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Binding and Killing of Bacteria by Bismuth Subsalicylate

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Bismuth subsalicylate (BSS) is a compound without significant aqueous solubility that is widely used for the treatment of gastrointestinal disorders. BSS was able to bind bacteria of diverse species, and these bound bacteria were subsequently killed. A 4-$\log_{10}$ reduction of viable bacteria occurred within 4 h after a 10 mM aqueous suspension of BSS was inoculated with 2×10^6 *Escherichia coli* cells per ml. Binding and killing were dependent on the levels of inoculated bacteria, and significant binding but little killing of the exposed bacteria occurred at an inoculum level of 2×10^9 *E. coli* per ml. Intracellular ATP decreased rapidly after exposure of *E. coli* to 10 mM BSS and, after 30 min, was only 1% of the original level. Extracellular ATP increased after exposure to BSS, but the accumulation of extracellular ATP was not sufficient to account for the loss of intracellular ATP. The killing of bacteria exposed to BSS may have been due to cessation of ATP synthesis or a loss of membrane integrity. Bactericidal activity of BSS was also investigated in a simulated gastric juice at pH 3. Killing of *E. coli* at this pH was much more rapid than at pH 7 and was apparently due to salicylate released by the conversion of BSS to bismuth oxychloride. It is proposed that the binding and killing observed for BSS contribute to the efficacy of this compound against gastrointestinal infections such as traveler's diarrhea.

Insoluble salts of bismuth have been extensively used as antimicrobial agents. A product containing bismuth subsalicylate (BSS) as the active ingredient has been shown to be clinically effective in the prevention and treatment of a number of gastrointestinal infections, including traveler's diarrhea (3, 4, 7) and *Campylobacter pylori* gastritis (16). Despite a long history of therapeutic use of BSS and other bismuth compounds, relatively little is known about their antimicrobial mode of action. The bacteriostatic (14–16) and bactericidal (7, 10) activities of these compounds have been reported previously. Also, electron microscopy of *C. pylori* present in gastric biopsies taken after oral administration of bismuth compounds indicated both intracellular and extracellular association of bismuth with *C. pylori* (14, 19). Concomitantly, bacteria lost adherence to the gastric epithelium, and structural degradation of the bacteria was evident. Although the antimicrobial mode of action of bismuth compounds has received relatively little attention, the actions of other metals have been examined in more depth. Mercury exerts antimicrobial activity as a result of covalent attachment to sulfhydryl groups on essential macromolecules (5). Several other metals such as uranium (20) and nickel (1) are taken up in large quantities by microorganisms; these metals do not become covalently bound to the cells, and viable cells are not needed for this uptake to occur (20). *Micrococcus luteus* and an *Azotobacter* sp. were reported to bind large amounts of lead (22); this uptake had no effect on growth rate or viability. In addition to containing a metal with antimicrobial properties, BSS also contains salicylate, an effective antimicrobial agent under acidic pH conditions (9).

Our work has focused on determining the events that occur during the killing of bacteria by BSS. This work demonstrates the bactericidal activity of BSS at both neutral and acidic pHs.

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MATERIALS AND METHODS

BSS. BSS, in the form of a 10% aqueous slurry, was manufactured by Procter and Gamble Co., Cincinnati, Ohio. BSS was present as needlelike crystals of about 2 by 7 μm.

Bacterial strains. Strain H10407, an enterotoxigenic *Escherichia coli* strain, was obtained from R. Leunk (Procter and Gamble). All other strains were obtained from the American Type Culture Collection, and strain designations are provided in the text.

Preparation of simulated gastric juice. A simulated gastric juice was prepared to mimic the composition of human gastric juice (11). This simulated gastric juice contained 0.1% pepsin (Sigma Chemical Co., St. Louis, Mo.), 0.1% porcine gastric mucin (Sigma), 20.5 mM NaCl, and 2.7 mM KCl. Concentrated hydrochloric acid was then added to a final level of 0.74%, and the pH was adjusted to the desired value with aqueous sodium hydroxide. Gastric juice was made fresh daily because preliminary experiments indicated that it developed bactericidal activity when allowed to age for several days.

Neutralization of antimicrobial activity. Preliminary experiments indicated that dilution of inoculated BSS suspensions into D/E neutralizing broth (Difco Laboratories, Detroit, Mich.) neutralized the bactericidal activity of BSS. Consequently, samples were diluted into D/E broth to halt bactericidal activity.

Determination of binding and killing. Bacterial cultures were grown overnight at 37°C with shaking in tryptic soy broth (Difco). Broth cultures were diluted in water, and A_{420} values were measured. Previously developed calibration data were used to adjust absorbances of these suspensions so that they contained the desired number of bacteria for the binding and killing assay. BSS was added to water or

simulated gastric juice and mixed thoroughly. The pH of the resulting suspension was adjusted as necessary, and the suspension was warmed to 37°C prior to addition of bacteria. At indicated times, the solutions were mixed thoroughly before removal of samples that were then diluted 1:10 in D/E broth. These samples were used to determine the total number of viable organisms present in the suspension. An additional sample was removed and centrifuged at 190 × g for 5 min (1,000 rpm in a TH-4 swinging-bucket rotor, TJ-6 centrifuge; Beckman Instruments, Inc., Palo Alto, Calif.). Preliminary experiments (data not shown) indicated that this centrifugation did not pellet bacteria free in suspension, but these conditions were sufficient to pellet BSS. Bacteria remaining in the supernatant were considered unbound, and those present in the pellet were considered bound to BSS. Immediately after centrifugation, the supernatant was diluted 1:10 in D/E broth. The pellet was also suspended with D/E broth. Samples in D/E broth were inoculated onto 150-mm-diameter plates of tryptic soy agar (Difco) with a model C spiral plater (Spiral Systems Instruments Inc., Bethesda, Md.) set to deliver 40 μl of sample. After overnight incubation at 37°C, plates were scanned with a Spiral Systems laser colony counter, and Spiral Systems CASBA bacterial enumeration software, version 1.2, was used to determine the number of viable bacteria in the original samples.

C. pylori ATCC 43504 was tested in the above procedure with the following exceptions. *C. pylori* was inoculated on Campy chocolatized agar (Remel Laboratories, Lenexa, Kan.) and incubated in a microaerophilic atmosphere (Campylobacter microaerophilic system; BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 5 days. Cells were suspended in water, and absorbances were adjusted as described above. Polysorbate 80 was found to be inhibitory to *C. pylori*, and *C. pylori* samples were diluted in D/E broth lacking Polysorbate 80 and lecithin. Samples were spiral plated onto brucella agar containing 3% laked sheep blood and 4% sheep serum and incubated in a microaerophilic atmosphere at 37°C for 7 days. The procedures described above were used for quantitation of *C. pylori* colonies on these plates.

Effects of albumin on binding. BSS (10 mM) was suspended in a 10-mg/ml solution of bovine serum albumin (Sigma). The suspension was mixed and then heated to 37°C in a water bath. Bacteria were added, and samples were assayed for binding and killing as described above.

Solubilization of BSS. In some experiments, bacteria bound to BSS were released by solubilizing the BSS with sodium tartrate (biotech grade; Fisher Scientific Co., Pittsburgh, Pa.). A volume of filter-sterilized 1 M sodium tartrate, pH 7, was added to an equal volume of 10 mM BSS suspension. This resulted in the solubilization of the BSS in approximately 1 min at 37°C.

Measurement of ATP. Intracellular and extracellular ATP levels were measured to determine the effects of BSS on cell physiology and integrity (17, 21). Samples of bacteria exposed to BSS were treated with sodium tartrate as described above to solubilize the BSS. A 50-μl sample was then mixed with 50 μl of 20 mM Tris hydrochloride (pH 7.7) and 100 μl of Microlyse (LKB-Wallac Instruments, Helsinki, Finland). A 100-μl sample of lysate was added to 100 μl of ATP assay mix (Sigma), and light production was quantitated with a photometer (SAI Instruments, San Diego, Calif.). ATP standard addition experiments were performed to determine the degree of quenching caused by sodium tartrate and tartrate-solubilized BSS. Results were corrected for quenching to determine the actual amounts of ATP present in the samples. Extracellular ATP was determined by filtering the tartrate-solubilized cell suspension through a 0.2-μm Dynagard filter (Microgon, Inc., Laguna Hills, Calif.) before the assay. Intracellular ATP was calculated by subtracting the extracellular ATP from the total ATP found in the sample.

Salicylate determination. Soluble salicylate levels were determined by passing samples through 0.2-μm Dynagard filters and assaying the filtrates with a salicylate diagnostic kit (Sigma).

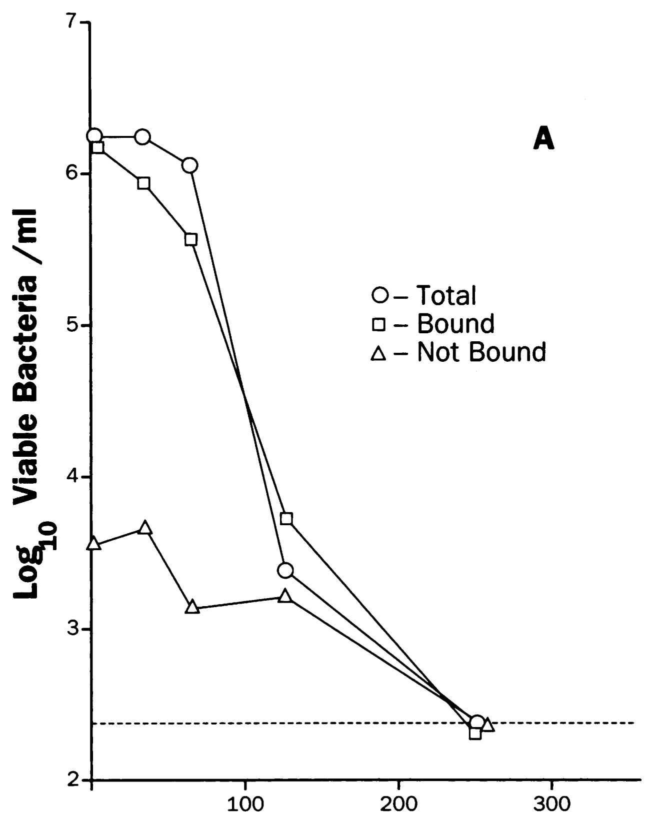
RESULTS

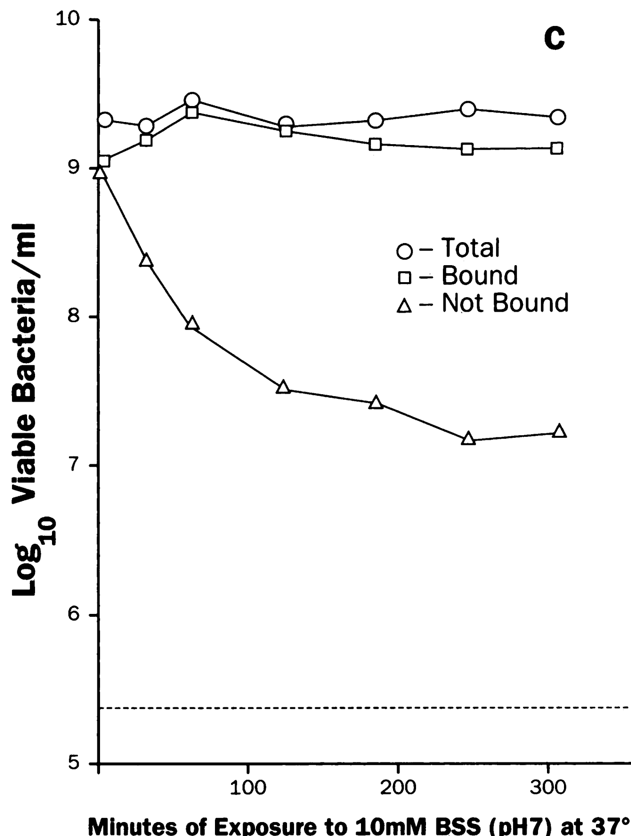
***E. coli* binding to BSS.** *E. coli* ATCC 10536 attached rapidly to BSS suspended in water (pH 7). At inoculum levels of 10^6 and 5×10^7 CFU/ml, binding was very rapid and was essentially complete before the first set of data was collected (Fig. 1A and B). At a greater inoculum level, most binding occurred within 30 min, but the number of unbound bacteria continued to decrease for an additional 90 min (Fig. 1C). At some of the time points, the sum of viable bound and unbound bacteria was slightly less than the total viable population observed. Some killing of bound bacteria by BSS continued during the centrifugation procedure prior to the addition of D/E broth, and this cell death during the centrifugation procedure accounted for the observed lack of additivity.

The rapid binding of bacteria was followed by a slower loss of viability of the bound cells. The extent of killing was highly dependent on the inoculum level, as demonstrated by the different amounts of killing observed for the three inoculum levels shown in Fig. 1. At high inoculum levels (2×10^9 CFU/ml), there was no detectable killing during the first 5 h of exposure despite most of the bacteria being bound to BSS. However, some killing of the exposed cells did occur with longer exposures. Exposure of 2.2×10^9 CFU/ml to BSS for 24 h resulted in a 90% loss in viability, compared with a 13% loss of viability in control bacteria exposed only to water (data not shown). In contrast, at an inoculum level of approximately 10^6 CFU/ml, a >4-$\log_{10}$ reduction in viable bacteria occurred within 4 h (Fig. 1A).

Since bacteria bound to BSS particles, it was possible that the bacterial colonies obtained in this work originated from BSS particles with one or more bacteria attached, rather than having developed from individual bacterial cells. This would have resulted in erroneously low values for the number of viable bacteria present in samples exposed to BSS. An alternative experimental approach was to add samples of suspensions of bacteria in BSS to 1 M sodium tartrate. Sodium tartrate solubilized the BSS, with concomitant release of the bound bacteria. Preliminary studies indicated that the resulting soluble bismuth solution did not have bactericidal activity for *E. coli*. Plating of these tartrate-solubilized samples yielded recoveries of viable bacteria very similar to those obtained in D/E broth (Table 1). Therefore, dilution of BSS-containing samples into D/E broth yielded accurate values for viable bacteria.

We examined the ability of D/E broth and its antimicrobial neutralizing components to release bacteria bound to BSS (Table 1). Addition of *E. coli* to 10 mM BSS in water resulted in only 0.1 to 1.0% of the bacteria remaining in suspension after low-speed centrifugation (second column). D/E broth, D/E broth components, or water (third column) was used to suspend the pellets of BSS and bacteria (fourth column). These suspensions were centrifuged again to determine the





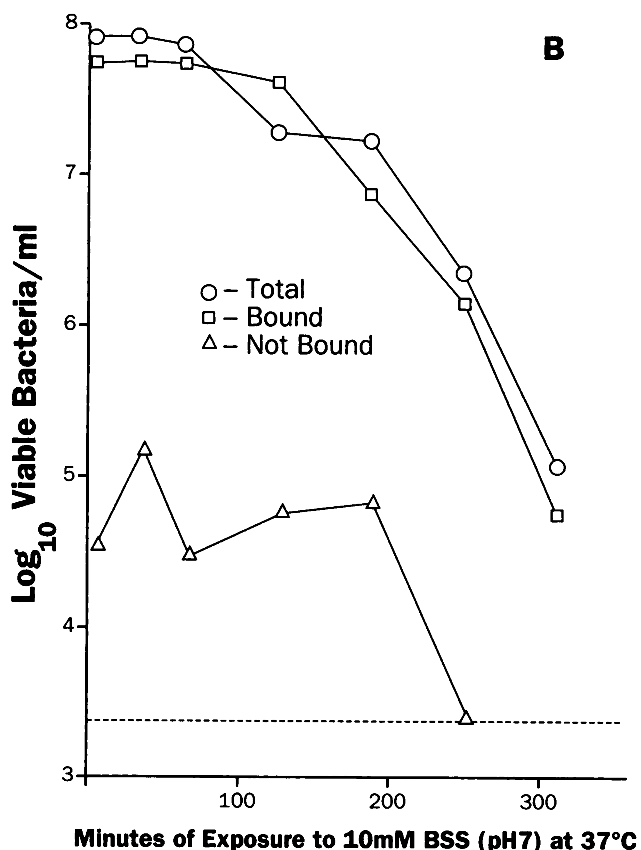


FIG. 1. Effects of inoculum level on binding and killing of *E. coli* ATCC 10536 by a 10 mM aqueous suspension of BSS. Limit of detection of procedure is indicated by the dashed line. Inoculum levels were 1.73×10^6 (A), 8.35×10^7 (B), and 2.17×10^9 (C) CFU/ml.

ability of the various components to release bound bacteria. The percentages of bacteria that remained in suspension after centrifugation, indicating release from BSS, are shown in parentheses in the fifth column. Exposure to D/E broth released about 44% of the bound bacteria. The combination of lecithin and Polysorbate 80 was largely responsible for release of the bound bacteria; these two components released about 38% of the bound bacteria. Lecithin alone could not be tested because of its immiscibility in water in the absence of an emulsifying agent. The nutrient components of D/E broth (tryptone and yeast extract) also contributed to the release. A solution of these materials at the concentrations found in D/E broth resulted in 32% release of the bound bacteria.

The effects of pretreatment of BSS with 10 mg of bovine serum albumin per ml on binding and killing of *E. coli* were examined (Fig. 2). Albumin pretreatment greatly decreased the extent of binding of bacteria and increased the length of time needed for killing of the exposed bacteria. However, exposure to albumin-pretreated BSS eventually resulted in killing of all of the exposed bacteria.

Binding of other bacteria to BSS. An enterotoxigenic strain of *E. coli* (H10407) showed binding and killing similar to that observed for *E. coli* ATCC 10536 (data not shown). Binding of *Salmonella typhimurium* and *Staphylococcus aureus* to BSS and their subsequent killing were also examined (Fig. 3). These strains showed binding to BSS similar to that

TABLE 1. Effects of D/E broth and components on release of bacteria bound to BSS[a]

Initial bacterial level (log_{10}/ml)	Bacteria in supernatant (log_{10}/ml)	Resuspending medium	log_{10}/ml (%)[b]		
			Bacteria in pellet	Bacteria in supernatant after second centrifugation	Bacteria in pellet after second centrifugation
6.51	4.60	1.0 M sodium tartrate	6.05	6.02	—[c]
6.50	3.73	D/E broth	6.09	5.82 (43.7)	5.93
6.44	3.60	0.5% Polysorbate 80	5.93	1.72 (<0.1)	6.00
6.50	3.70	37.9 mM sodium thiosulfate	6.14	3.54 (0.3)	6.07
6.48	3.65	8.8 mM sodium thioglycolate	6.01	4.84 (6.7)	5.98
6.50	3.61	0.7% Lecithin, 0.5% Polysorbate 80	6.07	5.84 (38.4)	6.06
6.48	3.40	Water	5.85	2.02 (<0.1)	5.45
6.46	3.42	D/E broth without neutralizers	6.02	5.58 (31.7)	5.92

[a] Bacteria (*E. coli* ATCC 10536) were added to 10 mM BSS suspended in water, pH 7. Samples were removed immediately and diluted in D/E broth to determine initial levels of viable bacteria. The remaining suspensions were centrifuged. The supernatants were removed and assayed for viable bacteria. The pellets were suspended to the original sample volume with the indicated media, and these were assayed for viable bacteria. The resuspended pellets were centrifuged again to determine the extent of release of bound bacteria. Supernatants and pellets (after resuspension in D/E broth) were assayed to determine the number of bacteria released during treatment.

[b] Percentages of total recovered bacteria found in the supernatant.

[c] —, 1.0 M sodium tartrate solubilized BSS; no material was present in pellet after centrifugation.

described above for *E. coli*. *Salmonella typhimurium* was killed more rapidly than *E. coli*; the increased sensitivity of *Salmonella typhimurium* was consistently observed in multiple experiments. Relatively little killing of *Staphylococcus aureus* occurred despite substantial binding to BSS. *C. pylori* ATCC 43504 was extremely sensitive to BSS, and the proportions of bound and unbound organisms could not be determined due to the rapid killing. The survival of equal quantities of *C. pylori* added to a BSS suspension or a water control is presented in Fig. 4. No viable organisms were detected 15 min after exposure of 2.3×10^6 CFU/ml to BSS.

BSS effects on ATP levels. ATP levels were determined in *E. coli* ATCC 10536 exposed to 10 mM BSS in water (pH 7) (Fig. 5). Total ATP levels decreased to about 10% of the initial levels after 30 min. Examination of extracellular ATP levels over time indicated a rapid increase in extracellular ATP after exposure to BSS, followed by a gradual decrease in extracellular ATP. After 30 min of exposure, most of the remaining ATP was extracellular. Other experiments (data not shown) indicated that exogenous ATP was fairly stable under these conditions, with 49% of the added ATP remaining after 3 h. The rapid decrease in total ATP after exposure to BSS was not due to instability of extracellular ATP.

Antimicrobial activity of BSS in simulated gastric juice. Addition of *E. coli* ATCC 10536 to 10 mM BSS suspended in simulated gastric juice (pH 3) resulted in much more rapid killing than was observed for BSS in water at pH 7 (Fig. 6). Since essentially all of the added bacteria were killed within a few minutes after addition to gastric juice containing BSS, it was not possible to assess the proportions of bound and unbound bacteria with the binding assay described above. Control experiments indicated that freshly prepared gastric juice at pH 3 had slow bactericidal activity against *E. coli*, and after 2 h at 37°C 57% of the added bacteria remained viable.

We examined the release of salicylate from a 10 mM BSS suspension in simulated gastric juice (pH 3) that was inoculated with 10^6 CFU of *E. coli* ATCC 10536 per ml (Fig. 7). Salicylate release was rapid and continued for at least 1 h. A concentration of 1.09 mM salicylate was present after 5 min of exposure; approximately 90% of the exposed bacteria were dead at this time. A subsequent experiment was performed in which bacteria were exposed to 1.09 mM salicylate in gastric juice to determine whether the levels of salicylate released in gastric juice could account for the rapid

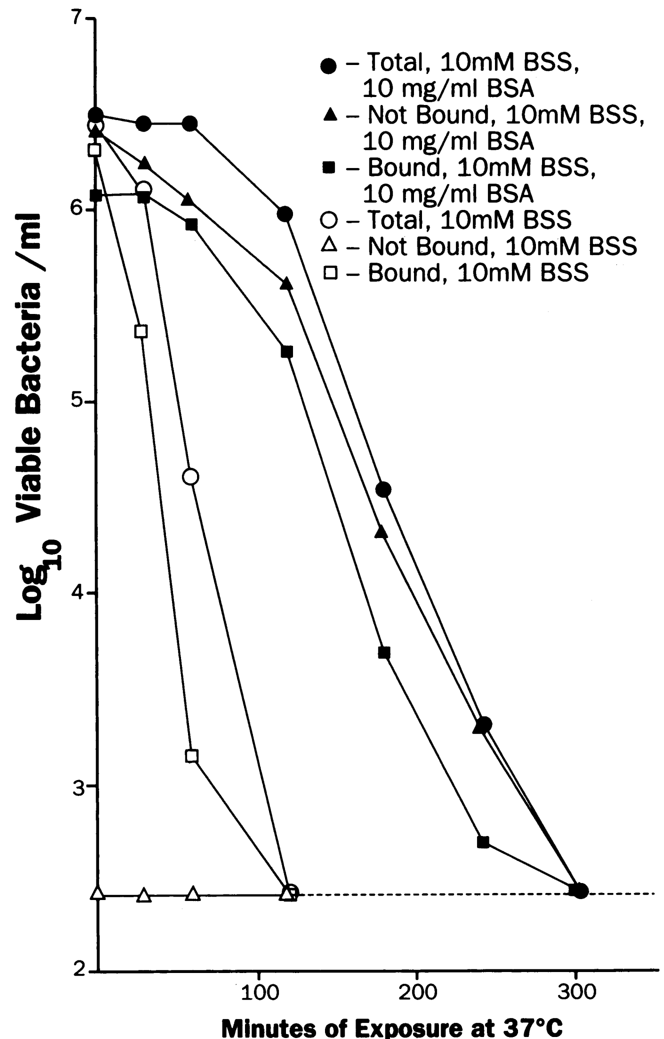


FIG. 2. Effects of pretreatment of 10 mM BSS with 10 mg of bovine serum albumin (BSA) per ml on binding and killing of *E. coli* ATCC 10536 by BSS.

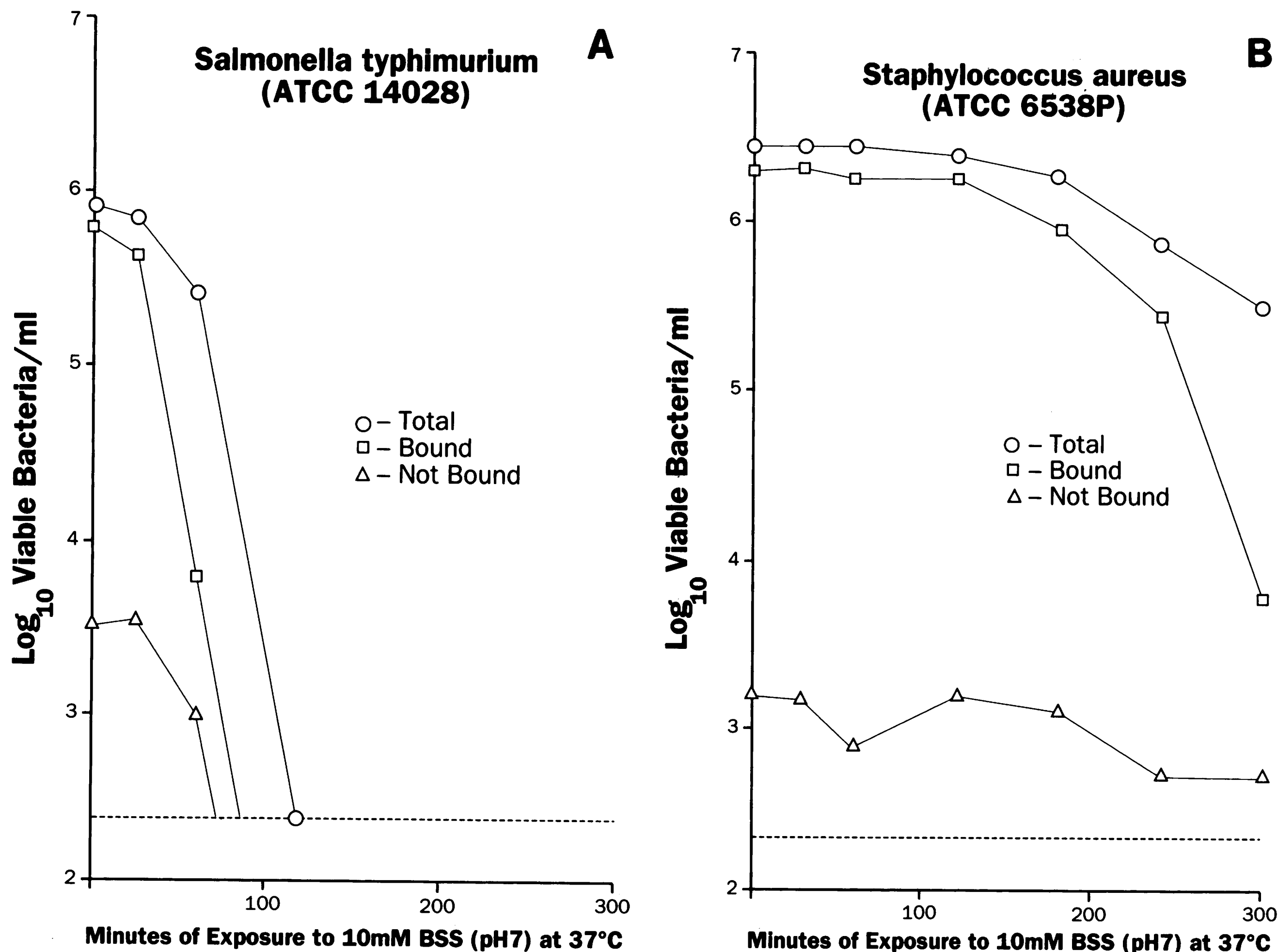


FIG. 3. Binding and killing of *Salmonella typhimurium* and *Staphylococcus aureus* by a 10 mM aqueous suspension of BSS.

killing observed (Fig. 8). The killing by salicylate alone at pH 3 was similar to that observed for bacteria exposed to 10 mM BSS in gastric juice at pH 3. Controls of 1.09 mM salicylate in water (pH 7) or gastric juice (pH 7) did not show any bactericidal activity.

DISCUSSION

The antimicrobial mode of action of BSS has received little attention. The effects of BSS we have described, including its ability to bind several species of bacteria and the subsequent killing of the bound organisms, may contribute to the observed in vivo efficacy of this compound. Our work utilized bacteria in stationary growth phase, and we did not examine the responses of bacteria in other growth phases. Extensive literature exists on the binding of microorganisms to other surfaces. Binding may occur as a result of binding to specific sites (lectinlike interactions) or nonspecific processes such as electrostatic and hydrophobic interactions, hydrogen bonds, and van der Waals interactions (12). The range of organisms observed to bind to BSS in this work suggests that nonspecific binding is occurring. Pretreatment of BSS with a protein (albumin) decreased the rate of binding and killing, but complete killing of the exposed *E. coli* was still observed. This suggests that albumin and bacteria compete to some extent for the same binding sites, and albumin may sterically inhibit binding of bacteria (13). Polysorbate 80 is reported (18) to be effective in desorbing bacteria attached to glass surfaces by hydrophobic interactions. Polysorbate 80 was ineffective in releasing *E. coli* bound to BSS, suggesting that hydrophobic interactions were not responsible for binding. It is unclear whether binding is essential for bactericidal activity of BSS. In the presence of 0.1% albumin (Fig. 2), all of the exposed bacteria were killed despite the incomplete binding observed. In the absence of albumin, more extensive binding occurred and the rate of killing was much greater. Hence, it appears that bound bacteria are killed more rapidly than unbound bacteria.

Although binding of microorganisms to surfaces has been extensively investigated, the rapid killing of bound bacteria we observed has not been reported previously. This may be a unique instance of bacteria binding to the surface of an antimicrobial but insoluble material. The killing of bacteria we observed at neutral pH is not due to the salicylate moiety, since binding and killing have also been observed with other insoluble bismuth compounds that do not have antimicrobial counterions (unpublished results). Also, salicylate lacked detectable bactericidal activity at pH 7. Bismuth therefore appears to be responsible for the bactericidal activity against the bound organisms.

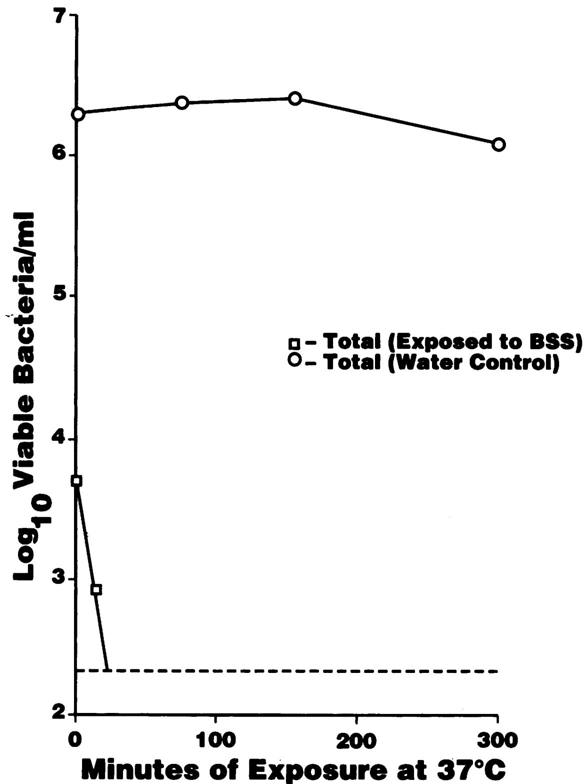


FIG. 4. Viability of *C. pylori* ATCC 43504 exposed to 10 mM BSS or water.

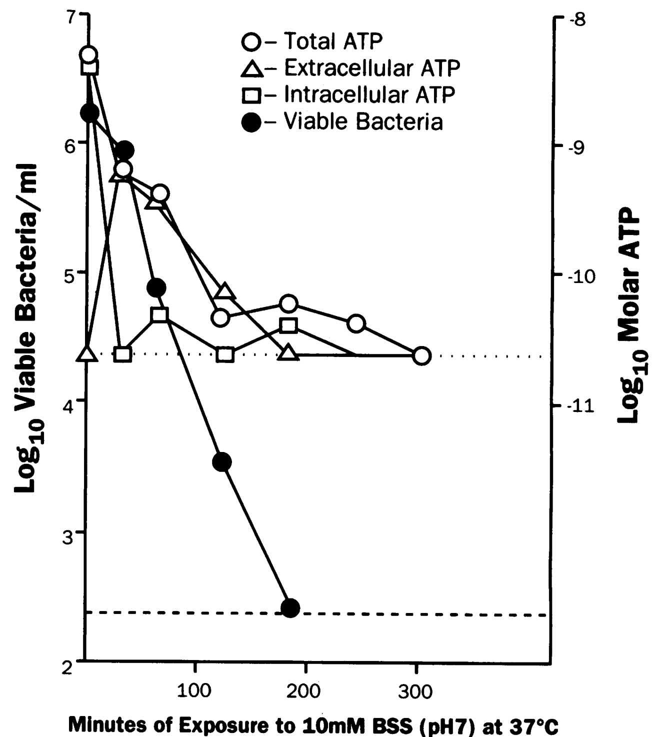


FIG. 5. Changes in ATP concentrations after exposure of *E. coli* ATCC 10536 to 10 mM BSS. Symbols: ·····, limit of detection of ATP assay; ----, limit of detection of viability assay.

Intracellular ATP levels in *E. coli* decreased very rapidly after exposure to BSS. Part of this loss of intracellular ATP resulted from leakage from the bacteria, as evidenced by a 40-fold increase in extracellular ATP during the first 30 min of exposure to BSS. However, the extracellular ATP present at 30 min represented only 13% of the ATP initially present in the cells, indicating that most of the decline in ATP was due to depletion of intracellular ATP reserves. Declines in total ATP have been reported after exposure to antibiotics such as doxycycline (8). Also, accumulation of extracellular ATP has been observed after exposure of *E. coli* to amikicin (17), and extracellular ATP increased in response to increasing levels of antibiotic. These ATP results suggest that exposure to BSS results in loss of membrane integrity, and the cellular processes for ATP synthesis, possibly including the proton gradient, are rapidly inactivated.

In addition to the bactericidal activity observed for BSS at neutral pH, this compound had an extremely rapid bactericidal activity when added to simulated gastric juice at pH 3. This rapid killing was due to the salicylate released by the conversion of BSS to bismuth oxychloride (7). Salicylate is antimicrobial in the protonated form (9), and the rapid killing produced by BSS at pH 3 may be largely due to the released salicylate. The antimicrobial mode of action of protonated salicylate is not known.

The antimicrobial properties of BSS described in this report may be important to its in vivo efficacy. BSS in the gastrointestinal tract may bind ingested bacteria, and these organisms subsequently may be killed. Also, bound organisms may be unable to attach to specific receptor sites in the gastrointestinal tract, a step apparently essential to the establishment of colonization and infection by many pathogens.

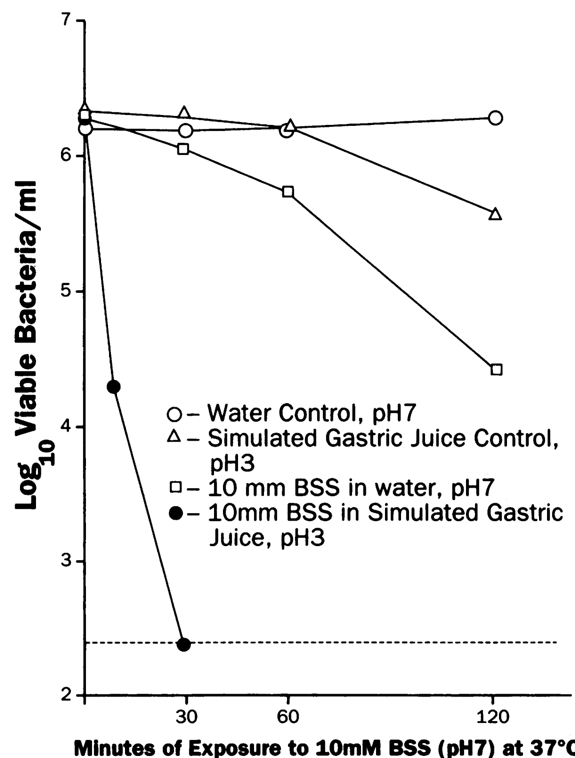


FIG. 6. Comparison of killing of *E. coli* ATCC 10536 by 10 mM BSS in water (pH 7) and simulated gastric juice (pH 3).

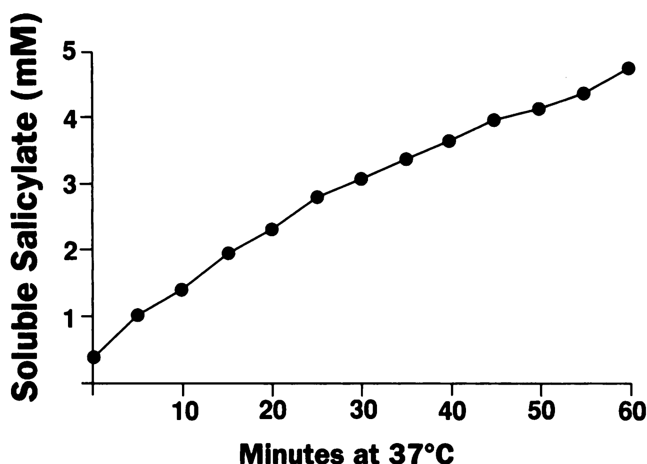


FIG. 7. Release of salicylate from 10 mM BSS suspended in simulated gastric juice (pH 3).

The observed properties of BSS indicate that it should be effective in vivo over a wide pH range, and this compound may have different antimicrobial modes of action at different pHs. At neutral or slightly acidic pH, the binding of bacteria by BSS and the subsequent killing of these organisms may be important. At the highly acidic pH characteristic of the resting stomach, the release of salicylate from BSS results in an extremely rapid bactericidal activity. BSS undergoes hydrolysis in the gastrointestinal tract, and the numerous hydrolysis products may contribute to the therapeutic efficacy of this compound (2).

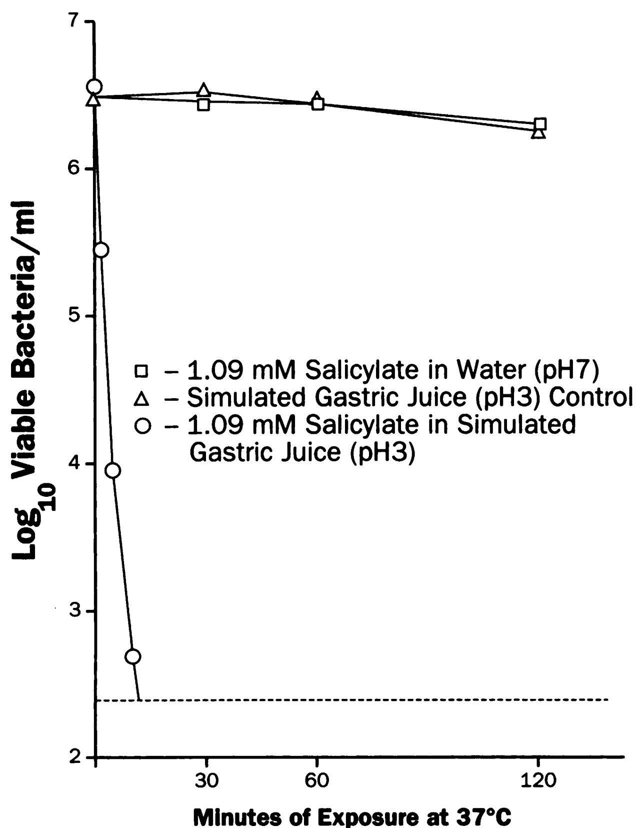


FIG. 8. Comparison of bactericidal activity of salicylate against *E. coli* ATCC 10536 in water and simulated gastric juice.

This work demonstrates that BSS is bactericidal in vitro against a range of organisms. Many previous investigators have used procedures such as agar diffusion or broth turbidity to assess the antimicrobial spectrum of BSS. Although these procedures are widely accepted for antibiotics, they may not be appropriate for an insoluble particulate antimicrobial agent such as BSS. This work is a step toward understanding the antimicrobial activity of a compound that has been used therapeutically for many decades.

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