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Reduction of capsular polysaccharide and potentiation of aminoglycoside inhibition in Gram-negative bacteria by bismuth subsalicylate

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Bismuth subsalicylate (BSS), sodium salicylate, and bismuth nitrate were compared with respect to their effects on capsular polysaccharide (CPS) production, bacterial growth inhibition, and potentiation of aminoglycoside inhibition on strains of Gram-negative bacteria. At 250 μM, BSS reduced CPS production in *Klebsiella pneumoniae* cultures by >90% in contrast to a 36% reduction by salicylate. At 500 μM, salicylate reduced CPS by 52%, versus a 70% reduction by bismuth nitrate. Substantial reduction of CPS production by BSS occurred before bacterial growth inhibition was observed. However, BSS at 250 μM decreased cell viability by 21%, and at 1 mM by 50%. Bismuth nitrate was equally inhibitory to cell growth. Salicylate at 1 mM did not affect bacterial cell counts. The susceptibility of selected Gram-negative bacteria to aminoglycoside antibiotics was studied in the presence of BSS or salicylate. Generally, salicylate at 2·5 mM reduced the concentration of aminoglycoside required to inhibit culture growth for 24 h (IC_{24}) by two-fold. In contrast, 700 μM BSS reduced the IC_{24} for amikacin four-fold for a resistant *K. pneumoniae* strain. At 500 μM, BSS reduced the IC_{24} of gentamicin seven-fold for *Salmonella typhimurium*. Inhibitory concentrations of amikacin or tobramycin for *Enterobacter cloacae* or *Serratia marcescens* were also reduced seven-fold with 500 μM BSS. Bismuth nitrate reduced the IC_{24} of tobramycin by four-fold for *E. cloacae*. Thus, the profound effects of BSS on CPS production and aminoglycoside potentiation were due to the additive effects of bismuth and salicylate ions, whilst its effects on growth inhibition were due to the bismuth ion.

Introduction

Recent reports have described several in-vitro effects of salicylate on bacteria, including the reduction of surface polysaccharides (Domenico, Schwartz & Cunha, 1989*b*), potentiation of aminoglycoside antibiotic activity (Aumercier *et al.*, 1990; Domenico *et al.*, 1990*b*), and antagonism of β-lactam antibiotic activity (Domenico, Hopkins & Cunha, 1990*a*; Foulds *et al.*, 1989). These clinically important effects may result from a range of actions on bacteria induced by salicylate, including chelation of cations required by polysaccharide synthetic enzymes (Sutherland, 1977; Domenico *et al.*, 1989*b*), uncoupling of oxidative phosphorylation (Brody, 1956; Haas *et al.*, 1985), intercalation into biological membranes (McLaughlin, 1973), and repression of outer membrane porin expression (Sawai *et al.*, 1987).

Reduction of capsular polysaccharide (CPS) and potentiation of aminglycoside antibiotics could improve the therapy of infections caused by encapsulated bacteria.

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The primary virulence factor of *Klebsiella pneumoniae* is the antiphagocytic polysaccharide capsule (Ehrenworth & Baer, 1956; Domenico, Johanson & Straus, 1982; Domenico & Straus, 1985; Simoons-Smith, Verweij-van Vught & MacLaren, 1986; Williams *et al.*, 1986). Salicylate, within therapeutic levels (0·5–2·5 mM), has been shown to reduce *K. pneumoniae* CPS expression *in vitro* by 50–75% (Domenico *et al.*, 1989*b*). CPS reduction may affect the outcome of therapy, since the degree of CPS production has been correlated to the degree of virulence (Ehrenworth & Baer, 1956; Domenico & Straus, 1985). Salicylate has also been shown to exhibit synergy with aminoglycoside antibiotics against several other Gram-negative bacteria. At high therapeutic levels (1·5–2·5 mM), salicylate has been shown to decrease the minimum inhibitory concentration (MIC) of aminoglycosides by two- to four-fold (Aumercier *et al.*, 1990; Domenico *et al.*, 1990*b*). At similar concentrations, salicylate reduced the effectiveness of β-lactam antibiotics against Gram-negative bacteria two- to four-fold (Domenico *et al.*, 1990*a*). The effectiveness of several other classes of antibiotic has been demonstrated to be hindered by salicylate (Rosner, 1985; Sawai *et al.*, 1987; Foulds *et al.*, 1989; Domenico *et al.*, 1990*a*).

Bismuth subsalicylate (BSS), the active ingredient in Pepto Bismol, is a highly insoluble salt of trivalent bismuth and salicylic acid. Therefore, it should share some of the biological effects imparted by sodium salicylate preparations. In this report, the contribution of bismuth to CPS reduction and aminoglycoside potentiation is presented.

Materials and methods

Bacterial strains and media

K. pneumoniae strain 52145, serotype O1 : K2 (Nassif *et al.*, 1989) was used for the studies of polysaccharide production. Quantitatively defined salts broth medium, with limiting nitrogen and excess glucose (Domenico *et al.*, 1989*b*) was employed for CPS production studies. Antimicrobial assays were performed in Mueller–Hinton II broth. *K. pneumoniae* KPCL4, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 8100, and *Enterobacter cloacae* ATCC 23355 were used in the aminoglycoside synergy studies. Strain KPCL4 produced the aminoglycoside-modifying enzymes AAC(6′)-IV and APH(3′)-III (Domenico *et al.*, 1990*b*). Starter cultures, grown to mid-log phase in a rotary incubator at 37°C for 4 h at 200 rpm, were used to prepare suspensions matched to a 0·5 MacFarland standard, which were further diluted 1 : 100 (approximately 5×10^5 cfu/mL) into pre-warmed medium. All cultures were incubated in an Avantage Analysis Module (Abbott Laboratories, Irving, Texas, USA) at 34·5°C.

Biochemical reagents

Stock solutions of 100 mM sodium salicylate (Sigma Chemical Co., St. Louis Missouri, USA), 100 mM bismuth subsalicylate (BSS; Proctor & Gamble, Cincinatti, Ohio, USA), and 100 mM bismuth nitrate (Sigma) were prepared fresh daily in propylene glycol containing 400 mM NaOH, pH 12·0. All solutions were kept at 4°C. Antibiotics used were sulphate-salt solutions of amikacin (Bristol Laboratories, Syracuse, New York, USA), gentamicin (Elkins-Sinn, Cherry Hill, New Jersey, USA), and tobramycin (Eli Lilly, Puerto Rico).

Extraction and quantitation of polysaccharides

Methods for CPS determination have been described previously (Domenico, Diedrich & Cunha, 1989*a*). Briefly, cultures grown for 18 h were mixed with a zwitterionic detergent in citric acid and heated gently to solubilize extracellular polysaccharides. Since glucuronic acid is an unique component of the serotype 2 capsule, the CPS content of extracts was assessed by measuring uronic acid (Blumenkrantz & Asboe-Hansen, 1973). Quantities of CPS were expressed as nanograms of uronic acid per 10^6 cfu (ng UA/10^6 cfu). Nutrient agar plates were used for enumerating viable bacteria from broth cultures. Detergent-extracted CPS was also loaded on a Sepharose 6B (Pharmacia, Uppsala, Sweden) gel filtration column (1·5 × 28 cm) equilbrated with 0·05% Zwittergent 3–14 (CalBiochem, San Diego, California, USA) in 10 mM citrate, pH 4·0, and 20 mM NaCl. Three millilitre fractions were collected and tested for uronic acid.

Antimicrobial assays

Combinations of salicylate solutions and antimicrobials were tested in broth medium for their ability to inhibit bacterial growth. Cultures were monitored for growth in the Avantage Microbiology Center. Inhibition was determined by monitoring lag times before initiation of culture growth (Domenico *et al.*, 1990*a*, *b*). Culture lag times were obtained from computer-generated growth curves. The 24 h inhibitory concentration (IC_{24}) was defined as the concentration of antibiotic necessary to produce a culture lag time of 24 h (1440 ± 60 min). The IC_{24} was expressed as the average of three antibiotic concentrations (mg/L) that produced precise 24 h lag times.

Results

Reduction in CPS production

The CPS-reducing potential of sodium salicylate for *K. pneumoniae* has been established (Domenico *et al.*, 1989*b*). In the present study, BSS proved to be a more potent CPS-reducing agent than salicylate, and this effect was seen at much lower concentrations. The effects of these compounds and of bismuth nitrate on CPS production are shown in Figure 1. When *K. pneumoniae* was exposed in culture to therapeutic levels (500 μM) of sodium salicylate, CPS production per cell was reduced by approximately 50% with no evidence of growth inhibition. Only a slightly greater reduction of CPS was achieved with higher levels of salicylate. In contrast, close to a 90% reduction of CPS/cell was effected by 250 μM BSS. At 1 mM BSS, CPS production was virtually absent in these cultures. $Bi(NO_3)_3$ produced nearly a 70% reduction of CPS at a concentration of 500 μM.

Addition of BSS or $Bi(NO_3)_3$ to cultures of *K. pneumoniae* inhibited growth substantially, as illustrated in Figure 2. When compared to untreated cultures, $Bi(NO_3)_3$ at 100 μM produced a substantial decrease in cfu/mL after 18 h of incubation. Although $Bi(NO_3)_3$ was consistently more detrimental to cell growth than was BSS, the high statistical variation in plating measurements obscured any significant differences. At 250 μM, BSS decreased the viable cell number from 1·4 to 1·1 × 10^9 cfu/mL. At higher concentrations of either bismuth salt, viable cell numbers decreased gradually to near

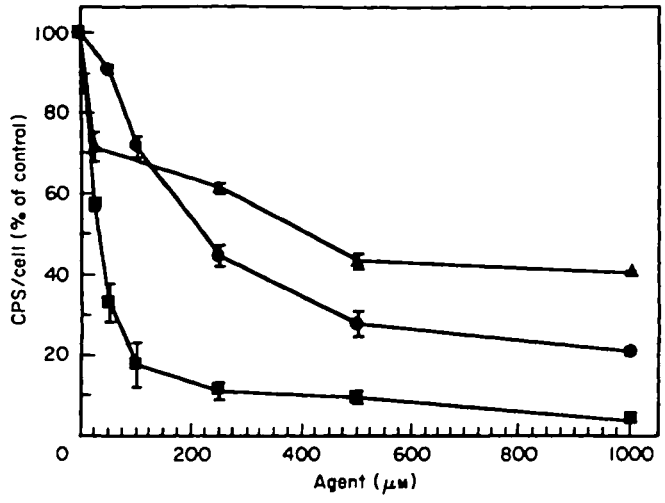

Figure 1. Effect of bismuth and salicylate on CPS production. CPS was extracted from 18 h cultures of *K. pneumoniae* 52145 treated with up to 1 mM sodium salicylate (▲), bismuth nitrate (●), or BSS (■). Uronic acid content was determined per cell and expressed as the percentage of CPS/cell found in untreated controls. Error bars represent the standard effor, derived from at least three independent determinations.

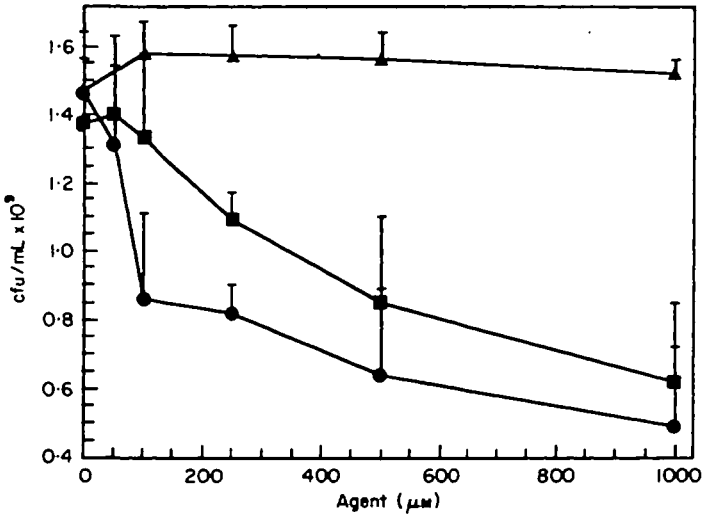

Figure 2. Effect of bismuth and salicylate on cell viability. Cultures of *K. pneumoniae* 52145, treated with up to 1 mM sodium salicylate (▲), bismuth nitrate (●), or BSS (■), were serially diluted in saline and plated on agar medium after 18 h of incubation. Viable count was performed in triplicate.

6×10^8 cfu/mL at 1 mM. In contrast, salicylate produced little effect on viable cell numbers at these concentrations. For $Bi(NO_3)_3$, the decrease in cell viability mirrored the decrease in CPS production. However, for BSS, CPS reduction occurred at lower concentrations than those reduced in cell viability.

Gel filtration profiles of detergent-extracted CPS from whole cultures of *K. pneumoniae* are shown in Figure 3. Only the void volume fraction is depicted, since

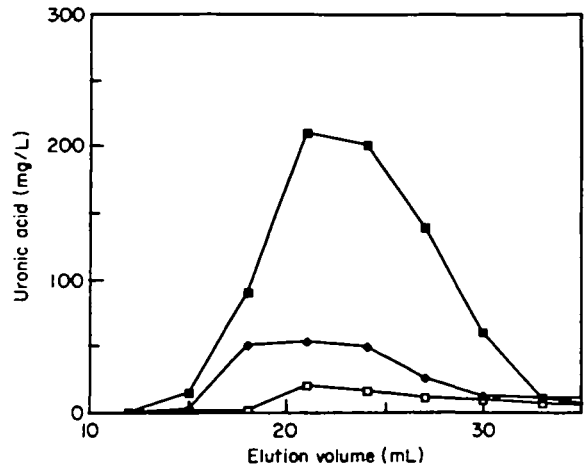

Figure 3. Gel filtration of CPS extracts. *K. penumoniae* 52145 cultures (10 mL), treated with 2·5 mM sodium salicylate (◆), 250 μM BSS (□), or untreated (■), were grown in a defined medium for 18 h. CPS extracts (1 mL) were loaded on a Sepharose 6B column (1·5 × 28 cm). Fractions of eluate collected were assayed for their uronic acid content. Only the void volume fraction, which contained all the uronic acid in the sample, is shown.

virtually all of the CPS eluted in this fraction. While the addition of salicylates to these cultures did not affect the molecular weight distribution of CPS, they did have a profound effect on the quantity of CPS eluted from the column. In the presence of 2·5 mM SAL, CPS was reduced to about 25% that of untreated (control) cultures. The quantity of CPS found in cultures treated with 250 μM BSS was about 4% that of untreated cultures.

Potentiation of antimicrobial activity

Whilst salicylate alone was shown to enhance aminoglycoside activity against several Gram-negative bacteria, BSS had an even more powerful effect, as illustrated in Figure 4. The amikacin-resistant strain of *K. pneumoniae* KPCL4 was cultured with various combinations of amikacin and BSS. The addition of 250 μM BSS increased the lag times of these cultures, but not significantly. More marked effects on amikacin activity were seen in the presence of 500 μM BSS, which reduced the IC_{24} from 21 to 12 mg/L. The addition of 700 μM BSS to these cultures decreased the IC_{24} to 6·5 mg/L. At these concentrations, the effects of BSS alone on culture lag time were minor.

Similar experiments were performed with an antibiotic sensitive strain of *S. typhimurium* cultured with BSS and gentamicin. Though this strain was sensitive to gentamicin (IC_{24} = 1·4 mg/L), addition of salicylate salts enhanced this sensitivity substantially, as shown in Figure 5. In the presence of 2·5 mM sodium salicylate, the IC_{24} for gentamicin was reduced to 0·7 mg/L. In the presence of 500 μM BSS, the IC_{24} was reduced to 0·2 mg/L.

A summary of these data, and the data obtained from cultures of *E. cloacae* and *S. marcescens* treated similarly, are presented in the Table. In general, 2·5 mM sodium salicylate produced a two-fold decrease in the IC_{24} for amikacin, gentamicin, or

Table. Effect of BSS and salicylate on bacterial inhibition

Strain	Antibiotic	Antibiotic IC_{24}* (mg/L)	Antibiotic IC_{24}* (mg/L) with: 2·5 mM salicylate	500 μM BSS
K. pneumoniae	amikacin	21·3 ± 0·20	16·0 ± 0·13	10·9 ± 0·42
S. typhimurium	gentamicin	1·35 ± 0·10	0·72 ± 0·05	0·2 ± 0·01
E. cloacae	amikacin	2·6 ± 0·06	1·6 ± 0·01	0·49 ± 0·0
	tobramycin	0·68 ± 0·04	0·27 ± 0·02	0·08 ± 0·01
S. marcescens	amikacin	5·9 ± 0·32	2·75 ± 0·25	0·79 ± 0·09
	tobramycin	7·2 ± 0·21	4·0 ± 0·08	1·1 ± 0·10

*IC_{24}, Mean (n = 3) concentration (± 1 S.D.) of antibiotic that inhibited culture growth for 24 ± 1 h.

tobramycin against all strains. On the other hand, 500 μM BSS generally decreased the IC_{24} for these antibiotics by about seven-fold.

The contribution of $Bi(NO_3)_3$ to aminoglycoside potentiation is illustrated in Figure 6. Tobramycin activity against *E. cloacae* was enhanced by all compounds tested. At 500 μM, BSS was twice as effective as $Bi(NO_3)_3$ and four times more effective than salicylate in reducing the IC_{24} for tobramycin. Generally, the addition of the bismuth cation reduced the IC_{24} by four-fold, while the salicylate anion caused another two-fold reduction.

The insolubility of bismuth salts prevented the testing of higher concentrations. Solubility was enhanced considerably when preparing 100 mM stock solutions of bismuth salts in alkaline propylene glycol. Bismuth compounds prepared in this manner were completely dissolved at a concentration of 100 mM and when diluted to 250 μM in culture media. However, stock solutions of BSS deteriorated rapidly, apparently due to the high alkalinity. Sodium salicylate stock solutions were also

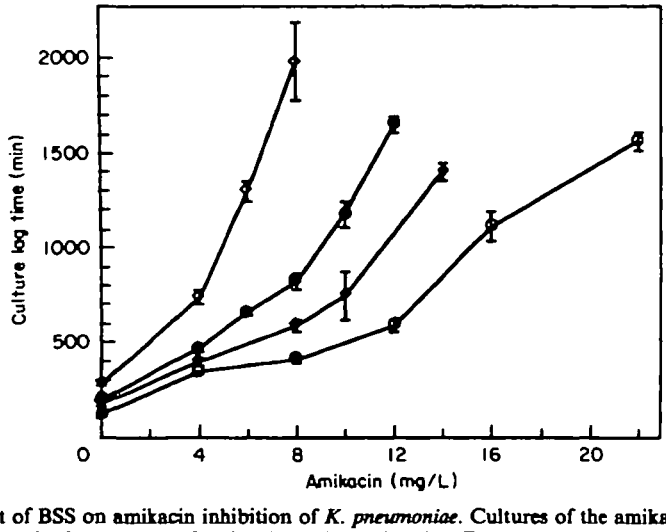

Figure 4. Effect of BSS on amikacin inhibition of *K. pneumoniae*. Cultures of the amikacin resistant strain KPCL4 were grown in the presence of amikacin to determine the effect on culture lag times. BSS was added to these cultures at final concentrations of 0 (○), 250 (◆), 500 (●), or 700 μM (◇).

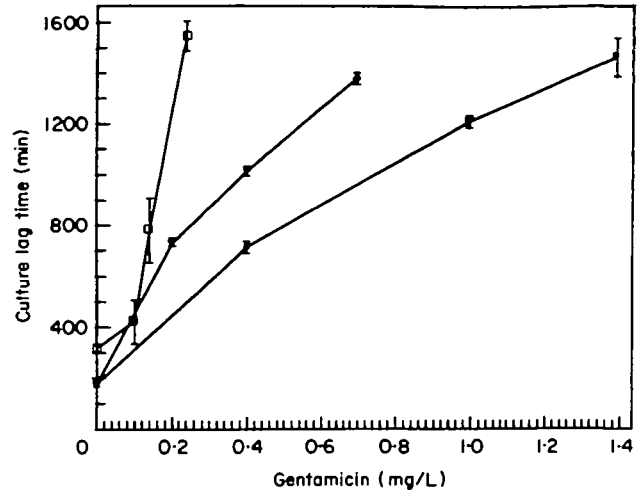

Figure 5. Effect of BSS or salicylate on the inhibition of growth by gentamicin of *S. typhimurium*. Cultures were treated with gentamicin to determine the effect on culture lag times. Cultures contained either gentamicin alone (■), or with 2·5 mM sodium salicylate (◆), or 500 μM BSS (□).

prepared in this manner to control the influence of high alkalinity or propylene glycol on test results. The effects of salicylate prepared in alkaline propylene glycol did not differ from its effects when prepared in water at physiological pH.

Discussion

Two potentially useful aspects of the action of BSS on selected, aerobic Gram-negative bacteria are addressed in this study. Repressing capsule production and potentiating

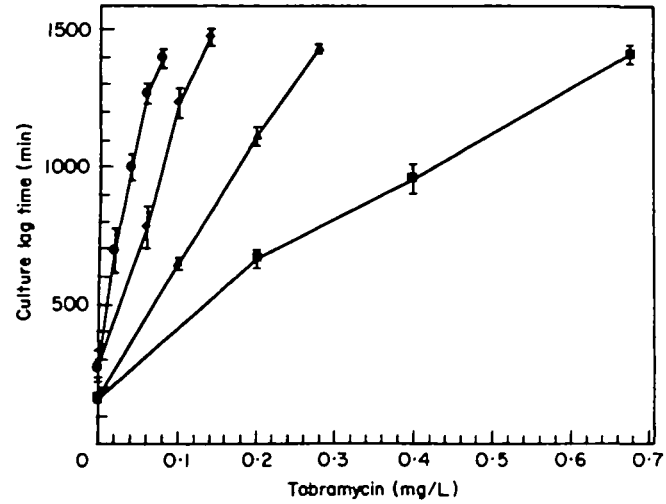

Figure 6. Effect of bismuth and salicylate on tobramycin activity against *E. cloacae*. Bacteria were cultured in the presence of various concentrations of tobramycin to determine the effect on culture lag time. Cultures contained either tobramycin alone (■), or with 2·5 mM sodium salicylate (▲), 500 μM $Bi(NO_3)_3$ (◆), or 500 μM BSS (●).

aminoglycoside activity may have far-reaching clinical implications. Bacterial biofilm formation *in vivo* results from copious CPS production, and may render bacteria refractory to host defences and to antibiotic therapy (Costerton, 1984; Simoons-Smit, Verweij-van Vught & MacLaren, 1986; Williams *et al.*, 1986). Although sodium salicylate at high concentrations reduced CPS production, BSS was significantly more active at much lower concentrations. $Bi(NO_3)_3$ exhibited an even more profound effect on CPS reduction than did salicylate. Thus the pronounced CPS-reducing effects of BSS at subinhibitory concentrations was due to the combined, and apparently independent actions of the bismuth cation and the salicylate anion.

The mechanisms of BSS action remain unclear, yet the role of salicylate as an uncoupler of oxidative phosphorylation (Brody, 1956; Haas, 1985) is probably important to its activity. Salicylate action has been likened to that of 2,4-dinitrophenol, a classic uncoupler that reduces ATP energy reserves in eukaryotic cells (Brody, 1956; Senior, 1990). The proton-translocating ATPase of *Escherichia coli* is nearly identical to the mitochondrial enzyme in structure and function (Senior, 1990), and is in large part responsible for bacterial energy production under aerobic conditions. Since CPS synthesis requires considerable energy resources (Sutherland, 1977). it is likely to be hindered by reduced ATP pools. Moreover, CPS production is not essential for bacterial growth or reproduction (Sutherland, 1977), and may be sensitive to repression when energy reserves are diminished. A recent study showed that intracellular levels of ATP in bacteria were decreased by 90% in the presence of 10 mM BSS, though no attempt was made to discern the individual effects of bismuth or salicylate (Sox & Olson, 1989).

The direct effect of salicylate may be the modulation of the electrical potential and pH gradient across the cytoplasmic membrane. Salicylate is an amphipathic compound, which can intercalate into phospholipid bilayers. In squid axons, salicylate concentrated in the interior lipid layer of membranes, with its negative charge exposed in the cell cytoplasm (McLaughlin, 1973). In isolated liver mitochondria, salicylate produced a decrease in protonmotive force and transmembrane potential (Haas, 1985). Because of its proton-carrying capacity, salicylate can dissipate the transmembrane proton gradient to by-pass ATP synthesis, thus lowering cellular energy reserves. Salicylate may also act to increase permeation of positively-charged molecules in general, as it does for the cadmium ion (Aumercier, 1990), which may explain its effect on the positively-charged aminoglycosides.

Both bismuth salts were comparable in their solubility but, relatively little is known about the degree and nature of dissociation of bismuth salts, or the biological effect of their undissociated forms. Less is known about the mode of action of bismuth. Synthetic enzymes such as ATPase and CPS-producing enzymes require metal cofactors for activity (Sutherland, 1977; Senior, 1990), so bismuth may act competitively with these metals to inhibit enzyme activity. Besides decreasing intracellular ATP levels, BSS has also been shown to increase extracellular ATP levels (Sox & Olson, 1989). Thus, bismuth may also disrupt bacterial cell integrity, causing leakage of intracellular components. Bismuth may displace magnesium ions that are important to the integrity of the Gram-negative outer membrane (Leive, 1968), thereby destabilizing the permeability barrier (Nikaido, 1989), and enhancing aminoglycoside permeation.

Whilst the use of BSS is limited largely to therapy of gut disorders, it is precisely in the gut where conditions are ideal for combined therapy. By itself, BSS possesses antimicrobial activity against Gram-negative bacteria at therapeutic concentrations. At

concentrations of 10–50 mM, BSS reduced Gram-negative cell viability by 3 to 6 $\log_{10}$ units (Sox & Olson, 1989; Manhart, 1990). When combined with aminoglycosides, the inhibitory capacity of BSS was greatly enhanced. Moreover, problems with aminoglycoside toxicity are avoided in the gut, since very little of the antibiotic is absorbed. Thus, antimicrobial chemotherapy against GI pathogens with this regimen may prove useful. Indeed, the combination of capsule elimination with potentiation of aminoglycoside antibiotics is intriguing, and should be investigated more fully in a range of disease states.

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