin was deaerated with nitrogen and reduced by addition of an equimolar amount (19.2 mg) of dithionite (pH 6, after mixing). A 0.2-ml volume of this mixture was given into a nitrogen-sparged ESR flat cell, and the ESR signal was recorded at 22°C.](image)
determine whether there is any interaction between quindoxin and DNA.

The first method used was simply to measure the absorption spectrum of quindoxin in the presence and absence of calf thymus DNA. The ratio of moles of DNA base pairs to moles of quindoxin was varied from 10 to 500. In all cases, neither a bathochromic shift nor a hypochromic effect in the absorption spectrum of quindoxin due to the presence of DNA could be detected in the quindoxin absorption spectrum. As a control, the spectrum of ethidium bromide was measured under the same conditions, with and without DNA. Changes identical to those previously reported were found (47).

The second method used was that of equilibrium dialysis using tritium-labeled quindoxin. Typical results are outlined in Table 2. Again, there was no indication of binding of quindoxin to DNA. This was true for both native or denatured DNA and irrespective of the presence of magnesium (4 mM) or a reducing system. Association constants greater than $10^{-1}$ (in base pairs) are ruled out by the data given in Table 2. Measuring the quindoxin association to intact cells, we excluded the binding of more than 10,000 quindoxin molecules per cell.

The third approach applied to detect binding was Sephadex G-25 fractionation of a reaction mixture containing $^3$H-labeled quindoxin, calf thymus DNA, and a reducing system, i.e., either dithionite or a cell-free extract of E. coli W 8 supplemented with NADH, alcohol dehydrogenase, and ethanol. Although the method was sen-
TABLE 2. Investigation of the binding of \(^3\)H-labeled quinoxin to DNA or intact cells of *E. coli* W8 with equilibrium dialysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>cpm (in)</th>
<th>cpm (out)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>15,900</td>
<td>15,800</td>
</tr>
<tr>
<td>DNA + 4 \times 10^{-3} M MgCl(_2)</td>
<td>16,000</td>
<td>16,000</td>
</tr>
<tr>
<td>DNA, heat denatured</td>
<td>15,500</td>
<td>15,500</td>
</tr>
<tr>
<td>DNA + reducing cell-free extract(^a)</td>
<td>18,400</td>
<td>18,400</td>
</tr>
<tr>
<td>DNA + cell-free extract(^b)</td>
<td>16,200</td>
<td>16,300</td>
</tr>
<tr>
<td><em>E. coli</em> KL 399 (OD(_{600} = 0.4))</td>
<td>13,800</td>
<td>13,700</td>
</tr>
<tr>
<td><em>E. coli</em> KL 399 (OD(_{600} = 4.0))</td>
<td>13,800</td>
<td>13,700</td>
</tr>
</tbody>
</table>

\(^a\) The samples were placed inside a dialysis bag and dialyzed against phosphate buffer or M9 medium (experiments with intact cells) containing 6.2 \times 10^{-6} M \(^3\)H-labeled quinoxin during 15 h at 25°C under anaerobic conditions (NADH, ethanol, and MgCl\(_2\) were also added outside when the sample inside contained them).

\(^b\) Cell-free extract from *E. coli* W8 supplemented with NADH, ethanol, and alcohol dehydrogenase.

\(^c\) Cell-free extract from *E. coli* W8 without supplements.

With quantitation of the degradation or breakdown of quinoxin-susceptible DNA or myxin (2). If the synthesis of DNA is inhibited either by thymine starvation or nalidixic acid (17), chloramphenicol completely prevents the degradation of DNA. The slightly stimulating effect of protein synthesis on quinoxin-induced DNA breakdown is similar to its influence on DNA decomposition induced by ultraviolet irradiation (17). Since DNA degradation in the presence of chloramphenicol was observed even at low quinoxin concentrations, it appears that quinoxin acts on and modifies the integrity and structure of preexisting DNA. Consequently, quinoxin affects both proliferating and nonproliferating cells. Therefore, quinoxin does not interfere with the synthesis of deoxynucleotides nor is the quinoxin-susceptible target a protein involved in DNA replication.

At present we do not know how quinoxin modifies the structure of DNA. Since no stable binding of tritiated drug molecules to DNA was observed, quinoxin cannot be an alkylating or intercalating agent. However, our results do not exclude a transient binding of quinoxin or an activated quinoxin derivative to DNA.

The increased susceptibility of *E. coli* repair mutants to QdNO parallels with their increased susceptibility to nitrofurantoin (28).

Generally there was a good correlation between the sensitivity to ultraviolet irradiation and the susceptibility to quinoxin, but *uvrA* strains, known to be extremely ultraviolet sensitive (20), were almost as resistant as the nonmutant parent strains. The pyrimidine-dimer-specific endonuclease, inactive in *uvrA* strains (4), seems not to be involved in repair of DNA exposed to quinoxin. Support for this assumption came from the observation that the degradation of DNA was normal in *uvrA* strains treated with quinoxin although no degradation of DNA was found after ultraviolet irradiation (40). Slightly increased susceptibility and a normal rate of DNA breakdown were also observed when *uvrA* mutants were exposed to X rays (13). Based on these results we suggest that DNA damaged by either X rays, nitrofurantoin, or QdNO is repaired by the same DNA repair systems.

The increased efficiency under anaerobic conditions found with quinoxin and related QdNO is unique for inhibitors of DNA synthesis. X rays (44), streptonigrin (49), phleomycin, and bleomycin (43) are observed to be more efficient in the presence of oxygen. In the case of X rays, there is good evidence for the presence of a polA-dependent repair system acting only under anaerobic conditions (44). Since all the repair-deficient strains tested were more susceptible to QdNO under anaerobic conditions, it may be

DISCUSSION

Since quinoxin inhibited the incorporation of \(^3\)H-thymidine at lower concentrations and to a greater extent than the incorporation of \(^3\)H-uridine or \(^3\)H-leucine and since most repair-deficient mutants were found to be more susceptible, it appears that the primary effect of quinoxin on *E. coli* is the inhibition of DNA synthesis. This inhibition renders the DNA vulnerable to attack by nucleases, and the genetic material of most strains exposed to quinoxin is ultimately degraded. The degradation of DNA is only a secondary phenomenon and cannot be responsible for the bactericidal effect of quinoxin because rapid loss of viability of *E. coli* JC 3272 recB21 was found in the absence of DNA breakdown. The conclusion that quinoxin inhibits the synthesis of DNA was further confirmed by the observation that, concomitant with loss of viability, the cells developed long, filamentous forms. Similar morphological changes have been observed after disturbing DNA metabolism by thymine starvation or treatment with nalidixic acid (17), 2,3-dihydroxyimethyl-quinoxaline-di-N-oxide (41), and mitomycin C (26).

Chloramphenicol stimulates DNA breakdown which is induced by DNA directly attacking agents like mitomycin C (7), streptonigrin (49), or myxin (2). If the synthesis of DNA is inhibited either by thymine starvation or nalidixic acid (17), chloramphenicol completely prevents the degradation of DNA. The slightly stimulating effect of protein synthesis on quinoxin-induced DNA breakdown is similar to its influence on DNA decomposition induced by ultraviolet irradiation (17). Since DNA degradation in the presence of chloramphenicol was observed even at low quinoxin concentrations, it appears that quinoxin acts on and modifies the integrity and structure of preexisting DNA. Consequently, quinoxin affects both proliferating and nonproliferating cells. Therefore, quinoxin does not interfere with the synthesis of deoxynucleotides nor is the quinoxin-susceptible target a protein involved in DNA replication.

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assumed that DNA damaged by quinoxin is equally well repaired in the presence and absence of oxygen.

The activity of many DNA synthesis-inhibiting agents depends on metabolic activation. Furthermore, the activation of streptonigrin, bleomycin, and phleomycin is stimulated by the presence of oxygen (25, 43, 48).

Bacteria reduced quinoxin and related QdNO only under anaerobic conditions, and by three different approaches we showed that quinoxin action was always accompanied by quinoxin reduction (35). This suggests strongly that analogous to the nitrofurans, where nitrofurazone resistance is coupled with a loss of nitrofurazone reductase activity (35), a metabolite of quinoxin is responsible for the antibiotic effect. Since QNO, which is the final product of the quinoxin reduction, displayed no antibiotic activity, an unstable intermediate product is probably the active agent.

ESR experiments demonstrated the generation of free radicals during the reduction of quinoxin. In the case of chemical reduction the hyperfine structure was discernible, and the free radical was identified to be QdNOH. Since hyperfine structure was missing in the ESR signal appearing in the presence of quinoxin and a biological reducing system, the free radical generated could not be identified. At present we do not know whether the absence of hyperfine structure resulted from a specific association between the free radical and a macromolecule or from the high viscosity of the surrounding medium.

Each hypothesis on the mechanism of action of quinoxin has to explain how quinoxin acts on preexisting DNA without being firmly bound to the target and why the activity of quinoxin is strictly dependent on a radical generating, reducing process.

We suggest that the radicals produced during the intracellular reduction of quinoxin or some decomposition product thereof damage the DNA without being bound to the target. As a consequence, DNA synthesis ceases, breakdown of the chromosome ensues, and the cell becomes nonviable.

More work is needed to give a more detailed picture of the reduction process, to identify the DNA-damaging molecule, and to characterize chemically the type of DNA damage induced by QdNO.

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LITERATURE CITED

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