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Antibacterial Action of 2-Bromo-2-Nitropropane-1,3-Diol (Bronopol)

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Patterns of growth inhibition of *Escherichia coli* in the presence of 2-bromo-2-nitropropane-1,3-diol (bronopol) indicate a period of biocide-induced bacteriostasis followed by growth at an inhibited rate. The length of the bacteriostatic period, but not the subsequent growth inhibition, was reduced by the addition of excess cysteine. Patterns of growth inhibition were unaffected by catalase or superoxide dismutase. The bactericidal concentrations (100 to 500 μg/ml) were considerably in excess of the MIC (13 μg/ml) and generally produced first-order reductions in viability. Bactericidal activity was considerably reduced by anoxic conditions and by the presence of catalase or superoxide dismutase. Results indicate that there are two distinct reactions between bronopol and thiols. Under aerobic conditions, bronopol catalytically oxidizes thiol-containing materials such as cysteine, with atmospheric oxygen as the final oxidant. By-products of this reaction are active oxygen species such as superoxide and peroxide, which are directly responsible for the bactericidal activity of the compound and for the reduced growth rate after the bacteriostatic period. The latter effect probably results from the oxidation of intracellular thiols such as glutathione and cysteine. Catalytic oxidation of thiols in the presence of excess thiol leads to the creation of an anoxic state. Under these conditions, the slower reaction with thiols, which consumes bronopol, predominates. Consumption of bronopol by its reaction with thiols, without the involvement of oxygen, leads to the eventual removal of bronopol from treated suspensions and the resumption of growth.

2-Bromo-2-nitropropane-1,3-diol (bronopol) has a broad spectrum of antibacterial activity (12) and is widely used, at concentrations of up to 0.1% (wt/vol), as a preservative for pharmaceutical and cosmetic products (4, 15). Previous studies of the mechanism of action of bronopol all conclude that the antibacterial activity of bronopol relates to its interaction with essential thiols within the cell (3, 16, 18). Such interaction is thought to lead to the oxidation of thiols through a radical anion intermediate (11). Unlike other thiol-interactive antimicrobial agents, bronopol possesses significant bactericidal activity that cannot be explained solely in terms of thiol oxidation.

This paper examines the hypothesis that separate actions are responsible for the growth inhibitory and bactericidal activities of the compound.

MATERIALS AND METHODS

Organisms, chemicals, and culture maintenance. *Escherichia coli* ATCC 8739 was used throughout the study. Cultures were maintained on nutrient agar (Oxoid CM 3) slopes at room temperature in the dark after incubation at 35°C. 2-Bromo-2-nitropropane-1,3-diol (bronopol) was obtained from the Boots Chemical Co., Nottingham, England. Catalase, superoxide dismutase, cytochrome *c*, cysteine, cystine, cysteine hydrochloride, and cystine dimethyl ester were obtained from the Sigma Chemical Co., St. Louis, Mo. All other reagents were of the purest available grade obtainable from BDH, Poole, England.

Growth inhibitory activity. To a series of Erlenmeyer flasks (250 ml) containing 98 ml of chemically defined simple salt medium (1) were added 1-ml portions of a similarly grown overnight culture. The flasks were shaken in an orbital incubator (35°C, 200 oscillations per min), and optical density measurements at 470 nm ($E_{470\ nm}$) were made at 15-min intervals. When the cultures were in the logarithmic phase of growth and $E_{470\ nm}$ was 0.15, various concentrations of a bronopol solution were added (1 ml). Growth was monitored in the flasks by optical density measurements for a further 2 h. In selected experiments, cysteine was added at various times following the addition of biocide to give molar ratios of cysteine to biocide of 10:1 and 1:1. In other experiments, catalase (50 U/ml) or superoxide dismutase (60 U/ml) was added simultaneously with bronopol. Experiments were performed in duplicate.

Preparation of washed cell suspensions. Erlenmeyer flasks (250 ml) containing 100 ml of a chemically defined simple salt medium (1) were inoculated from nutrient agar slopes and incubated at 35°C in an orbital incubator (200 oscillations per min) for 16 h. The cells were harvested by centrifugation (10,000 × *g*, 15 min, 35°C), washed twice in sterile saline (0.9%, wt/vol), and suspended to an appropriate optical density in phosphate-buffered saline (pH 7.0, 0.1 M). Suspensions were used within 1 h of preparation.

Bactericidal activity. Washed suspensions of *E. coli* (19 ml, 5×10^8 cells per ml) in Erlenmeyer flasks (100 ml) were equilibrated at 35°C in an orbital incubator (200 oscillations per min) for 30 min prior to the addition of biocide (1 ml). At appropriate times, 1-ml portions were removed and serially diluted in thioglycolate medium (Oxoid CM173). Suitable dilutions (0.1 ml) were spread on the surfaces of triplicate predried (50°C, 30 min) nutrient agar plates. Viable counts were determined following incubation of the plates at 35°C for 16 h. Preliminary experiments confirmed that dilution (1:10) of bronopol, at the concentrations used, in thioglycolate medium completely neutralized the bactericidal effect. Experiments were repeated at various temperatures at pH 7.0 and at various pH values at 35°C. Selected experiments were performed in the presence of catalase (100 U/ml) or superoxide dismutase (200 U/ml) or under anoxic conditions obtained by flushing the culture media with nitrogen before inoculation and performing the experiment in static tubes sealed from the atmosphere by a layer of light liquid paraffin.

Chemical analyses. Chemical analyses of the reaction

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products of bronopol-thiol mixtures were conducted by performing the reactions in 5-mm-diameter nuclear magnetic resonance (NMR) tubes and obtaining ^{1}H and ^{13}C spectra by using a Fourier transform NMR spectrometer at 80 MHz (Bruker WP80 SY). Proton and two-dimensional ^{1}H-^{13}C (HETCOR) spectra of isolated reaction products were obtained by using a 300-MHz, Fourier transform spectrometer (Varian XL-300). Infrared and mass spectroscopy were conducted with a Pye Unicam SP 3-300 spectrometer and Kratos MS 25 spectrometer, respectively.

Thin-layer chromatographic (TLC) analysis of reaction products was conducted on Kiesel gel 60 TLC plates (Macherey-Nagel, Duren) with butan-1-ol-glacial acetic acid-water (60:15:25). Plates were developed either with ninhydrin in acetone (0.2%, wt/vol) (13) or with sodium carbonate solution (10%, wt/vol), followed by 0.7 N Folin-Ciocalteau reagent (14). The latter stain was superior for visualization of the bronopol spots.

Gas chromatographic (GC) analysis of reaction products was performed with a Hewlett-Packard 5840A gas chromatograph, 7671A automatic sampler, and 5840 GC terminal and an E301 silicone elastomer (10%)-celite column. Temperatures were increased at 10°C/min up to 250°C. Peaks were detected by flame ionization.

RESULTS

Growth inhibition studies. After the addition of biocide (4 to 20 μg/ml) to actively growing cultures of *E. coli*, growth immediately ceased. Bronopol-induced bacteriostasis persisted for up to 90 min, and when growth was resumed, it was at a lower rate than that of the controls (Fig. 1). The length of the bronopol-induced bacteriostasis was proportional to the applied biocide concentration, as was the degree of growth inhibition measured immediately after recovery from this bacteriostasis. Plots of growth rate inhibition after the induced bacteriostasis against bronopol concentration allowed estimates of the MIC to be made (13 μg/ml; Fig. 1).

The ability of thiols to neutralize and reverse the growth inhibitory action of bronopol was examined by the addition of cysteine to growth-inhibited cultures at various times after the addition of biocide (7 μg/ml). Bronopol concentrations of one-half the MIC were used so that both activation and neutralization of the growth inhibitory effects might be observed. When the molar ratio of cysteine to bronopol was 1:1, cysteine failed to alter the pattern of inhibition. However, at a 10:1 molar ratio the length of the induced bacteriostatic period was substantially reduced provided that the addition of cysteine was made less than 40 min after that of the biocide. In no case was the inhibited growth rate following the shortened bacteriostatic period increased by the presence of a neutralizer (Fig. 2). The addition of the enzyme catalase or superoxide dismutase to the growth-inhibited cultures caused no change in the pattern of inhibition.

Bactericidal activity. Bactericidal activity was approximated to first-order kinetics for concentrations of bronopol greater than 100 μg/ml (Fig. 3a). The compound had a concentration exponent of 0.9 (Fig. 3b) and a temperature coefficient (θ_{10}) of 2.9 (Fig. 3c), and activity increased with increasing pH (Fig. 3d).

Time-survival data were redetermined for bronopol at 500 μg/ml under anoxic conditions and under aerobic conditions in the presence of catalase and superoxide dismutase. All three sets of conditions significantly reduced the degree of bactericidal activity. Such effects were particularly marked under anoxia and in the presence of superoxide dismutase (Fig. 4).

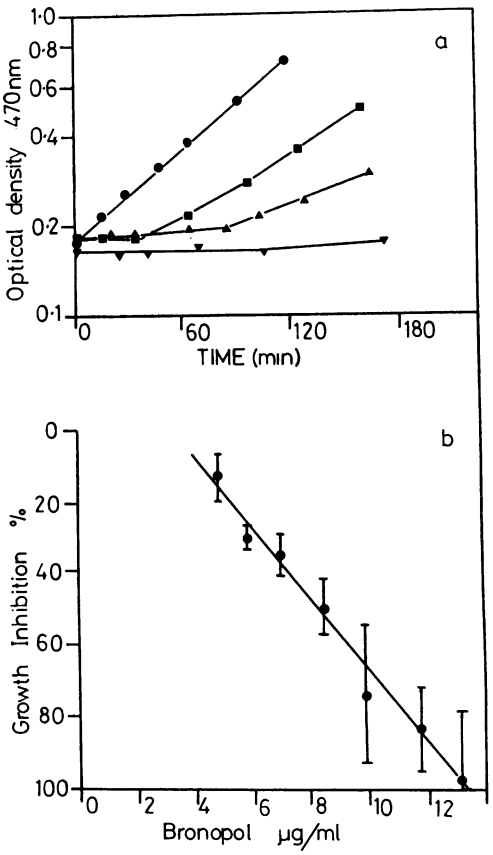

FIG. 1. Growth inhibitory activity of bronopol towards *E. coli* ATCC 8739. (a) Cultures were grown in a simple salt, chemically defined medium until logarithmic phase and $E_{470\ nm}$ of 0.15. Bronopol (●, 0 μg/ml; ■, 5 μg/ml; ▲, 10 μg/ml; ▼, 12.5 μg/ml) was added, and growth was monitored as $E_{470\ nm}$ for up to 2 h. (b) Plot of the inhibition of growth rate relative to untreated controls after the biocide-induced bacteriostatic period versus bronopol concentration for the determination of the minimum growth inhibitory concentration (13 μg/ml).

Reactions of bronopol with thiols. The reactions of bronopol with cysteine, cysteine methyl ester, and glutathione were assessed in D_2O in 5-mm-diameter NMR tubes. The results of these studies were checked by TLC, and when necessary, larger scale reactions were conducted with a view to separate and identify the reaction products.

The results of previous TLC studies suggest that in the presence of air the major reaction product of cysteine and bronopol is cystine (16). This result was readily confirmed with equimolar amounts of the two reactants by using a combination of ^{1}H NMR and TLC, with the additional observation that consumption of cysteine was not accompanied by any measurable loss of bronopol. Even with molar ratios of cysteine to bronopol of 10:1, the NMR signals for bronopol were clearly seen when the cysteine had been completely consumed. In the absence of bronopol, cysteine hydrochloride was essentially unchanged after several hours in solution in an NMR tube. With cysteine methyl ester, essentially similar results were obtained. In this case, the differences in the NMR spectra of the free bases of the thiol and disulfide were more marked, and the conversion was confirmed without the need for TLC. With the methyl ester,

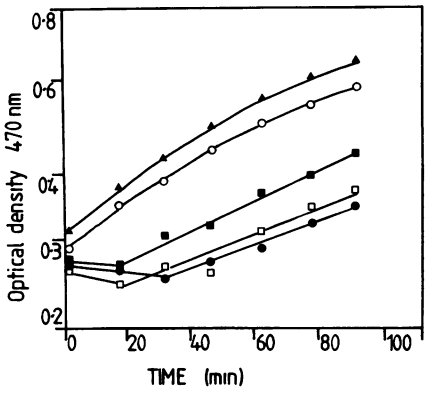

FIG. 2. Effect of cysteine on the growth inhibitory activity of bronopol toward *E. coli* ATCC 8739. Cultures were grown in a simple salt, chemically defined medium until logarithmic phase and $E_{470\ nm}$ of 0.3. Bronopol (7 μg/ml) was added to all cultures except the control (▲), followed by cysteine (62 μg/ml) at various times (○, 0 min; ■, 20 min; and □, 40 min). ●, No cysteine. Growth was monitored as $E_{470\ nm}$ for up to 2 h.

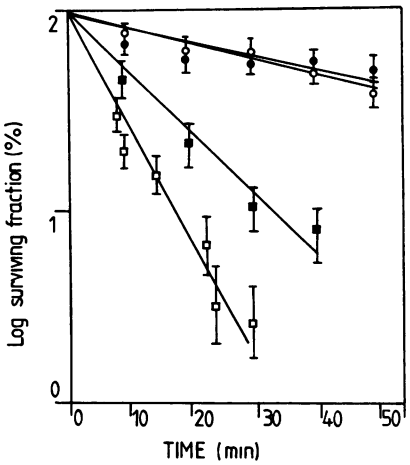

FIG. 4. Bactericidal activity of bronopol (500 μg/ml toward washed suspensions of *E. coli* ATCC 8739 (□) in phosphate-buffered saline (0.1 M, pH 7.0) in the presence of catalase (●, 100 U/ml) or superoxide dismutase (■, 200 U/ml) and under anoxic conditions (○).

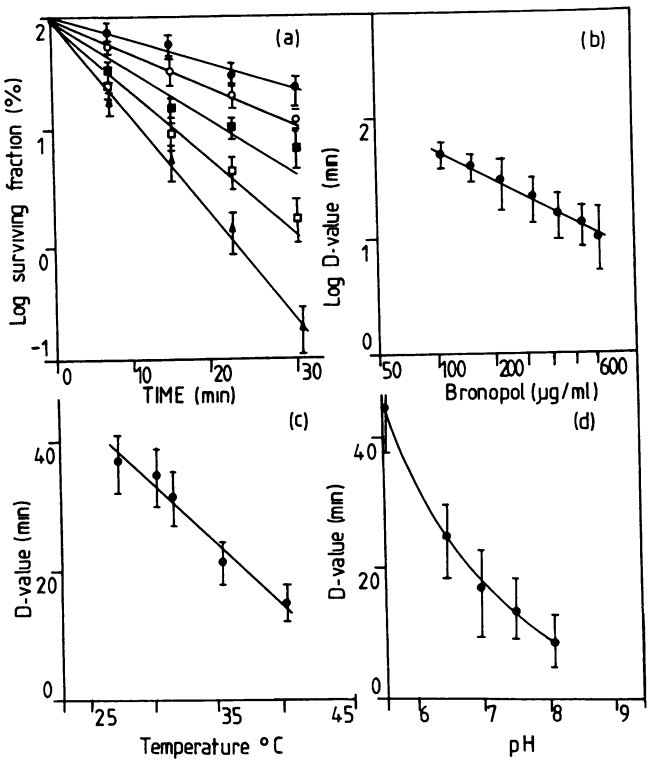

FIG. 3. Bactericidal activity of bronopol toward washed suspensions of *E. coli* ATCC 8739 in phosphate-buffered saline (0.1 M). (a) Time-survival data determined at 35°C and pH 7.0. Bronopol concentrations: ●, 100 μg/ml; ○, 200 μg/ml; ■, 300 μg/ml; □, 400 μg/ml; and ▲, 500 μg/ml. (b) Plot of decimal reduction times following bronopol treatment (pH 7, 35°C) versus bronopol concentration for the determination of the concentration exponent (0.9). (c) Effect of temperature on the bactericidal activity of bronopol (500 μg/ml) at pH 7.0 (θ_{10} = 2.9). (d) Effect of pH on the bactericidal activity of bronopol (500 μg/ml) at 35°C.

it was possible to make the solution alkaline and to extract the disulfide with chloroform. Comparison of the extract with an authentic sample confirmed that the disulfide was the sole significant reaction product.

At the relatively low field of 80 MHz, the reaction of bronopol with glutathione gave spectra with considerable areas of overlapping peaks that did not permit assignments to be made with confidence. However, comparison with spectra of authentic material suggested that glutathione was oxidized to the disulfide, as were cysteine and cysteine methyl ester. This result was readily confirmed by TLC. Again, the disulfide was the only significant reaction product, with the bronopol remaining unchanged.

In the presence of air, the reactions of bronopol with all the above-mentioned thiols were extremely rapid even at the lower bronopol/thiol ratios and despite the relatively poor aeration that is expected in a 5-mm-diameter NMR tube. The most likely oxidant for consumption in these reactions is oxygen because the bronopol itself clearly plays a catalytic role. Oxygen consumption after mixing bronopol (50 μg/ml in phosphate buffer, 0.1 M, pH 7.0) and cysteine (0.15 mg/ml in phosphate buffer, 0.1 M, pH 7.0) was assessed by conducting the reaction in a metabolic chamber with an oxygen electrode (Rank Bros., Cambridge, United Kingdom) and a strip chart recorder. Immediately after bronopol addition, oxygen was rapidly consumed and a state of anoxia was achieved within 2 min (Fig. 5).

In the absence of air, the reactions took a different course. Two observations were made in the NMR experiments. First, the bronopol was consumed; second, the reactions were considerably more complex than those observed in the presence of air. The reaction was considerably slower than that observed previously, with significant levels of cysteine remaining after 2 h when a bronopol/cysteine ratio of 1:10 was used. In the case of cysteine methyl ester, there was also evidence from both NMR and TLC data for the formation of the disulfide, but this was no longer the exclusive product. In view of the complexity of the reaction, no attempt was made to follow the reaction with glutathione under anoxic conditions. Instead, cysteine methyl ester was used in a relatively large-scale reaction to allow the isolation of at least some of the reaction products.

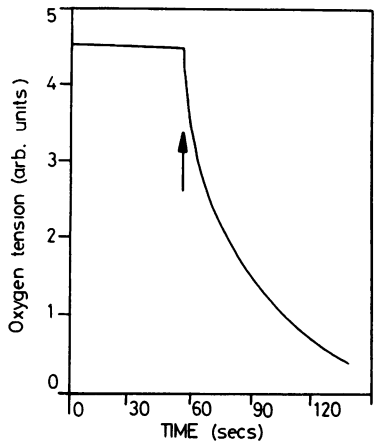

FIG. 5. Oxygen consumption following the interaction of solutions of bronopol (50 μg/ml in phosphate buffer, 0.1 M, pH 7.0) and cysteine (0.15 mg/ml in phosphate buffer, 0.1 M, pH 7.0) in a metabolic chamber with oxygen electrode. Arrow denotes time of bronopol addition. arb., Arbitrary.

When cysteine methyl ester hydrochloride (8.6 g) was treated with bronopol (10 g) in phosphate buffer at pH 7.0 after removal of oxygen by bubbling nitrogen in, the solution became cloudy. After 2 days, a yellow solid and a yellow oil separated. The solid was collected by filtration, and the oil was removed by solvent extraction. The solid was insoluble in all common solvents. Mass spectrometry showed the sequential loss of 32 mass units, and simple chemical tests confirmed the solid to be sulfur.

The oil was a complex mixture, but flash chromatography followed by GC-mass spectometry and two-dimensional NMR allowed the identification of the most volatile components. These components were two closely related isomers, which at first were difficult to separate on GC, and were not physically separable for NMR analysis. They were therefore examined as a mixture, and the NMR spectrum in $CDCl_3$ at 300 MHz showed resonances that were assigned as follows: δ 1.64, CH_3; δ 1.81, CH_3; δ 2.02, impurity; δ 2.82 to 3.31, a complex region of multiplets due to nonequivalent protons of CH_2 coupled to CH; δ 3.67, 3.73, 3.74, and 3.75, four OCH_3 groups; δ 4.0, CH; and δ 4.32, CH. The ^{13}C NMR spectrum was easier to interpret and showed very clearly that the sample was a mixture of two very similar compounds. The spectrum showed the following resonances in $CDCl_3$: δ 24.96 (CH_3), 27.78 (CH_3), 38.53 (CH_2), 39.62 (CH_2), 51.99 (OCH_3), 52.64 (OCH_3), 65.12 (CH), and 65.31 (CH). The multiplicities of hydrogen resonances (CH, CH_2 and CH_3) were determined by the DEPT pulse sequence.

A two-dimensional (HETCOR) NMR experiment confirmed the assignments and correlated the proton multiplets at δ 4.0 and 4.32 with the carbon resonances at δ 65.12 and 65.31, respectively. The proton and carbon resonances for the methoxy groups correlated as expected. The carbon with signal at δ 39.62 correlated with multiplets and δ 3.3 and 2.8 in the proton spectrum, and the carbon with signal at δ 38.53

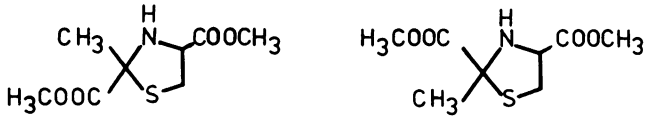

FIG. 6. Proposed structures for the anoxic reaction products of cysteine and bronopol.

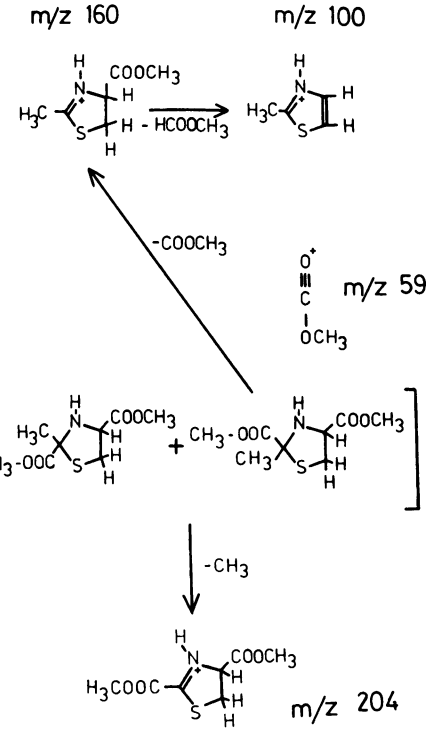

FIG. 7. Proposed fragmentation pattern for the anoxic reaction products of cysteine and bronopol subjected to GC-mass spectrometry.

correlated with multiplets at δ 3.3 and 3.0 in the proton spectrum. The carbon resonances at δ 27.78 and 24.96 of the remaining methyl groups correlated with proton resonances at δ 1.64 and 1.81, respectively.

These observations are entirely consistent with the *cis* and *trans* structures given in Fig. 6. The fragmentation pattern after GC-mass spectrometry was also consistent with the proposed structures. Although there was no molecular ion, there was a small peak at *m/z* 204, representing the loss of a CH_3 group, and the remainder of the anticipated fragmentation pattern was easily reconciled (Fig. 7). Structures for smaller peaks at *m/z* 119 and 87 have not yet been assigned.

The formation of thiazolidines has been observed when aqueous solutions of cystine are exposed to air for prolonged periods (5). Presumably, the reaction proceeds by the oxidation of cystine to pyruvic acid and the condensation of pyruvic acid with a molecule of cysteine (Fig. 8), which is also formed by the breakdown of cystine.

In the present study, a reaction involving the methyl ester gave reaction products analogous to those depicted in Fig. 6. Presumably, bronopol acts as an oxidizing agent in the absence of oxygen, although no breakdown products of bronopol were isolated from the very complex reaction mixture.

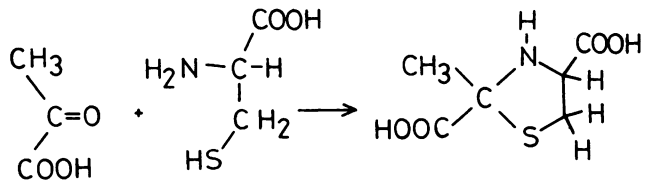

FIG. 8. Thiazolidine formation by condensation of pyruvic acid and cysteine.

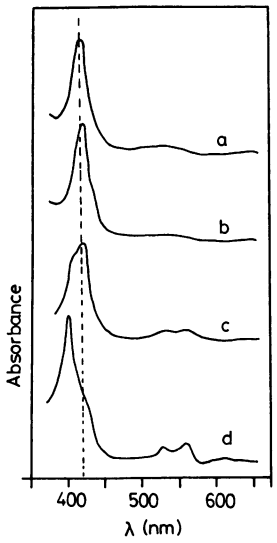

FIG. 9. Absorption spectra of cytochrome *c* (2 μg/ml in phosphate buffer, 0.1 M, pH 7.0) alone (a) and following addition of bronopol (200 μg/ml) (b) or cysteine (100 μg/ml) (c) or the simultaneous addition of bronopol and cysteine (d).

Cytochrome *c*. The absorption spectra of cytochrome *c* (2 μg/ml in phosphate buffer, 0.1 M, pH 7.0 [Fig. 9a]) were determined with a Pye Unicam SP8-500 spectrophotometer following addition of bronopol (200 μg/ml [Fig. 9b]) or cysteine (100 μg/ml [Fig. 9c]) or both (Fig. 9d). The addition of bronopol alone caused no change in the cytochrome *c* spectrum, but the addition of cysteine caused a gradual shift in the spectrum, indicating the formation of the reduced form of cytochrome *c* (Fig. 9c). Simultaneous addition of bronopol and cysteine caused an immediate change in the cytochrome *c* spectrum to that of the reduced form (Fig. 9d).

DISCUSSION

Chemical studies of the interactions of bronopol with cysteine, cysteine methyl ester, and glutathione demonstrated that in the presence of air bronopol acts as a catalyst for the oxidation of thiol groups to disulfides, with the rapid consumption of oxygen. The observation that bronopol was not consumed in this reaction allows an extension of the reaction mechanism proposed by Stretton and Manson (16), according to which a radical anion intermediate is formed from bronopol during the conversion of cysteine to cystine and of glutathione to its disulfide (Fig. 10). NMR spectroscopy is a more convenient and more informative technique for observing such reactions than TLC because it unambiguously shows that bronopol is not destroyed while thiol is consumed. In cell suspensions, such catalysis leads to an alteration in the redox state, oxidation of glutathione to its disulfide, and inhibition of enzyme function and growth. These consequences of catalysis are evidenced by the immediate cessation of growth on bronopol addition to actively growing cultures and the generation of anoxic conditions. The length of the induced bacteriostatic period depended on bronopol concentration and was shortened by the addition of exogenous thiols such as cysteine. This observation suggested that there is a second slower reaction that did not require oxygen and that consumed or neutralized the bronopol within the cell. This neutralization occurred at a rate that depended on the concentration of accessible thiols. Presumably, restoration of cellular function follows bronopol consumption and occurs through the resumption of respiration and the reduction of glutathione disulfide to glutathione via glutathione reductase. Reductions in the rate of growth, relative to those of control cultures, following the induced bacteriostasis probably reflect irreversible damage to the cell caused by bronopol treatment, possibly through the generation of oxygen radicals (6) during the catalytic period (Fig. 10) and from the continued diffusion of oxygen into the suspensions during bacteriostasis. When the slower reactions of bronopol with thiols were examined by maintaining anoxia, a number of reaction products other than the disulfide were, indeed, produced and bronopol was consumed. The production of elemental sulfur in the reaction of bronopol with cysteine methyl ester indicates an oxidative degradation of the cysteine derivative that is comparable with the slow autoxidation of cysteine observed in air (5). In this study, the oxidation of the thiol occurred in the absence of oxygen and presumably resulted in the chemical reduction of bronopol. The reaction was complex, and the observed products in this work should not be taken as reflective of the products formed in vivo under anoxic conditions.

The bactericidal activity of bronopol was expressed at concentrations greatly in excess of the MIC and was suppressed by the absence of oxygen or the presence of catalase or superoxide dismutase. The estimated concentration exponent (0.92) agreed with that reported by Hurwitz and McCarthy (9). The activity of bronopol increased with increasing pH from 5.5 to 8.0. This result is consistent with the published data (17) and suggests that the presence of the thiolate anion contributes to the reactivity of the thiol (10). The enzymes superoxide dismutase and catalase scavenge

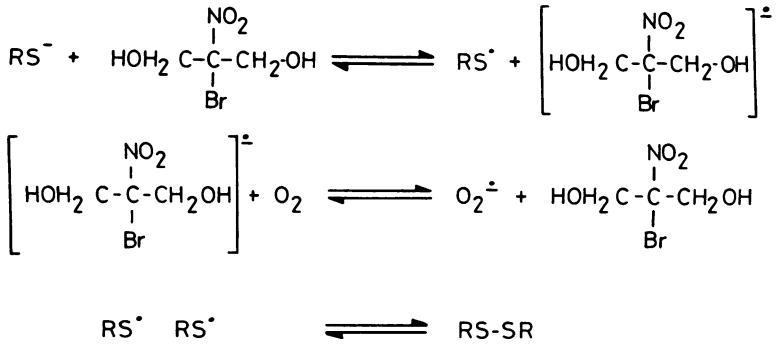

FIG. 10. Formation of radical anion intermediate from bronopol.

peroxide and superoxide from suspension supernatants (8). The protection against the bactericidal activity of bronopol afforded by these enzymes suggests that the activity stems from the aerobic interaction and the generation of active oxygen species from oxygen diffusing into the suspensions during bronopol treatment. Such reactions would act as a continuous source of superoxide for as long as bronopol remained. The generation of superoxide by the aerobic reaction of bronopol with thiols was demonstrated by the reduction of cytochrome *c* by superoxide (Fig. 9) (2, 7).

The results therefore suggest a dual action of bronopol, with catalytic oxidation of accessible thiols being responsible for the growth inhibition and generation of free radicals causing cell death. The extent of killing reflects the number of free radicals generated from oxygen diffusing into the suspension during the biocide-induced bacteriostasis and therefore relates to bronopol concentration and the length of the bacteriostatic period.

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