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Potentiation of Susceptibility to Aminoglycosides by Salicylate in *Escherichia coli*

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Susceptibility of *Escherichia coli* to kanamycin and seven other aminoglycosides has been found to be strongly potentiated by salicylate. At pH 7.5, in the presence of 15 mM salicylate and 0.5 μg of kanamycin per ml, the efficiency of plating of the bacteria was 2×10^{-5}, whereas there was no significant killing in the presence of kanamycin or salicylate alone. With 0.75 μg of kanamycin per ml, the addition of 2.5 mM salicylate was sufficient to reduce the efficiency of plating by more than 10^4-fold. Synergistic effects were found also at pHs 6.5 and 8.5. To determine whether the action of salicylate resulted from its behavior as a weak acid or its salicyl structure, similar experiments were carried out with acetate and salicyl alcohol. Acetate, a membrane-permeating weak acid, showed a synergistic effect on kanamycin susceptibility at pH 6.5 that was comparable to the effect seen with salicylate at pH 6.5. However, acetate had no synergistic effect with kanamycin at pH 7.5 or 8.5. This is consistent with the ability of acetate to increase the membrane potential of cells and the dependence of susceptibility to kanamycin and other aminoglycosides on the membrane potential. Salicyl alcohol, which has a hydroxyl group in the place of the carboxyl group that is present in salicylate, was an effective synergist with kanamycin. It was equally effective at pHs 6.5 and 7.5 and somewhat more effective at pH 8.5. These results support the hypothesis that two effects are involved in the synergy between aminoglycosides and salicylate: a weak acid effect, possibly to increase the membrane potential, and an uncharacterized effect related to the salicyl structure.

Previous reports showed that salicylate and other membrane-permeating weak acids, such as acetate and benzoate, increase the resistance of *Escherichia coli* to several antibiotics, including ampicillin, tetracycline, chloramphenicol, nalidixic acid, and cephalosporins (5, 12). These compounds were found to decrease the rates of permeation of cephalosporins through the outer membrane of *E. coli* by three- to fivefold (5). Sawai et al. (13) found that the OmpF content of the outer membrane was drastically reduced in cells grown in salicylate. Since OmpF forms a major porin channel for the antibiotics mentioned above, its absence can explain, at least in part, the increased antibiotic resistance of salicylate-grown cells.

The aminoglycosides are positively charged antibiotics whose entry into the cell is not known to require a specific outer membrane protein or other specific channels but is dependent on the membrane potential (2, 3) and, possibly, a quinone-related transporter (1, 2). Certain membrane-permeating weak acids can increase the membrane potential of bacteria during growth at a low external pH (pH_{ext}) (11, 18), presumably in the following way (11). The protonated form of the acid enters the cell and dissociates, thereby reducing the internal pH (pH_{int}) of the cell and the delta pH ($pH_{int} - pH_{ext}$). Continued pumping of protons out of the cell by the H^+-ATPase or the electron transport system (which then works against a lower pH gradient) increases the membrane potential. It was thus of interest to determine whether salicylate affected susceptibility to the aminoglycosides.

In this report we describe the effects of salicylate on the susceptibility of *E. coli* to kanamycin and other aminoglycosides. A potentiation of killing by all eight aminoglycosides tested was found. From quantitative studies on the potentiation of kanamycin susceptibility at several pHs with salicylate, acetate (another weak acid), and salicyl alcohol (a nonacidic salicylate analog), it was possible to distinguish two different effects of salicylate on increasing the susceptibility of *E. coli* to aminoglycosides.

(A preliminary report of this work was presented at the Twelfth Mid-Atlantic Extrachromosomal Genetic Elements Meeting, 1988 [M. Aumercier and J. L. Rosner, Plasmid 21:163, 1989].)

MATERIALS AND METHODS

Bacteria. The *E. coli* K-12 strains used in these experiments were N99 [F^- *rpsL200 galK2* IN(*rrnD-rrnE*)*1*] from the collection of the National Institutes of Health and RW51 (F^- *galK*, spectinomycin resistant, streptomycin susceptible; from Robert A. Weisberg).

Chemicals. Chemicals used (and their sources) were sodium salicylate (pK_a 2.97), salicyl alcohol (saligenin), acetylsalicylic acid (aspirin) (pK_a 3.5), sodium benzoate (pK_a 4.2), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-Tris(hydroxymethyl)-3-amino-propanesulfonic acid (TAPS), neomycin sulfate, spectinomycin dihydrochloride, kasugamycin hydrochloride, tobramycin, kanamycin sulfate, and gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.), nalidixic acid, streptomycin sulfate, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (Calbiochem-Behring, La Jolla, Calif.), sodium acetate (pK_a 4.76; Fisher Scientific Co., Pittsburgh, Pa.), and amikacin Sensi-Disk (30 μg; BBL Microbiology Systems, Cockeysville, Md.). Aqueous stock solutions were prepared at 1 to 5 M. Acetylsalicylic acid was dissolved in ethanol at 0.94 M.

Media. Tryptone broth (TB) contained the following, per liter: 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of NaCl, and 1 μg of thiamine per ml. The pH was adjusted to 7.4 with NaOH. It was supplemented with 1.1% Bacto-Agar (Difco) for plates and 0.6% agar for top agar. The media

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were buffered (15) with 100 mM PIPES at pH 6.5, 100 mM HEPES at pH 7.5, or 100 mM TAPS at pH 8.5 (using NaOH). Plates were made by combining 1 volume of 4.4% molten agar and 1 volume of 400 mM buffer (filter sterilized) with 2 volumes of double-strength TB and kept at 55°C while appropriate supplements of antibiotic or other chemicals were added, as indicated. Each plate contained 32 ml that was dispensed with a pipette. Dilutions of cells were made in TMG buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM $MgSO_4$, 0.01% gelatin).

Determination of EOP. A fresh overnight culture of N99, which was grown at 37°C in TB (pH 7.4), was diluted in TMG buffer and plated in 2.5 ml of TB top agar on the indicated plates. The plates were incubated at 37°C for at least 24 h. In the case of slow growth because of high amounts of antibiotic, chemicals, or both, incubation was continued until there was no increase in the number of colonies (usually no longer than 6 days). At that time, the final counts reported here were made. The efficiency of plating (EOP) was the titer of CFU obtained from the test plates divided by the titer obtained from the control plates lacking both the antibiotic and the test chemical.

The 50% inhibitory concentration (IC_{50}) was the concentration of antibiotic that reduced the EOP to 0.5 and was estimated from the survival curves.

Agar double-diffusion tests. About 10^6 bacteria (from a fresh overnight culture in TB at 37°C) were plated in 2.5 ml of TB top agar on TB plates (pH 7.4). A sterile paper disk (diameter, 0.5 in. [1.27 cm]; Difco) was placed at the center of the plate, and 17 μl of a 3.1 M solution of sodium salicylate was added. Other substances tested were sodium benzoate (20 μl of a 3 M solution) and acetylsalicylic acid (30 μl of a 1 M ethanolic solution). Sterile paper disks (diameter, 0.25 in. [0.64 cm]) were placed at a distance of 20 mm (center to center) from the central disk, and 2 to 18 μl of a 1- to 10-mg/ml solutions of the test aminoglycoside was added to these disks. As a control, to demonstrate salicylate-induced resistance to nalidixic acid, 5 μl of an 8-mg/ml solution of nalidixic acid was added to one 0.25-in. (0.64-cm)-diameter sterile paper disk on each plate as a control (5). In the case of amikacin, a commercial Sensi-disk (diameter 0.25 in. [0.64 cm]) containing 30 μg of this antibiotic was used. After overnight incubation at 37°C, the plates were examined for the effects of salicylate. Synergy was indicated by asymmetric zones of inhibition surrounding the aminoglycoside-containing disks, with more inhibition on the side facing the central disk than on the side away from the central disk.

RESULTS

Increased susceptibility to aminoglycosides during growth in the presence of salicylate. Salicylate renders *E. coli* resistant to normally inhibitory levels of several antibiotics (5, 12), including nalidixic acid. To determine the effect of salicylate on resistance to aminoglycosides, double-diffusion agar tests with strain N99 and several aminoglycosides, including kanamycin, tobramycin, gentamicin, amikacin, neomycin, spectinomycin, and kasugamycin, were performed. Streptomycin was tested by using strain RW51, a streptomycin-susceptible strain. A disk with nalidixic acid was placed on each plate as a control. It was observed that the zones of growth inhibition surrounding the disks containing each of the aminoglycosides tested were much greater on the side facing the central disk containing salicylate than on the other side. Thus, in the areas where subinhibitory concentrations of both salicylate and aminoglycoside were present, there was increased inhibition of growth. In contrast, the inhibitory zone surrounding the disk containing nalidixic acid was asymmetrically smaller on the side facing salicylate, indicating less susceptibility to nalidixic acid in the presence of salicylate, as was found previously (12). In general, the more positively charged aminoglycosides showed greater enhancement of susceptibility by salicylate, which suggests that the charge has a role in this phenomenon (data not shown).

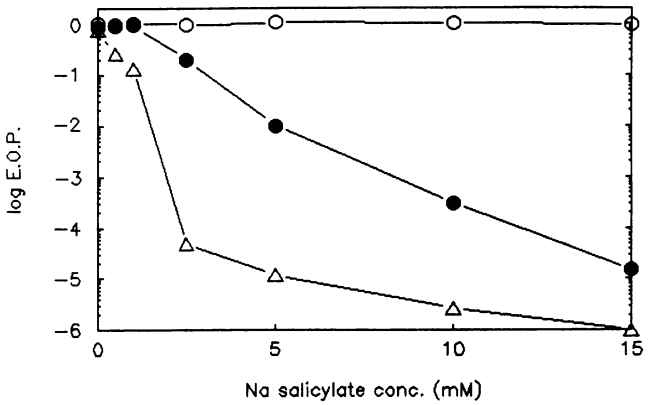

FIG. 1. EOPs of strain N99 on TB (pH 7.4) plates with 0 (○), 0.50 (●), or 0.75 (△) μg of kanamycin per ml as a function of the sodium salicylate concentration.

These observations were extended by quantitative plating experiments of strain N99 on TB agar (pH 7.4) containing different salicylate concentrations and 0, 0.5 or 0.75 μg of kanamycin per ml (Fig. 1). In the absence of kanamycin, salicylate, even up to 15 mM, did not affect the EOP. In the absence of salicylate, neither concentration of kanamycin resulted in significant killing. EOPs were 0.85 and 0.78 with 0.5 and 0.75 μg of kanamycin per ml, respectively. With increasing concentrations of salicylate, a strong synergistic effect on killing was found. For example, with 15 mM salicylate, the survival was 2×10^{-5} in the presence of 0.5 μg of kanamycin per ml and 1×10^{-6} with 0.75 μg of kanamycin per ml. Thus, salicylate strongly increases the killing by kanamycin. Additional experiments showed that 5 mM acetylsalicylate or benzoate decreased colony formation in the presence of 1 μg of kanamycin per ml by over 200-fold.

Susceptibility to aminoglycosides in the presence of salicyl alcohol. If the synergism between kanamycin and salicylate is due to the behavior of salicylate as a weak acid, a nonacidic analog should not behave synergistically. Salicyl alcohol (*o*-hydroxybenzyl alcohol) is similar to salicylate except that the carboxyl group is replaced with a hydroxyl. Figure 2 shows the results of plating of strain N99 in the presence of 0, 0.5, or 0.75 μg of kanamycin per ml and various concentrations of salicyl alcohol. Virtually no effect was seen for any concentration of salicyl alcohol in the absence of kanamycin or in the presence of only 0.5 μg/ml. However, a moderately strong effect was seen with 15 and 20 mM salicyl alcohol in the presence of 0.75 μg of kanamycin per ml. The EOP was reduced by 10- and 10^4-fold, respectively. It seems, therefore, that even the nonacidic alcohol has an effect on kanamycin susceptibility. Nevertheless, this effect was weaker than that seen with salicylate. With 0.75 μg of kanamycin per ml, it could be estimated that about nine times more salicyl alcohol (18 mM) was required to reduce the EOP by 10^3-fold compared with the concentration

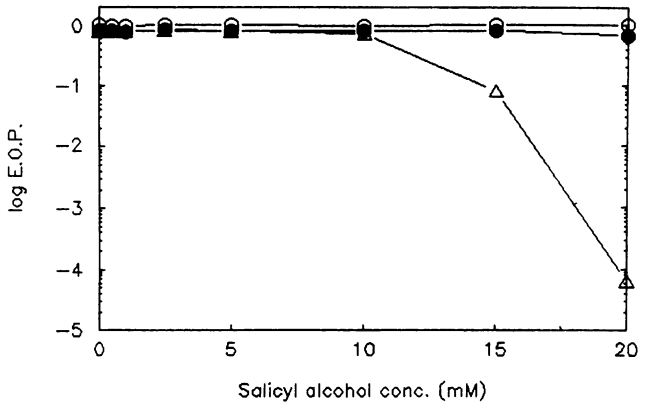

FIG. 2. EOPs of strain N99 on TB (pH 7.4) plates with 0 (○), 0.50 (●), or 0.75 (△) μg of kanamycin per ml as a function of salicyl alcohol concentration.

of salicylate (2 mM) that was required. This suggests that salicyl alcohol has only a part of the synergistic activity of salicylate.

Effects of pH on inhibition of growth by salicylate and salicyl alcohol. The pH_{ext} plays a crucial role in the effects of weak acids on the cell. Because of pH homeostasis, the pH_{int} of *E. coli* is maintained at about 7.6 to 7.8 over a wide range of pH_{ext}s (10, 17). For an ideal weak acid, the ratio of the concentration of the dissociated form of the weak acid ($[A^-]$) to that of the undissociated (protonated) form ([HA]) depends on the difference between the pK of the acid and the pH of the solvent: $\log([A^-]/[HA]) = pH - pK$. Therefore, there is relatively more [HA] at lower pHs. Since HA is the membrane-permeating form for acetic, benzoic, and salicylic acids (pK_as of 4.75, 4.2, and 2.97, respectively), the pH_{ext} determines the amount of acid that enters the cell. However, inside the cell, the acid dissociates according to the pH_{int}. Thus, if the pH_{int} is higher than the pH_{ext}, relatively more $[A^-]$ is present inside than outside the cell, even though the HA concentrations do not differ. For the acids used in this study, a change in pH_{ext} from 6.5 to 7.5 or from 7.5 to 8.5 was expected to decrease the concentrations of both the dissociated and the protonated forms inside the cell by about 10-fold for each unit of increase. The $[A^-]$ in the medium increased insignificantly over that range of change in pH_{ext}s.

To compare the effects of salicylate and salicyl alcohol on kanamycin toxicity at different pH_{ext}s, it was necessary to find concentrations that did not inhibit colony formation at those pHs. The EOP of strain N99 at three different pH_{ext}s (buffered with 100 mM PIPES [pH 6.5], HEPES [pH 7.5], or TAPS [pH 8.5]) is shown for different concentrations of salicylate (Fig. 3A) and salicyl alcohol (Fig. 3B). The concentrations of salicylate required for toxicity decreased with lower pH_{ext}s. Salicyl alcohol was not lethal at any concentration or pH_{ext} tested, but from the slow growth of colonies, it was clear that it affected the cells. The IC_{50}s for salicylate are estimated as 3.1, 17.5, and 55.5 mM for pH_{ext}s of 6.5, 7.5, and 8.5, respectively. Interestingly, the ratios of these IC_{50}s is about 1:6:18 and not 1:10:100, as would be expected to compensate for decreasing internal concentrations of both the dissociated and undissociated acids with a unit increase of the pH_{ext}. This suggests that either salicylate does not behave as an ideal weak acid at higher pH_{ext}s or that its toxicity is augmented at pH_{ext} 8.5 by some additional mechanism. The observation that salicyl alcohol is not lethal under the conditions tested indicates strongly that the compound was not significantly contaminated with salicylate.

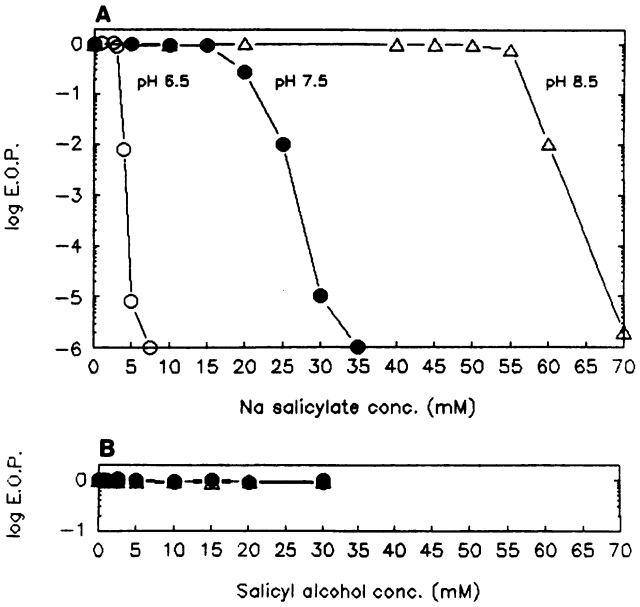

FIG. 3. EOPs of strain N99 on TB with 100 mM PIPES buffer (pH 6.5) (○), 100 mM HEPES buffer (pH 7.5) (●), or 100 mM TAPS buffer (pH 8.5) (△) as a function of the sodium salicylate (A) or salicyl alcohol (B) concentration.

Effects of pH on the synergy between kanamycin and either salicylate or salicyl alcohol. The effects on viability of either salicylate or salicyl alcohol with kanamycin were compared at pH_{ext}s 6.5, 7.5, and 8.5 to see whether the weak acid effects could be distinguished from the effects of the salicyl structure. The concentrations of salicylate used at the different pHs were chosen to reflect the ratios obtained above for toxicity by salicylate alone, i.e., 0.5 mM at pH 6.5, 2.5 mM at pH 7.5, and 9 mM at pH 8.5. The concentration of salicyl alcohol used was 15 mM, regardless of pH. At pH 6.5 (Fig. 4A), both salicylate and salicyl alcohol showed synergistic effects on kanamycin susceptibility, but the effect of salicyl alcohol was less pronounced. For kanamycin alone, the IC_{50} was estimated to be 7.6 μg/ml. In the presence of 0.5 mM salicylate, this decreased sixfold, to about 1.2 μg/ml. (Interestingly, the decrease in EOP of salicylate-treated cells occurred over a wide range of kanamycin concentrations.) In the presence of 15 mM salicyl alcohol, only about a 30% decrease, to about 5.2 μg/ml, was found. Salicyl alcohol was much less effective than salicylate at pH 6.5.

At pHs 7.5 (Fig. 4B) and 8.5 (Fig. 4C), the IC_{50}s for kanamycin alone were 1.1 and 0.7 μg/ml, respectively. This is consistent with the known greater bacterial susceptibility to kanamycin that occurs at the higher membrane potentials found at higher pHs. Even so, either 2.5 mM salicylate or 15 mM salicyl alcohol showed reasonably strong synergistic effects with kanamycin at pH 7.5, reducing the IC_{50}s from 1.1 to 0.3 and 0.7 μg/ml, respectively. At pH 8.5, the effect of 9 mM salicylate was similar to that of 2.5 mM salicylate at pH 7.5. However, 15 mM salicyl alcohol was slightly more effective at pH 8.5 than it was at pH 7.5 and was comparable to 9 mM salicylate (Fig. 4C). Thus, as the pH_{ext} increased, the difference in the effect on kanamycin susceptibility between salicylate- and salicyl alcohol-treated cells diminished. Salicylate appears, then, to have two modes of action: one is due to its structure and is shared by salicyl alcohol, particularly as seen at higher pH_{ext}s; the other one is

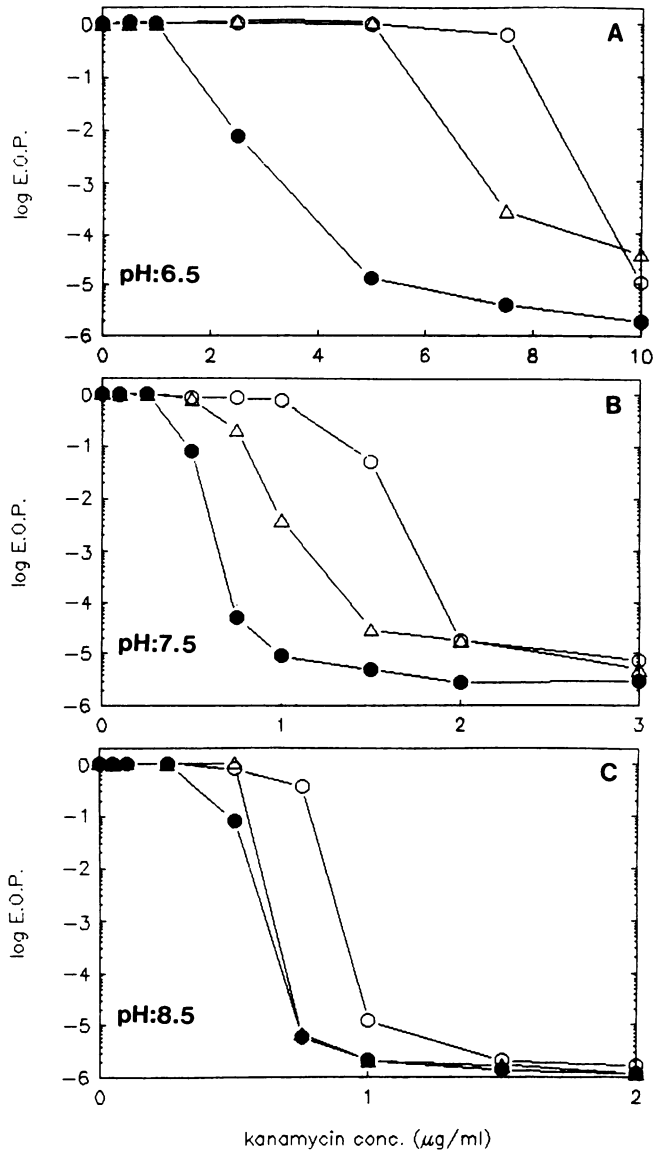

FIG. 4. EOPs of strain N99 as a function of kanamycin concentration in the absence (○) or presence (●) of sodium salicylate or 15 mM salicyl alcohol (△). The concentrations of sodium salicylate at pH 6.5, 7.5, and 8.5 were 0.5, 2.5, and 9 mM, respectively. Cells were plated on TB with 100 mM PIPES buffer (pH 6.5) (A), 100 mM HEPES buffer (pH 7.5) (B), or 100 mM TAPS buffer (pH 8.5) (C). Note different abscissas.

possibly due to its behavior as a weak acid to increase the membrane potential, especially at low pH_{ext}s.

Synergy of acetate with kanamycin at pH 6.5. If the interpretation presented above is correct, part of the effect of salicylate at pH 6.5 on membrane potential might be achieved by a weak acid that does not have the salicyl structure. For this reason, the effect of acetate on kanamycin susceptibility was determined at pHs 6.5, 7.5, and 8.5. Table 1 shows that 10 mM acetate reduced the EOP of strain N99 in the presence of 7 μg of kanamycin per ml to 2×10^{-5} at pH 6.5, an effect comparable to that of salicylate at pH 6.5. However, at pH 7.5 or 8.5, 50 or 200 mM acetate, respectively, had essentially no effect on kanamycin-treated cells. Thus, acetate does indeed show a strong synergism with

TABLE 1. EOPs of strain N99 on TB plates with pH and additions as indicated[a]

pH 6.5			pH 7.5			pH 8.5		
Kanamycin concn (μg/ml)	Addition (concn [mM])	EOP	Kanamycin concn (μg/ml)	Addition (concn [mM])	EOP	Kanamycin concn (μg/ml)	Addition (concn [mM])	EOP
0	None (0)	1.0	0	None (0)	1.0	0	None (0)	1.0
7	None (0)	0.75	1	None (0)	0.75	0.7	None (0)	0.6
7	Sal (0.5)	5×10^{-6}	1	Sal (2.5)	1×10^{-5}	0.7	Sal (10)	8×10^{-6}
7	Ace (10)	2×10^{-5}	1	Ace (50)	0.45	0.7	Ace (200)	0.5
7	SalOH (15)	2×10^{-3}	1	SalOH (15)	5×10^{-3}	0.7	SalOH (15)	1×10^{-5}
7	Ace (10) + SalOH (15)	7×10^{-6}	1	Ace (50) + SalOH (15)	4×10^{-3}	0.7	Ace (200) + SalOH (15)	2×10^{-5}

[a] EOPs were determined as described in the text. Abbreviations: Sal, salicylate; Ace, acetate; SalOH, salicyl alcohol.

kanamycin, but only at the lower pH. The combination of salicyl alcohol and acetate at pH 6.5 marginally enhanced the effect of acetate alone on kanamycin susceptibility (Table 1).

DISCUSSION

Salicylate has a profound effect on the resistance of *E. coli* to a number of negatively charged or neutral antibiotics (5, 12). One basis for this resistance is that salicylate down-regulates the expression of the outer membrane porin OmpF (13; J. L. Rosner and J. Foulds, unpublished data), which serves as a channel for the entry of these antibiotics into the periplasmic space (9). In contrast, we reported here the increased susceptibility to aminoglycosides of *E. coli* in the presence of salicylate. We also observed the potentiation of kanamycin by benzoate and acetylsalicylic acid (aspirin). The efficacies of other positively charged antimicrobial agents, bleomycin (M. Aumercier and J. L. Rosner, unpublished data) and cadmium cation (J. L. Rosner and M. Aumercier, submitted for publication), are also potentiated by salicylate. In the case of Cd^{2+}, the mechanism is somewhat different from that observed in this study, since acetate did not enhance killing by Cd^{2+} at pH 6.4, 7.4, or 8.4. Thus, it may be anticipated that, when administered together, salicylates and related compounds could improve the therapeutic action of aminoglycosides and other positively charged antibiotics. It should be noted, however, that salicylates reduce the susceptibility to a number of beta-lactams, chloramphenicol, nalidixic acid, and tetracycline (5, 12), and so would be contraindicated when these antibiotics are administered.

Our results suggest that at least two properties of salicylate are responsible for the potentiation of aminoglycoside susceptibility. The first, which is also found with acetate, is that of a weak acid acting at low pH_{ext}s. Since weak acids increase the membrane potential of cells at low pHs (11, 18) and since the low membrane potential observed at low pHs (7) appears to limit aminoglycoside susceptibility (3), the increased membrane potential could be the basis of this synergism. The second property, which is also found in salicyl alcohol, is seen at low and high pH_{ext}s. It seems similar to that found for the synergy of salicylate or salicyl alcohol with Cd^{2+} (Rosner and Aumercier, submitted). The basis for this activity is not known, but it must reside in the salicyl or benzyl structure. We consider the following to be possible mechanisms for enhancing aminoglycoside susceptibility. (i) EDTA can act to chelate divalent cations that are antagonistic to aminoglycoside activity in *Pseudomonas aeruginosa* (4, 6). Perhaps salicylate and salicyl alcohol can have similar effects on *E. coli*.

(ii) At low pHs, high concentrations of salicylate have been found to decrease the membrane potential of *E. coli*, as though it were a protonophoric uncoupler of oxidative phosphorylation (14, 16). Normally, uncoupling of oxidative phosphorylation would be expected to decrease the susceptibility to aminoglycosides by blocking the energy-dependent uptake steps (6). However, certain H^+-ATPase mutants (*uncA* or *uncB*) that are uncoupled (by an unknown mechanism) have increased respiratory activity and are hypersensitive to streptomycin (8). Bryan (1) and Bryan and Kwan (2) have argued that, in addition to a requirement for membrane potential, a quinone or related part of the electron transport system is needed as an anionic transporter for aminoglycosides (although this has been challenged [19]). Conceivably, salicylate and salicyl alcohol could also increase such respiratory chain-related activity.

(iii) The salicyl structure may have a regulatory effect on the activity of some other cellular element involved in aminoglycoside uptake or target susceptibility, e.g., the ribosomes. The regulation of OmpF (13; Rosner and Foulds, unpublished data) discussed above and of *ina-1* (15) are examples of genes whose expression is regulated by salicylate.

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