Lethal Action and Metabolic Effects of Streptonigrin on *Escherichia coli*

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SUMMARY

Streptonigrin is lethal to cultures of *Escherichia coli* at concentrations that allow synthesis of DNA, RNA, and protein to continue. At higher concentrations DNA synthesis is preferentially inhibited. The lethal event is accompanied by DNA degradation, an effect enhanced if protein synthesis is also inhibited by witholding a required amino acid or by adding chloramphenicol. The degradation products, which are released into the culture medium, include nucleotides and bases, but no unusual products were detected. Both an electron source and oxygen are required for streptonigrin to exert its greatest lethal effect. This suggests that a reaction product of oxygen and intracellularly reduced streptonigrin is the lethal agent. This agent is likely to be a peroxide or peroxy free radical but is not hydrogen peroxide.

INTRODUCTION

Streptonigrin is an antitumor agent having the structure shown in Fig. 1 (1). It has been found to cause chromosomal breaks and other abnormalities during mitosis (2) and meiosis (3). Mizuno (4) reported that streptonigrin causes inhibition of DNA synthesis in tissue cultures and that tritiated streptonigrin is associated with a DNA-containing fraction obtained from such cultures. Levine and Borthwick (5) reported that streptonigrin causes inhibition of DNA synthesis in *Salmonella typhimurium* and induction of phage formation in lysogenic bacteria. Szybalski (6) detected the presence of many single-strand breaks in DNA isolated from streptonigrin-treated *Bacillus subtilis*. Radding (7) observed the breakdown of bacterial DNA into acid-soluble material in the presence of streptonigrin.

Our previous work (8) has shown that the bactericidal action of streptonigrin on exponential cultures of *Escherichia coli* is greatly enhanced by certain compounds that affect electron transport, including cyanide and carbonyl cyanide phenylhydrazone. Phenazine methosulfate, which is an autoxidizable electron scavenger, completely prevents killing by streptonigrin. Lethality is also strongly decreased in the absence of an electron source, such as glucose. White and Dearman (9) found that in cultures of *E. coli* streptonigrin can be metabolically reduced to a semiquinone whose electron spin resonance spectrum is readily observed. These results indicate that a source of electrons is necessary in order

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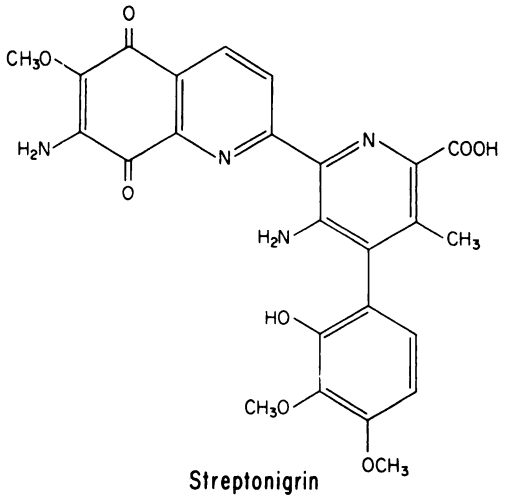

FIG. 1. *The structure of streptonigrin as determined by Rao, Biemann, and Woodward (1)*

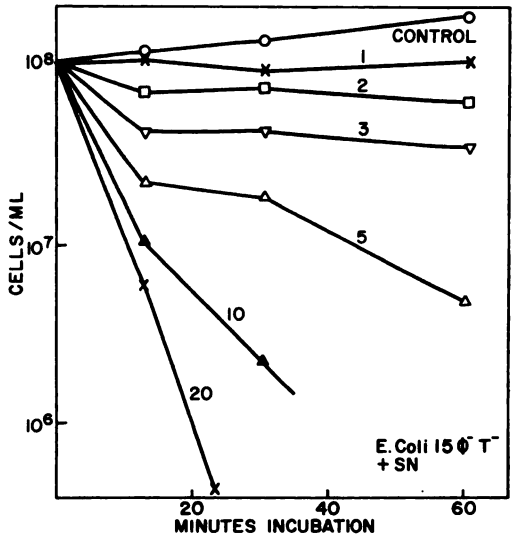

FIG. 2. *Loss of viability of E. coli cells in the presence of streptonigrin*

Streptonigrin (SN) was added at zero time to cultures growing exponentially at a concentration of 10^8 cells/ml. The number near each curve represents the concentration of streptonigrin in micrograms per milliliter.

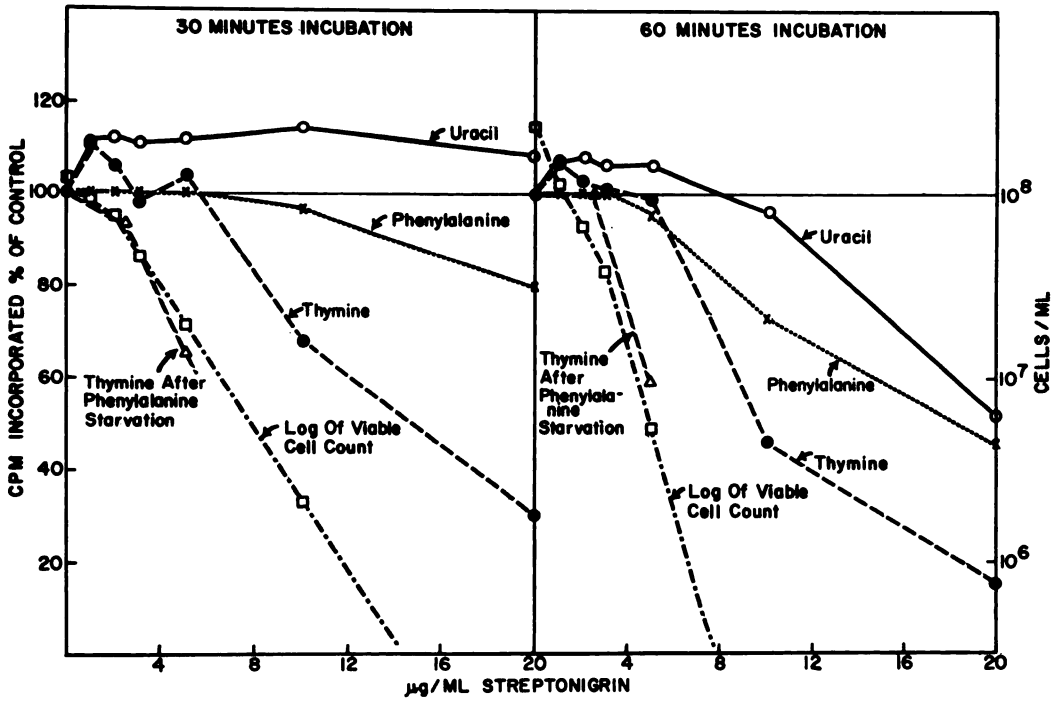

FIG. 3. *Effect of streptonigrin on net incorporation of labeled precursors of DNA, RNA, and protein into acid-insoluble fraction of E. coli.*

Streptonigrin (5 μg/ml) was added at zero time to exponential cultures that had been incubated for 5 min in radioactive medium. Assays for acid-precipitable material were performed as described in MATERIALS AND METHODS. Radioactivity in controls was at least 500 cpm/ml at zero time and at least 6000 cpm/ml at 65 min.

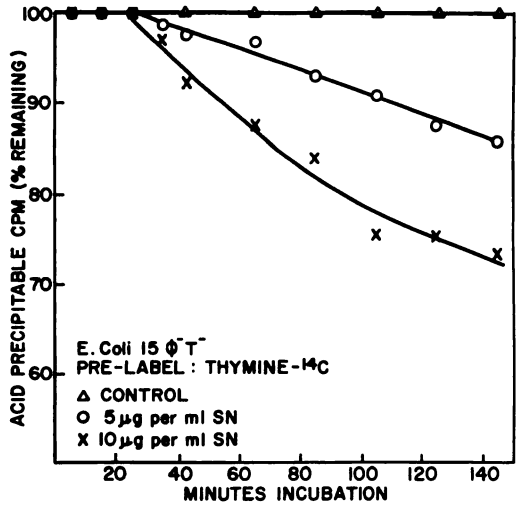

FIG. 4. *Stability of labeled DNA-¹⁴C in streptonigrin-inhibited cultures of E. coli*

Cells were grown for two generations in medium containing thymine-¹⁴C and then chilled, washed, and resuspended at zero time in fresh, unlabeled medium with streptonigrin (SN).

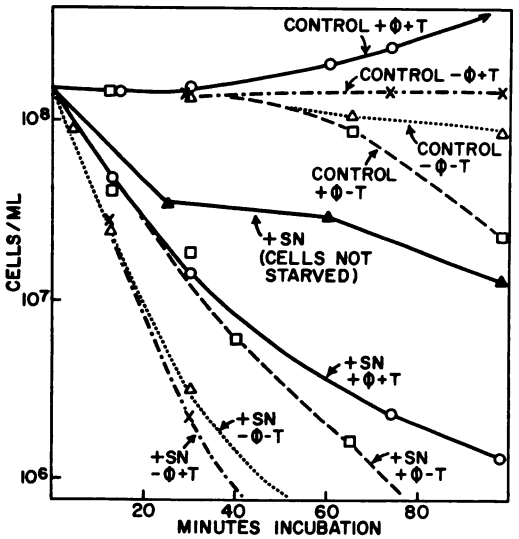

FIG. 5. *Loss of viability of phenylalanine-starved E. coli cells in the presence of streptonigrin under various poststarvation conditions*

An exponential culture of *E. coli* 15 T⁻Phe⁻ was resuspended in the absence of phenylalanine and incubated for 90 min. Cells were then resuspended in fresh medium either with phenylalanine (φ) ± thymine (T), or without phenylalanine ± thymine. The +φ, −T condition leads to thymineless death after 1 hr. Some loss of viability also occurs in the −φ, −T experiment because this strain is able te synthesize a small amount of phenylalanine. A curvo for unstarved cells is included for comparison. The concentration of streptonigrin (SN) was 5 μg/ml.

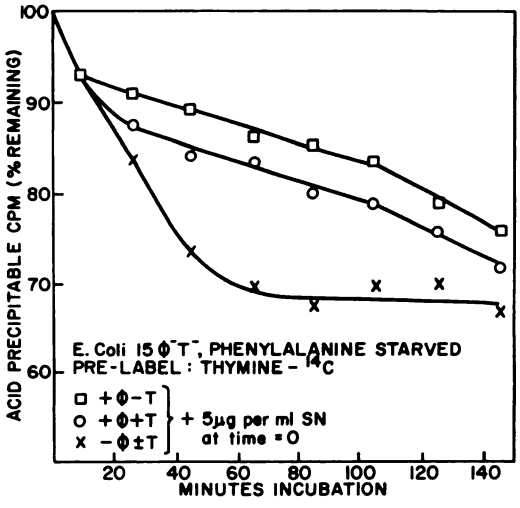

FIG. 6. *Stability of labeled DNA-¹⁴C in streptonigrin-inhibited cultures of phenylalanine-starved E. coli strain 15 T⁻Phe⁻*

Cells were treated as described in the legend of of Fig. 5, which illustrates viabilities determined with the same cultures. Thymine-¹⁴C was present both before and during the starvation period. SN, streptonigrin.

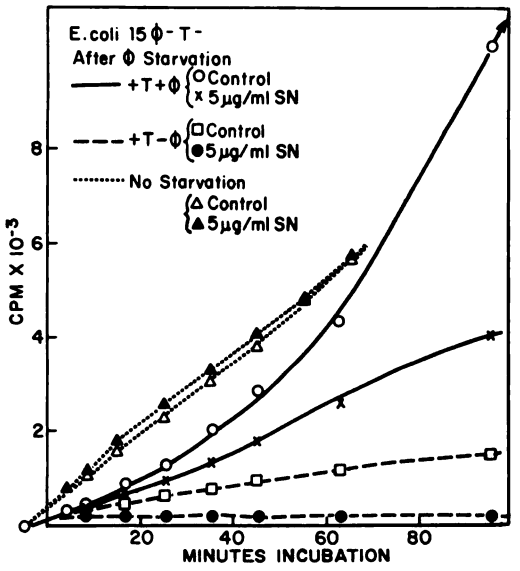

FIG. 7. *Effect of streptonigrin on DNA synthesis in phenylalanine-starved E. coli 15 T⁻Phe⁻*

After a 90-min incubation period in unlabeled medium without phenylalanine, cells were chilled, washed, and resuspended in fresh medium containing thymine-¹⁴C, either with or without phenylalanine. After 5 min streptonigrin (SN) was added (zero time in the figure). Controls received no antibiotic. Viabilities for these cultures are shown in Fig. 5. Curves for nonstarved cells are included for comparison.

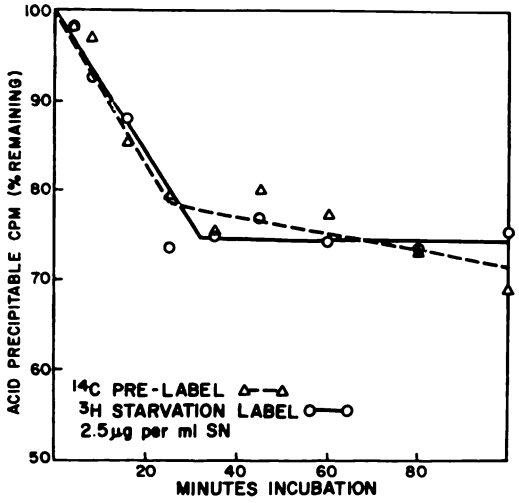

FIG. 8. *Relative stabilities of DNA synthesized before and during phenylalanine starvation in streptonigrin-inhibited cultures of E. coli 15 T⁻Phe⁻*

Cells in the exponential phase were grown for two generations in medium containing phenylalanine and thymine-^{14}C. They were chilled, washed, and resuspended in fresh medium containing thymine-^{3}H but no phenylalanine. After 90 min they were chilled, washed, and resuspended in fresh medium containing neither phenylalanine nor thymine. Streptonigrin (SN) at 2.5 μg/ml was added at zero time. Assays for acid-precipitable radioactivity were performed as described in MATERIALS AND METHODS. The extrapolated radioactivity at zero time was 7200 cpm of ^{3}H per milliliter and 9500 cpm of ^{14}C per milliliter. Assays of both labels in a control culture (not shown) remained at 100%.

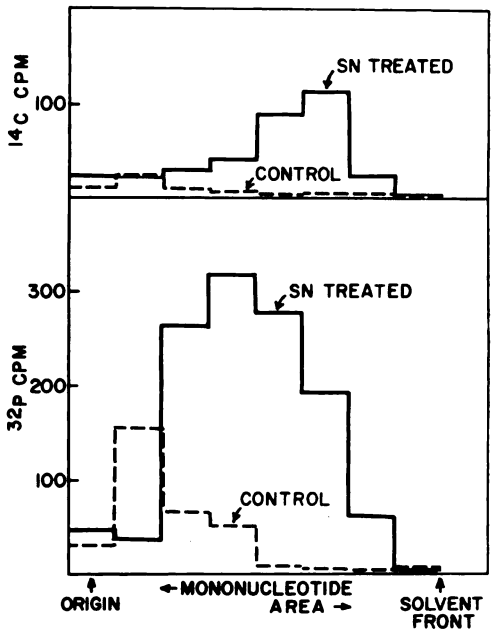

FIG. 9. *Chromatographic fractionation of lyophilized cell supernatant from streptonigrin-inhibited cells*

Aliquots representing approximately 0.1 ml of the original cell supernatants, labeled with thymine-^{14}C and phosphate-^{32}P, were spotted at the origin of a polyethyleneimine-cellulose thin layer plate. Unlabeled 5′-deoxymononucleotides were used as internal ultraviolet markers. The plate was developed by triple stepwise saline elution (see MATERIALS AND METHODS). After radioautography, equivalent areas were scraped from the plate, eluted with 0.5 M saline, and centrifuged, and the supernatants were counted in Triton scintillation fluid. Radioactivity found in each fraction is shown for both ^{14}C and ^{32}P. In this system relative R_F values for the deoxynucleotides were: 5′-dGMP < 5′-dAMP < 5′-dCMP < 5′-TMP; also monophosphates > diphosphates > triphosphates. Unlabeled monophosphate controls were not well differentiated in chromatograms of cell supernatants, and therefore only a "mononucleotide area" is labeled in the figure. SN, streptonigrin.

that streptonigrin may be intracellularly reduced to a lethal form, possibly the semiquinone or a product of it.

We have also found *in vitro* a physical interaction between streptonigrin and DNA that causes an increase in the temperature of the melting transition (T_m) of DNA at low ionic strength (10). When chemically reduced in the presence of DNA, the antitumor agent causes single-strand breaks in DNA, as shown by a decrease in its rate of sedimentation through a sucrose gradient, especially after denaturation of the treated DNA samples.

The present paper further describes the effects of streptonigrin on cultures of an *E. coli* strain (15 T⁻Phe⁻) that requires both thymine and phenylalanine. These effects are correlated with the interactions between streptonigrin and DNA *in vitro*. A mechanism of bactericidal action consistent with the experimental evidence is presented.

MATERIALS AND METHODS

Sources of materials. Streptonigrin (NSC-45383) was obtained from Dr. T. J. McBride of Charles Pfizer and Company.

A stock solution at 500 μg/ml in 0.10 M Tris-chloride buffer at pH 7.4, if kept refrigerated and dark, retained its antibacterial activity for several months. The aqueous solubility of streptonigrin could be increased by raising either ionic strength or pH.

Radiochemicals were purchased from New England Nuclear Corporation; snake venom phosphodiesterase, from Worthington; hydrogen peroxide, "Baker analyzed" reagent grade, 31.4% assay, from J. T. Baker Chemical Company; sucrose, from Baker and Adamson; cesium chloride (99.9%), from Varlacoid; nutrient agar, from Difco; Triton X-100, from Palmetto; 2,5-diphenyloxazole (PPO), from Nuclear-Chicago; 1,4-bis[2 - (4 - methyl - 5 - phenyloxazolyl)] benzene (dimethylPOPOP), from Packard; Carbowax 20 M (polyethylene glycol), from Union Carbide; MN-cellulose powder 300 for thin layer chromatography, no binder, from Macherey, Nagel and Company, distributed by Brinkmann Instruments; polyethyleneimine (50% aqueous), from Borden; Millipore filter sheets, from Millipore Filter Corporation; Whatman No. 1 chromatography paper and Whatman No. 1 filter paper discs, 2.4 cm, from W. and R. Balston, Ltd.; medical X-ray film (royal blue, Estar base), from Eastman Kodak; and mitomycin C and phenazine methosulfate, from Nutritional Biochemicals. Actinomycin D was a gift from Lederle Laboratories. Tris buffers were prepared from solutions of Trizma base (Sigma) and brought to the desired pH at 25° with HCl. Water was glass-distilled. All other chemicals were of commercially available reagent grade.

Scintillation counting. Two scintillation mixtures were used: (a) "toluene scintillation fluid" (5.0 g of PPO and 0.30 g of dimethylPOPOP per liter of toluene for samples collected on Millipore filters and for chromatographic assays in which the label was on filter paper or on cellulose scraped from thin layer plates) and (b) "Triton scintillation fluid" (4.0 g of PPO and 0.10 g of dimethylPOPOP per liter of a mixture of toluene and Triton X-100, 2:1 by volume) (11). The scintillators were first dissolved in toluene and then Triton was added. Fifteen milliliters of this mixture gave a stable suspension with 1 ml of aqueous sample.

Scintillation counting was done in a Nuclear-Chicago Mark I scintillation spectrometer. With optimum settings, efficiencies were as follows, using toluene scintillation fluid and Millipore filters: for single isotopes, ^{3}H, 29%; ^{14}C, 72%; and ^{32}P, 100%; for double labeling, ^{3}H, 29%, and ^{14}C, 46.3%; ^{14}C, 70%, and ^{32}P, 88%; and for triple labeling, ^{3}H, 28%; ^{14}C, 41%; and ^{32}P, 88%. In the Triton scintillation fluid only the ^{3}H efficiency was lower (22%). Counting times were chosen to give at least 2% accuracy except when values near background were obtained, as in certain fractions of gradients. At the concentrations used in this work no quenching by streptonigrin was observed in either scintillation mixture.

Chromatography. Polyethyleneimine-cellulose thin layer plates were prepared according to the method of Randerath (12, 13). A Desaga apparatus with its gauge set at 0.5 mm was used to spread the suspension on glass plates. These were stable for at least 2 months if kept desiccated and dark in a refrigerator. One centimeter of adsorbent was scraped from the top and sides of each plate before use in a Camag sandwich chamber apparatus. Plates were first washed in the sandwich chamber by allowing distilled water to rise to the top edge. Since R_F values were a function of age and thickness of each plate, internal standards were used when available. Samples in aliquots of 2 μl were spotted by means of capillary pipettes. Elution of these plates with distilled water moved bases and nucleosides; for separation of nucleotides, chromatograms were developed by a triple stepwise method (12) with the use of NaCl solutions (0.6 M for 5 min, 0.9 M for 10 min, and 1.1 M to 10 cm). Air-dried plates were exposed in darkness in direct contact with medical X-ray film for 2 days in the case of ^{32}P and 7 days for ^{14}C. Ultraviolet absorbance of internal controls on the original chromatograms indicated the identity of dark spots obtained on X-ray films.

Thymine was distinguished from thy-

mine glycols on Whatman No. 1 chromatographic paper with water-saturated phenol as developing solvent. In this system the R_F of thymine is 0.86; that of the *cis*-glycol is 0.56; and that of the *trans*-glycol, 0.48 (14).

Cultivation of bacteria. E. coli 15 T⁻Phe⁻, a strain requiring thymine and phenylalanine, was used except where otherwise indicated. Cultures were grown at 37° with forced aeration in Tris-buffered minimal medium at pH 7.4 (15). Normally 10 μg of L-phenylalanine per milliliter, 2 μg of thymine per milliliter, and 2 mg of glucose per milliliter were present. Aliquots of streptonigrin were introduced during exponential growth at cell concentrations between 1 and 5×10^8 cells/ml. For studies of streptonigrin effects under anaerobic conditions, nitrogen containing less than 0.001% oxygen was bubbled through cultures for 20 min before addition of the antibiotic. Viable cells were determined by serial dilution of culture aliquots in 0.15 M saline and plating on Difco nutrient agar. Doubling time of control cultures in the above medium was 50 min.

Rates of incorporation of radioactive precursors were determined by growing bacteria with uniformly labeled phenylalanine-^{14}C (0.01 μC/μg), thymine-methyl-^{3}H (0.20 μC/μg), thymine-2-^{14}C (0.012 μC/μg), or uracil-2-^{14}C (0.0036 μC/μg) in the incubation medium. Overnight cultures were resuspended and grown for at least two generations, at which time they were in exponential growth at a density of 4×10^8 cells/ml. Aliquots were then diluted to 10^8 cells/ml in separate aerated culture tubes containing radioactive precursors. Two isotopes were used in each experiment, thymine-^{3}H to label DNA and either phenylalanine-^{14}C or uracil-^{14}C to label protein or RNA. In this way a control was established that related experiments done with different cultures on separate days. Five minutes after dilution of the cells in radioactive medium, antibiotic was added to appropriate culture tubes. For radioactivity assays, 1.00-ml samples were pipetted into 2.0 ml of cold 7.5% trichloracetic acid and filtered through 2.3-cm squares of Millipore HA, and the precipitates were washed with 9 ml of cold 5% trichloracetic acid. The Millipore squares were dried in air overnight before counting in 10 ml of toluene scintillation fluid.

Breakdown of DNA into acid-soluble material was followed by resuspending an overnight culture in fresh medium containing thymine-^{14}C (0.025 μC/μg) and allowing the cells to grow for approximately two generations. Culture tubes were then transferred to an ice-water bath with continued aeration for 2 min, centrifuged immediately at 4° and $5900 \times g$ for 5 min, washed once with cold Tris minimal medium, and resuspended in Tris medium containing thymine-^{12}C and other requirements. Aliquots were placed in separate culture tubes and aerated at 37° for 5 min before addition of antibiotic at zero time of the ensuing incubation. At intervals, 1.00-ml samples were pipetted from each culture tube into 2.0 ml of cold 7.5% trichloracetic acid, filtered, and counted as above.

For experiments in which cells were starved for phenylalanine before antibiotic treatment, an overnight culture was resuspended in fresh Tris medium containing all requirements. After two generations the cultures were chilled, centrifuged, and washed as in the above procedure. They were then resuspended at the same cell concentration (5×10^8 cells/ml) in Tris medium lacking only phenylalanine. Incubation was continued for 90 min. This incubation allows the DNA replication cycle to come to completion and protects the cells from thymineless death (16). After starvation the cells were harvested, washed twice, and resuspended in fresh Tris medium containing glucose and one of the following: (a) both phenylalanine and thymine, (b) neither phenylalanine nor thymine, (c) thymine but no phenylalanine (continued starvation), or (d) phenylalanine but no thymine. After 5 min antibiotic was added to appropriate culture tubes. DNA in these experiments was labeled in various ways: (a) both before and during starvation with thymine-^{14}C (0.025 μC/μg), (b) before starvation with thymine-^{14}C (0.025 μC/μg) and during starvation with thymine-^{3}H (0.25

μC/μg), or (c) after starvation and during antibiotic treatment with thymine-^{14}C (0.025 μC/μg).

Characterization of DNA from streptonigrin-treated cells. Cell fractions were obtained from phenylalanine-starved cells containing DNA labeled both before and during starvation with thymine-^{14}C (0.025 μC/μg) and/or phosphate-^{32}P (0.0063 μC/μg at time of assay). Cells were incubated with streptonigrin for a given length of time, chilled with aeration, harvested, and washed twice with Tris medium. Cell pellets were either frozen for later DNA isolation or resuspended in 5% cold trichloracetic acid and allowed to remain at 4° overnight, after which the acid suspension was centrifuged and the supernatant was extracted with ether to remove trichloracetic acid before being assayed in the Triton scintillation mixture for acid-soluble radioactivity.

The original cell supernatants, usually 5 ml from each culture tube, were lyophilized to reduce volume to about 0.5 ml and were chromatographed on polyethyleneimine-cellulose thin layers and on Whatman No. 1 chromatography paper. Aliquots were also studied by electrophoresis on cellulose polyacetate strips in the Gelman Deluxe chamber No. 51170 with a Buchler power supply at a constant 350 V. After radioautography as described above, supporting media were either cut up or scraped from plates for scintillation counting under 5 ml of toluene scintillation fluid. Elution of polyethyleneimine-cellulose scrapings (12) for 30 min with 1.0 ml of 0.5 M NaCl, in the case of nucleotides, or 1.0 ml of 0.1 N HCl, for bases and nucleosides, followed by counting in Triton scintillation fluid, gave similar results.

Cell pellets, washed and frozen after harvesting of bacteria, were used to study the nature of undegraded DNA present in cells after treatment with streptonigrin. The procedure of Marmur (17) was used to isolate DNA for analysis in CsCl gradients and sucrose gradients. Cell pellets were also thawed, lysed, and digested with papain by the method of Pritchard and Lark (18). Lysates were dialyzed overnight against 0.02 M Tris, pH 7.4, and centrifuged in a CsCl solution (4.360 g of CsCl per 3.36 ml of aqueous sample) for 72 hr at 30,000 rpm and 20° in a Spinco model L ultracentrifuge with the SW 50 rotor. Fractions of 16 drops each were collected through the bottoms of the tubes. With a capillary pipette, 10 μl of each fraction were spread on a numbered Whatman No. 1 filter paper disc and submerged immediately in a beaker containing 1 liter of cold 5% trichloracetic acid. The filter discs were washed by a Bollum batch process (19) as follows: twice with 500 ml of cold 5% trichloracetic acid, twice with cold 95% ethanol, and twice with ether. The discs were air-dried and counted under 5 ml of toluene scintillation fluid. Fractions containing DNA labeled both with ^{32}P and ^{14}C (three fractions from each gradient) were combined, dialyzed against Tris buffer (0.10 M, pH 8.8, containing Carbowax 20 M to reduce volume), and digested with snake venom phosphodiesterase for 4 hr at 36°. Digests were chromatographed on polyethyleneimine-cellulose thin layers with triple stepwise saline elution (13). Unlabeled 5′-deoxynucleoside monophosphates were used as standards.

In order to ascertain whether a significant number of single-strand breaks were present in DNA isolated by gradient centrifugation from streptonigrin-treated cells, samples of the above lysates, diluted to 30 μg of DNA per ml, were denatured at 95–100° for 8 min and immediately quenched in an ice-water bath. Combined with 1 μg of denatured DNA-^{3}H as a marker and adjusted to ionic strength of 0.1 M, these samples were layered in a volume of 0.20 ml on 4.80 ml of a 5–20% sucrose gradient (in 0.05 M sodium phosphate and 0.10 M NaCl, pH 6.70). Centrifugation was performed at 10° for 2 hr at 42,500 rpm in a Spinco model L ultracentrifuge with the SW 50 rotor. Ten-drop fractions were taken from tube bottoms directly into scintillation vials. Distilled water was added to make 1 ml, and then 15 ml of Triton scintillation fluid were added.

Rate of oxygen consumption. A Gilson Medical Electronics Oxygraph fitted with

a Clark oxygen electrode was used to study the rate of oxygen consumption by metabolizing suspensions of *E. coli* in the the presence of streptonigrin (25–40 μg/ml), cyanide, and phenazine methosulfate.

Hydrogen peroxide assay. Production of hydrogen peroxide in *E. coli* cells as a result of treatment with streptonigrin or phenazine methosulfate was assayed by a method in which titanium sulfate is converted to the yellow oxide, which absorbs at 410 mμ (20). Absorbances were read on a Gilford recording spectrophotometer. A standard plot of absorbance vs. H_2O_2 concentration was linear up to 5×10^{-4} M with a slope of 0.5/mM and a lower limit of detection at about 2×10^{-5} M. Standard plots, determined in the presence of 10 μg of streptonigrin or 100 μg of phenazine methosulfate per milliliter, were also linear but had somewhat lower slopes.

Polarography. Polarographic determinations of half-wave potentials ($E_{1/2}$ values) of antibiotics vs. a saturated calomel electrode were accomplished using a Heath Polarography system, model EUW-401. Solutions were bubbled with nitrogen for 15 min prior to analysis and kept under a nitrogen cover during the polarographic run. Plots of log $[i/(i_d - i)]$ against voltage V (where i_d = diffusion current and i = current at voltage V) gave values for n, the number of electrons involved in the electrode reaction (21).

Mutants of altered ultraviolet sensitivity. *E. coli* strain B and several of its derivatives (22) were obtained from the American Type Culture Collection. Strain B/r is more radiation-resistant than the wild type. Ultraviolet-sensitive mutants include Bs3, Bs8, and Bs12, which are damaged at different loci and are unable to perform host cell reactivation (hcr⁻); Bs2, which is hcr⁺ but is damaged at the *exr* locus and is X-ray-sensitive; Bs1, a double mutant damaged at the *exr* and Bs8 loci; and Bs11, which is probably deficient in its ability to bring about genetic recombination (rec⁻) (23).

RESULTS

Viability studies. The bactericidal action of streptonigrin on *E. coli* 15 T⁻Phe⁻ is illustrated in Fig. 2. Several observations (Table 1) implicate the intracellular oxidation-reduction environment in the mechanism of killing by streptonigrin. Under anaerobic conditions, when electron carriers are converted to a reduced state, lethality is much less. On the other hand, in the absence of an energy and electron source (glucose), lethality is also decreased.

TABLE 1

Bactericidal action of streptonigrin in the presence of other inhibitors

Streptonigrin (5 μg/ml) and other inhibitors were added at zero time to cells growing exponentially with an initial cell population of 10^8/ml. In the anaerobicexperiment, nitrogen bubbling was begun 1 min before addition of streptonigrin.

Inhibitors	Cells viable after 15 min
	%
None	120
Streptonigrin	31
Phenethyl alcohol (0.02 M)	100
Streptonigrin + phenethyl alcohol (0.02 M)	0.29
Carbonyl cyanide phenylhydrazone (100 μg/ml)	100
Streptonigrin + carbonyl cyanide phenylhydrazone (100 μg/ml)	0.001
Sodium cyanide (0.01 M)	100
Streptonigrin + cyanide (0.01 M)	0.014
Phenazine methosulfate (50 μg/ml)	100
Streptonigrin + phenazine methosulfate (50 μg/ml)	87
Streptonigrin (glucose washed out at −5 min)	90
Streptonigrin (anaerobic)	75
None (anaerobic)	100

Bacteriostatic concentrations of phenazine methosulfate, an autoxidizable electron scavenger, prevent killing by streptonigrin. Compounds that synergize the bactericidal effect of streptonigrin are cyanide, carbonyl cyanide phenylhydrazone, and phenethyl alcohol. Dinitrophenol, which strongly synergizes the lethal action of mitomycin, has no significant effect on the lethal action of streptonigrin.

Polarography. Because of the above observations and also because spectrophotometric studies (24) showed that streptonigrin was reduced by borohydride and

reoxidized by air in a reversible manner, the polarographic half-wave potential was determined. Table 2 shows for several inhibitors the half-wave potentials vs. a saturated calomel electrode in 0.10 M Tris buffer, pH 7.4. All waves were cathodic in nature; that is, reduction occurred at the dropping mercury electrode. Upon borohydride reduction the streptonigrin wave at —0.32 V became anodic. Air was allowed to enter the vessel, and the solution was then deaerated with nitrogen and polarographed. An identical cathodic wave was again observed, indicating reversibility. A plot of log $[i/(i_d - i)]$ vs. voltage gave an n value of 1.8 for the number of electrons involved in the electrode reaction. This would indicate a reduction involving 2-electron transfer, typical of many quinones. Mitomycin reduction at —0.38 V also involved 2 electrons.

TABLE 2

Half-wave potentials vs. the saturated calomel electrode

Compounds were studied at 10^{-4}–10^{-3} M in 0.1 M Tris buffer, pH 7.4, using a Heath Polarography system, model EUW-401.

Compound	$E_{1/2}$	Relative diffusion current
		$\mu amp/M$
Streptonigrin (2 waves)	−0.32	4.7×10^3
	−1.08	7.0×10^3
Mitomycin (2 waves)	−0.38	6.0×10^3
	−0.75[a]	
Actinomycin (2 waves)	−0.36	1.5×10^3
	−0.58	2.9×10^3
Menadione[b]	−0.28	8.4×10^3
Phenazine methosulfate	−0.17	7.6×10^3

[a] Indistinct wave.

[b] 2-Methyl-1,4-naphthoquinone.

Oxygen consumption. Oxygen consumption of *E. coli* cells, measured with a Clark oxygen electrode, was not significantly affected by streptonigrin. Cyanide (0.01 M) completely inhibited respiration of these cells, both with and without streptonigrin at concentrations up to 100 μg/ml. Phenazine methosulfate (100 μg/ml) increased oxygen consumption, and cyanide did not inhibit oxygen consumption when phenazine methosulfate was present.

Hydrogen peroxide production. The absence of significant quantities of hydrogen peroxide in streptonigrin-inhibited cultures was established by means of the titanium oxide assay. Log phase cultures at a concentration of 10^8 cells/ml were treated for 45 min with streptonigrin at 10 μg/ml in the presence and absence of cyanide (which inhibits catalase), or with phenazine methosulfate at 100 μg/ml, also with and without cyanide. Results shown in Table 3 indicate that no significant amount of H_2O_2 was detected in the presence of streptonigrin at a highly lethal concentration, while phenazine methosulfate at a bacteriostatic concentration caused significant production of H_2O_2.

TABLE 3

Concentration of hydrogen peroxide in cultures of E. coli

Concentrations were: streptonigrin, 10 μg/ml; sodium cyanide, 0.01 M; phenazine methosulfate, 100 μg/ml; 10^8 cells/ml.

Compound	Concentration of H_2O_2 after 45 min
	M
Streptonigrin	N.D. ($<0.2 \times 10^{-4}$)[a]
Streptonigrin + cyanide	N.D. ($<0.2 \times 10^{-4}$)
Phenazine methosulfate	0.9×10^{-4}
Phenazine methosulfate + cyanide	3.0×10^{-4}

[a] N.D. = none detected.

Fate of labeled precursors. Figure 3 shows the incorporation of thymine-^{3}H, L-phenylalanine-^{14}C, and uracil-^{14}C into acid-precipitable material by streptonigrin-inhibited cultures of *E. coli* strain 15 T$^-$Phe$^-$, reflecting net synthesis of DNA, protein, and RNA, respectively. Incorporation was followed with time for six concentrations of streptonigrin, and results are plotted for 30 and 60 min of incubation. With 10 μg of streptonigrin per milliliter at 30 min, net DNA synthesis was inhibited, whereas protein synthesis was not strongly affected and RNA synthesis was apparently stimulated. However, net DNA synthesis was not inhibited with less than 5 μg of streptonigrin per milliliter, although at this concentration only about 10% of

the cells were viable. In fact, there appeared to be a stimulation of net DNA synthesis with very low streptonigrin concentrations.

When cells were first incubated for 90 min in the absence of phenylalanine and resuspended with streptonigrin and all nutritional requirements, some inhibition of DNA synthesis occurred even at the lowest streptonigrin concentration used (1 μg/ml). Net protein synthesis was not affected until relatively high concentrations of streptonigrin were reached, and uracil incorporation was stimulated at 30 min with all concentrations used. After 60 min both phenylalanine and uracil incorporation had ceased.

Loss of acid-precipitable material in cultures previously labeled with thymine-^{14}C (Fig. 4) indicated extensive DNA breakdown after 30 min of incubation.

In order to study the effect of streptonigrin on cells in the absence of DNA synthesis, *E. coli* 15 T$^-$Phe$^-$ labeled with thymine-^{14}C was incubated without phenylalanine, but with thymine-^{14}C, for 90 min. This preliminary incubation renders cells immune to thymineless death (25). Then they were treated with streptonigrin under various conditions, and the breakdown of labeled DNA and decline in viable cell count were followed (Figs. 5 and 6). Both the bactericidal effect and the rate of DNA breakdown were greatest when phenylalanine was absent from the medium, either in the presence or absence of thymine. When phenylalanine was present without thymine, thymineless death began after 1 hr of incubation as expected, and streptonigrin did not prevent this. During the first hour, however, there seemed to be less killing by streptonigrin, possibly because the presence of phenylalanine would allow synthesis of DNA repair enzymes, or of proteins that are normally associated with the DNA and might have a shielding effect. When both requirements were present, the lethal effects and extent of DNA breakdown were less than in the absence of phenylalanine, but still much more pronounced than in unstarved cells.

The effect of streptonigrin on the rate of thymine-^{14}C incorporation into acid-precipitable material by phenylalanine-starved cells was also studied. Figure 7 shows that when phenylalanine starvation was continued in the presence of thymine-^{14}C, there was some uptake of thymine by the control culture; that is, some DNA was being made, perhaps because starvation was not completely effective in preventing reinitiation of the DNA replication cycle. When streptonigrin was added under these conditions, no incorporation of thymine-^{14}C was detected, and lethality was maximal. When phenylalanine was present in the above experiment, there was a lag of about 40 min before cell count increased in the control culture (Fig. 5), followed by rapid division. In the presence of streptonigrin, incorporation of thymine-^{14}C was inhibited after about 4 min with 5 μg of antibiotic per milliliter (Fig. 7). This experiment clearly shows that streptonigrin inhibited DNA synthesis more strongly in cells previously incubated without phenylalanine than in unstarved cells.

Because DNA made during the starvation period, in the absence of protein synthesis, does not have its normal complement of associated protein (25), an experiment was devised to ascertain whether in the presence of streptonigrin this "unprotected" DNA was more susceptible to breakdown than DNA synthesized under normal conditions. Cells were first incubated with thymine-^{14}C to label normal DNA and then, during the 90-min starvation period, with thymine-^{3}H. They were then resuspended in the presence of streptonigrin, but without phenylalanine or thymine, and acid-precipitable radioactivity was determined for both isotopes as a function of time. Figure 8 shows that there was no significant difference in rate or extent of breakdown of DNA labeled by the two isotopes. After incubation with 2.5 μg of streptonigrin per milliliter for 2 hr, 31.9% of incorporated ^{14}C label and 29.5% of incorporated ^{3}H label had become acid-soluble.

Since certain other mutants of strain 15 have been shown to contain a defective lysogenic bacteriophage (26), it was desirable to confirm that the degradation of DNA observed here was not related to

the possible induction of such a bacteriophage. In several experiments with *E. coli* strain B, the extent of DNA degradation in the presence of streptonigrin was comparable to that obtained with 15 T^-Phe^-. Also, the inhibition of protein synthesis (with chloramphenicol) synergized the lethal action of streptonigrin and enhanced DNA degradation, as with 15 T^-Phe^-.

DNA isolated from streptonigrin-treated cells. Both Marmur's procedure (17) for DNA separation and the preparation of cell lysates by the Pritchard-Lark procedure (18) were used to prepare samples for CsCl gradient analysis and for sucrose gradient analysis before and after denaturation. There was no difference between DNA from control cultures and DNA from streptonigrin-treated cultures.

Because cells initially incubated without phenylalanine gave the biggest changes in rates of synthesis and breakdown of DNA during streptonigrin treatment, DNA from such cultures was isolated and studied. Bacteria were grown in the presence of thymine-^{14}C and phosphate-^{32}P, both before and during a phenylalanine starvation interval. After treatment with streptonigrin (10 μg/ml) of half the culture for 1 hr, cell lysates were prepared and DNA was isolated by preparative CsCl gradient centrifugation to give 33 fractions for each sample. An assay of the radioactivity of each fraction showed that most of the DNA was present in fractions 16–18, in both the control and streptonigrin-treated cultures. There was no observable difference in buoyant densities. The ^{14}C:^{32}P ratios, determined from samples of the peak fractions 17, were 1.44 both for the control and the streptonigrin-treated samples. This indicated that thymine was not preferentially lost from DNA during treatment with the antibiotic.

After digestion of the DNA with snake venom phosphodiesterase and thin layer chromatography of the digest on polyethyleneimine-cellulose, followed by radioautography, only four spots were obtained on the X-ray film. These corresponded to the four 5′-deoxynucleotides, as indicated by ultraviolet absorption of unlabeled internal standards. Radioactive areas were scraped from the plates and counted either (a) by placing the cellulose scrapings directly under 5 ml of toluene scintillation fluid or (b) by first eluting the scrapings with 0.5 M saline, centrifuging, and counting the supernatants in Triton scintillation fluid. Both methods gave results in close agreement with those listed by Chargaff for the composition of DNA of *E. coli* strain 15 T^- (27). Table 4 shows these results, calculated as percentage of total ^{32}P radioactivity, which was between 1500 and 2000 cpm for each 20-μl sample.

TABLE 4

Composition of DNA isolated from streptonigrin-treated E. coli

Details of methods (a) and (b) are given in the text.

Digestion product	Streptonigrin-treated		Control		Values of Chargaff (27)
	(a)	(b)	(a)	(b)	
	mole %		*mole %*		*mole %*
5′-dGMP	27.2	27.5	28.1	28.1	27.5
5′-dAMP	23.4	23.7	23.8	24.2	23.6
5′-dCMP	24.4	24.9	23.2	26.6	26.1
5′-dTMP	24.3	24.1	24.3	21.1	22.4
Pyrimidine to purine ratio	0.96	0.96	0.92	0.91	0.95

From the centrifugation experiments and the above chromatographic assays, it was concluded that DNA from streptonigrin-treated cells, as isolated in the CsCl preparative gradient, does not differ significantly from normal DNA.

Breakdown products resulting from inhibition of E. coli *cells by streptonigrin.*

Comparison of the activities of ^{14}C and ^{32}P in all fractions of CsCl gradients of cell lysates showed no radioactive breakdown products from streptonigrin-treated DNA in other portions of the gradient. However, assays of cell supernatants obtained after centrifugation of cells showed that supernatants from cultures treated with streptonigrin for 1 hr contained twice as much ^{32}P and 34 times as much ^{14}C as the control. The excess activities found in streptonigrin supernatants represent approximately 21% of the incorporated ^{32}P and 33% of the incorporated ^{14}C. A comparison of the latter number with the data in Fig. 8 shows that DNA degraded during inhibition by streptonigrin can be accounted for as units that appear in the cell supernatant and are lost in washing procedures before lysate preparation.

Cell supernatants were lyophilized to reduce their volumes and then radioautographed in various ways to determine the nature of the labeled material. On polyethyleneimine-cellulose thin layers, developed with distilled water to move bases and nucleosides, 10 cpm of ^{14}C were found in the thymine area ($R_F = 0.73$) for the control, while for the streptonigrin-treated sample 170 cpm were found in the thymine spot. On polyethyleneimine-cellulose plates eluted with NaCl solutions by triple stepwise elution, most of the ^{32}P of the antibiotic-treated sample was found in the mononucleotide area. The control showed very little activity in this area, but did exhibit radioactivity at lower R_F values. A diagram of radioactivity obtained by eluting areas of chromatograms with 0.5 M NaCl is shown in Fig. 9. These samples represent equivalent volumes of original cell supernatants.

In another experiment, *E. coli* cultures were labeled with thymine-^{14}C before and during phenylalanine starvation. After treatment of half the culture with 10 μg of streptonigrin per milliliter for 90 min, cells were centrifuged and various fractions were prepared to determine the location of labeled products of DNA breakdown. These were: fraction 1, culture supernatants, lyophilized; 2, fraction 1 submitted to snake venom phosphodiesterase digestion; 3, an acid-soluble extract of the cell pellet; (4), the aqueous portion of fraction 3 after ether extraction; and 5, fraction 4 submitted to snake venom phosphodiesterase digestion.

Only fractions 1 and 2 showed significant radioactivity when assayed in Triton scintillation fluid. In the original culture, with 5×10^8 cells/ml, 0.0442 μC of ^{14}C per milliliter was incorporated in acid-precipitable material before treatment with streptonigrin. After 90 min in the presence of antibiotic, this had decreased to 0.0320 μC/ml with 1×10^6 viable cells/ml, while the radioactivity in the control remained constant. Therefore 0.0122 μC of ^{14}C per milliliter had become acid-soluble. In fraction 1, 0.0125 μC/ml, based on the original volume of the culture, was obtained. Therefore essentially all solubilized ^{14}C-product was released from the cells and appeared in the culture supernatant when centrifuged at 4°. This represented 27.5% of the incorporated ^{14}C label.

The above fractions were chromatographed on polyethyleneimine-cellulose thin layer plates and eluted stepwise with saline solutions, and radioautograms were prepared. No significant activity was detected on the film for fractions from uninhibited cultures. One labeled spot was detected on the X-ray film for fractions 1 and 2 from streptonigrin-inhibited cultures. This corresponded to either thymine or thymidylic acid and indicated that no observable quantity of oligonucleotides was present in the supernatant. In acid-soluble fractions 3 and 4, there was activity at the origin and an ill-defined spot in the thymine or thymidylic acid area. On ether extraction and digestion, all activity in fraction 5 was removed from the origin, the indication being that oligonucleotides of larger than 10 nucleotides were present in the acid-soluble fraction after treatment with streptonigrin. Their concentration, however, represented less than 1% of the ^{14}C activity in fraction 1. Chromatography and electrophoresis in three different systems showed that at least 94% of the activity was due to thymine or thymidine.

Inhibition of mutants having altered ultraviolet sensitivity. Table 5 shows the

effects of streptonigrin inhibition on viability and DNA degradation of a number of mutants of *E. coli* strain B, in both the presence and absence of chloramphenicol. The viability data show that the radiation-resistant strain B/r is also more resistant to streptonigrin. The rec⁻ strain Bs11 and the double mutant Bs1 are Strain B/r also shows this pattern. Bs1, Bs2, and Bs11 degrade their DNA whether chloramphenicol is present or not.

DISCUSSION

Cultures of *E. coli* exposed to streptonigrin exhibit an initial first-order decline in viability that implies a single-hit

TABLE 5

Degradation of DNA and loss of viability of ultraviolet-sensitive cultures of E. coli strain B in the presence of streptonigrin

DNA in these strains was labeled by 2 hr of exponential growth in minimal medium containing thymine-^{14}C (0.03 μC/ml; 0.4 μg/ml) and deoxyadenosine (500 μg/ml) (28), followed by washing and resuspension in minimal medium for 10 min prior to addition of antibiotics. Cultures in exponential growth at 1–2 × 10^8/ml were inhibited at zero time with streptonigrin (2 μg/ml) with or without chloramphenicol (200 μg/ml). Samples of 0.04 ml were taken for viability assay every 30 min. Samples of 0.1 ml were taken at 10-min intervals for assay of acid-precipitable radioactivity. Uninhibited control cultures continued in exponential growth. Chloramphenicol by itself was bacteriostatic. No DNA degradation was detected in control cultures either with or without chloramphenicol.

E. coli strain	Phenotypic characteristic	Chloramphenicol	Viability[a]	DNA undegraded[b]
			%	%
B	Wild type	−	60	98
		+	11	36
Bs1	hcr⁻; X-ray-sensitive	−	11	45
		+	0.018	28
Bs2	hcr⁺; X-ray-sensitive	−	68	59
		+	3.4	32
Bs3	hcr⁻	−	23	100
		+	0.26	56
Bs8	hcr⁻	−	23	95
		+	0.60	51
Bs11	Probably rec⁻	−	0.62	16
		+	0.0035	28
Bs12	hcr⁻	−	77	92
		+	12	31
B/r	Radiation-resistant	−	120	96
		+	28	42

[a] Expressed as percentage of an uninhibited control culture at 0 min.

[b] Expressed as percentage of an uninhibited control culture at 120 min.

the most sensitive to the antibiotic. Chloramphenicol, an inhibitor of protein synthesis, clearly enhances both the lethal action of streptonigrin and its effect in promoting DNA degradation. Bs3, Bs8, and Bs12 are similar to the wild type in the extent to which they degrade their DNA in the presence of streptonigrin: in the presence of chloramphenicol, but not in its absence, the DNA is extensively degraded. mechanism; that is, only one hit per cell is required for killing (5). This points to the bacterial chromosome as the site of action, since one chemical modification ("hit") of the single DNA molecule could render the cell nonviable. The inactivation of enzymes or other cellular components, multiple copies of which are present in the cell, could not lead to the observed first-order kinetics. Destruction of the membrane is

excluded as the mechanism of lethal action by the continuation of metabolism after the cells are nonviable.

Streptonigrin must be reduced intracellularly in order to become lethal (8). Observations that support this argument are: (a) streptonigrin can be reduced intracellularly in the presence of an electron source such as glucose (9); (b) an electron source must be present for full expression of the lethal effect; (c) cyanide, which facilitates the reduction of streptonigrin, enhances lethality; and (d) phenazine methosulfate, which competes for electrons intracellularly, antagonizes lethality.

Oxygen is also necessary in order for streptonigrin to be lethal. Hochstein *et al.* (29) suggested that in their mitochondrial system this antibiotic was repeatedly reduced and reoxidized with concomitant formation of hydrogen peroxide, which these authors thought could account for its damaging effects on bacterial cells. However, it was shown here that in bacterial cultures there is no detectable production of hydrogen peroxide, even with highly lethal quantities of streptonigrin. Moreover, bacteriostatic concentrations of phenazine methosulfate, although causing significant production of hydrogen peroxide, actually protect against killing by streptonigrin. Therefore hydrogen peroxide cannot contribute significantly to the lethal action of the antibiotic. Yet both reduction of streptonigrin and availability of oxygen are necessary to its lethal action. Consequently we assume that a reduced form of streptonigrin must react with oxygen to give a lethal species that is not hydrogen peroxide. This lethal species may be a peroxy free radical or a peroxide modification of the antibiotic. Like streptonigrin, this species would probably have an affinity for DNA. Following its association with DNA it might react with the DNA and initiate degradation.

The results obtained with *E. coli* by viability assays and by incorporation of labeled macromolecular precursors in the presence of streptonigrin are similar to those obtained with another aminoquinone antitumor agent, mitomycin C (30). This agent requires intracellular reduction to a lethal form, which can then alkylate and cross-link DNA (31). As with streptonigrin, the antibacterial effects of mitomycin C (8) are antagonized by phenazine methosulfate and synergized by cyanide, effects which could be explained by the influence of these compounds on the intracellular oxidation-reduction environment. However, there are certain important differences between the two antibiotics. The intracellular reduction of mitomycin is not reversible, presumably because reduced mitomycin readily loses a methoxy group and rearranges to a very reactive species (32). Ishizu *et al.* (33) found that mitomycin C, unlike streptonigrin, does not give rise to detectable concentrations of free radical while undergoing reduction in bacterial cultures. Comparison of the structures of mitomycin C and streptonigrin shows that mitomycin contains two or three possible alkylating groups—a carbamate moiety, an aziridine ring, and an aminoquinone ring—whereas in streptonigrin only the aminoquinone moiety might be considered an alkylating group. Szybalski and Iyer (34) stated that, unlike mitomycin, streptonigrin did not cross-link DNA *in vitro*. They were also unable to detect binding of tritiated streptonigrin to DNA when the antibiotic was reduced with borohydride in the presence of DNA (32). Thus, after intracellular reduction, mitomycin and streptonigrin apparently attack DNA in quite different ways.

Our studies of the incorporation of radioactive precursors into bacterial macromolecules show that net DNA synthesis is inhibited at antibiotic concentrations that do not inhibit either net RNA or protein synthesis. However, inhibition of DNA synthesis per se is not the lethal event, since net DNA synthesis is not inhibited at concentrations of streptonigrin that kill 90% of the cells. After phenylalanine starvation, during which the DNA replication cycle is brought to completion (16), DNA synthesis is much more strongly inhibited by streptonigrin, and DNA degradation and lethality are likewise increased.

Experiments in which streptonigrin was chemically reduced in the presence of DNA indicate that after reduction the anti-

biotic can cause single-strand breaks in DNA *in vitro* (10). However, the extensive degradation of DNA observed in streptonigrin-inhibited bacterial cultures is probably entirely enzymatic, since no significant quantity of dialyzable fragments could be detected in samples of DNA treated *in vitro* with reduced antibiotic. The type of DNA breakdown observed with streptonigrin is quite analogous to that found when bacteria are exposed to ionizing radiation. Single-strand breaks are present in DNA treated with gamma irradiation (35). After X-irradiation of *E. coli*, DNA is broken down to units that appear in the medium. Approximately 30% of labeled DNA-^{3}H becomes acid-soluble in 20–30 min after exposure of cells to 50 kr of X-irradiation. At the same time normal DNA synthesis is completely inhibited (36). Such damage is thought to occur primarily by the attack of free radicals which are produced in irradiated water. The presence of oxygen allows the formation of the hydroperoxy radical (HO_2) and peroxy derivatives of organic free radicals, which are more damaging to biological systems than are the nonoxygenated species (37).

Degradation of DNA has also been observed following treatment of *E. coli* with mitomycin (38) and sulfur mustard (39). In both cases breakdown was enhanced when protein synthesis was inhibited during or following treatment, as was observed here with streptonigrin. The significance of this effect of inhibiting protein synthesis is not yet understood. By way of contrast, Huston and Pollard (40) found that following a preliminary incubation without leucine to complete the chromosome, bombardment of *E. coli* strain 15 T$^-$Leu$^-$ with fast protons led to a decreased rate of degradation in the continued absence of leucine, whereas in our case a comparable procedure led to an enhanced rate of degradation.

Further information can be obtained by studying the streptonigrin-induced degradation of DNA in ultraviolet-sensitive mutants. These mutants are probably all defective in their ability to repair DNA damage sustained during irradiation (23, 41). Strains B3, B8, and B12 are all hcr$^-$ and are thought to lack the ability to excise pyrimidine dimers from DNA as well as the ability to excise DNA bases alkylated with mitomycin C (23, 42, 43). Our results suggest that the genetic defect in these strains is not relevant to streptonigrin-induced DNA degradation, since they are like the wild type in this respect. (Nevertheless, strains B3 and B8 are somewhat more sensitive to the *lethal* action of streptonigrin.) In strains Bs1, Bs2, and Bs11 the degradation of DNA is much more extensive than in the wild type, suggesting that in these mutants part of the enzymatic apparatus utilized in DNA repair has been inactivated. Unlike the wild type, they lack control over the extent to which DNA is degraded during the repair process.

The very extensive degradation observed in the presence of chloramphenicol seems to imply that protein synthesis is required in order to control DNA degradation. Since extensive degradation occurs in strains Bs1, Bs2, and Bs11, even in the absence of chloramphenicol, the proteins that must be synthesized to achieve control might be defective in these strains. However, other interpretations of our results are possible. Blocking protein synthesis causes many secondary changes, such as in the degree of charging of the tRNA and in the proportion of ribosomes that exist as subunits. Such changes, rather than absence of a particular protein, may bring about the effect of chloramphenicol on DNA degradation.

The polarographic determinations indicate that streptonigrin with an $E_{1/2}$ near that of menadione, might be able to receive electrons enzymatically from any of several intracellular components, including reduced pyridine nucleotides, lactate, and various flavoproteins (44). Enzymes have been identified in bacterial and mammalian organisms that are able to catalyze the reversible reduction of naturally occurring quinones by oxidation of reduced pyridine nucleotides (45). The interaction of streptonigrin with ferrous and cupric ions, which we observed spectrally (24), suggests that the antibiotic may interact with such metal ions on cytochromes and flavo-

proteins. Rajagopalan[2] found that streptonigrin is reduced by xanthine oxidase, an iron-molybdenum flavoprotein.

There are similarities between the bactericidal action of streptonigrin and that of simple quinones. Many quinones are readily reduced to the semiquinone forms in bacterial cultures under the same circumstances as streptonigrin (33). Among these are 1,4-naphthoquinone and benzoquinone. Both are quite lethal against *E. coli* at 10 μg/ml, but the lethal action is greatly reduced in the absence of oxygen.[3] McCarty (46), working with ascorbic acid and hydroquinone, found that these agents inactivate pneumococcal transforming DNA in the presence, but not in the absence, of oxygen. Catalase nullified the effect, suggesting the participation of hydrogen peroxide. However, the quantity of hydrogen peroxide formed was much too small to account for the observed inactivation (although it caused inactivation at sufficiently high concentrations). McCarty concluded that intermediate peroxides, formed during oxidation of ascorbic acid and hydroquinone, might be responsible for inactivation of transforming DNA.

The foregoing observations suggest that in their bactericidal action streptonigrin and some of the simpler quinones may initiate a similar sequence of events, beginning with intracellular reduction to a semiquinone that reacts with O_2 to form a peroxide or peroxy free radical. This species may directly damage the DNA. As a consequence the cell becomes nonviable and, at least in the case of streptonigrin, DNA synthesis ceases and breakdown of the chromosome ensues.

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