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Potentiation by Salicylate and Salicyl Alcohol of Cadmium Toxicity and Accumulation in *Escherichia coli*

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The toxicity of Cd^{2+} in *Escherichia coli* K-12 was potentiated by salicylate and several related compounds. The efficiency of plating on Luria broth plates was reduced by more than 10^5-fold when 10 mM salicylate and 200 μM $CdCl_2$ were present simultaneously but was unaffected when either compound was present by itself. Synergistic effects were found at pH 7.4 with certain other weak acids (acetyl salicylate [aspirin], benzoate, and cinnamate) and with a nonacidic salicylate analog, salicyl alcohol, but not with acetate or *p*-hydroxy benzoate. Thus, the synergism with Cd^{2+} is determined by the structure of the compounds and not merely by their acidity. The kinetics of $^{109}Cd^{2+}$ uptake by cells grown and assayed in broth indicated the presence of two uptake systems with K_ms of 1 and 52 μM Cd^{2+} and V_{max}s of 0.059 and 1.5 μmol of Cd^{2+} per min per g of cells, respectively. The kinetics of uptake for cells grown and assayed with 20 mM salicyl alcohol showed 2.5-fold increases in the V_{max}s of both systems but no change in the K_ms. Salicylate-grown cells also exhibited increased rates of $^{109}Cd^{2+}$ uptake by both systems. Thus, enhanced uptake of Cd^{2+} may be responsible for the potentiation of Cd^{2+} toxicity by salicylate and salicyl alcohol.

Previous work showed that salicylate strongly potentiates the toxicity of kanamycin and other aminoglycosides in *Escherichia coli* (1). Two different mechanisms of salicylate action were distinguished: one due to its behavior as a weak acid and the other due to its salicyl structure. The weak-acid effect could be demonstrated with acetate at pH 6.5 but not at pH 7.5 or 8.5. Since (i) the uptake of the positively charged aminoglycosides depends on the cell's membrane potential (4), (ii) the membrane potential normally is low at low external pH (pH_{ext}) (4, 10), and (iii) acetate and other membrane-permeating weak acids increase the membrane potential of *E. coli* at low pH_{ext} (20, 25), it seems reasonable that the synergy between aminoglycosides and these weak acids at low pH_{ext} is due (at least in part) to the increase in membrane potential. However, a nonacidic salicylate analog, salicyl alcohol, acted synergistically with kanamycin at pH_{ext}s of 6.5, 7.5, and 8.5. Thus, the salicyl structure itself must play an important role in the synergy.

Griffith et al. (7, 8, 16) have shown that the *tet* gene product increases the susceptibility of *E. coli* to aminoglycosides and to Cd^{2+} yet does not significantly raise the membrane potential. This suggests that Cd^{2+} and kanamycin susceptibilities are related. It was of interest, therefore, to test salicylate, salicyl alcohol, and acetate for their effects on susceptibility to Cd^{2+}.

Incubation of *E. coli* with low concentrations of cadmium salts results in a prolonged growth lag (19) and cell death (7, 18), apparently because of single-strand breaks of the DNA (18). In addition, heat shock, oxidation stress, and SOS regulons are induced (26), as is the appearance of a cadmium-binding protein which may help detoxify the cell (11).

Laddaga and Silver (14) have shown that in *E. coli*, Cd^{2+} uptake occurs by active transport, possibly via a Zn^{2+} porter: the uptake followed Michaelis-Menten kinetics in a minimal (but not a complex) medium and was energy dependent. According to the chemiosmotic theory of Mitchell (17), the membrane potential provides the energy for active cation uptake. The experiments reported here show that the salicylate and salicyl alcohol, but not acetate, potentiate Cd^{2+} toxicity and increase the uptake of Cd^{2+}. Thus, the synergies seen here do not seem to depend on increases in membrane potential.

MATERIALS AND METHODS

In general, the materials and methods used were as described previously (1) and are only briefly repeated here for clarity or to emphasize exceptions.

Bacteria. The *E. coli* K-12 strain used in these experiments was N99 (1).

Chemicals. Chemicals used (and their sources) were $CdCl_2$ and sodium acetate (Fisher); $^{109}Cd^{2+}$ (500 mCi/mmol; New England Nuclear Corp.); acetyl salicylic acid, N-acetyl-*p*-amino-phenol (acetaminophen), *p*-hydroxy benzoic acid, salicyl alcohol, sodium benzoate, sodium salicylate, trans-cinnamic acid (β-phenyl acrylic acid), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and *N*-tris(hydroxymethyl) methyl-3-amino-propanesulfonic acid (TAPS) (Sigma Chemical Co., St. Louis, Mo.); 2,4-dinitrophenol, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (Calbiochem-Behring, La Jolla, Calif.).

Media. The Luria broth (LB) medium used in these experiments contained the following (per liter): 10 g of Bacto-tryptone (Difco Laboratories, Detroit, Mich.), 5 g of Bacto-yeast extract, and 5 g of NaCl. For overnight growth of cells and for the uptake assays, the pH was adjusted to 7.4 with NaOH. Plates and top agar contained 1.5 and 0.6% Bacto-agar, respectively, and they were buffered with 100 mM PIPES at pH 6.4, with 100 mM HEPES at pH 7.4, or with 100 mM TAPS at pH 8.4 (23) as described previously (1).

Determination of EOP. The method for determination of efficiency of plating (EOP) was as described previously (1) except that LB media were used. The EOP was the titer of CFU obtained from the test plates divided by the titer obtained from the control plates lacking both $CdCl_2$ and the

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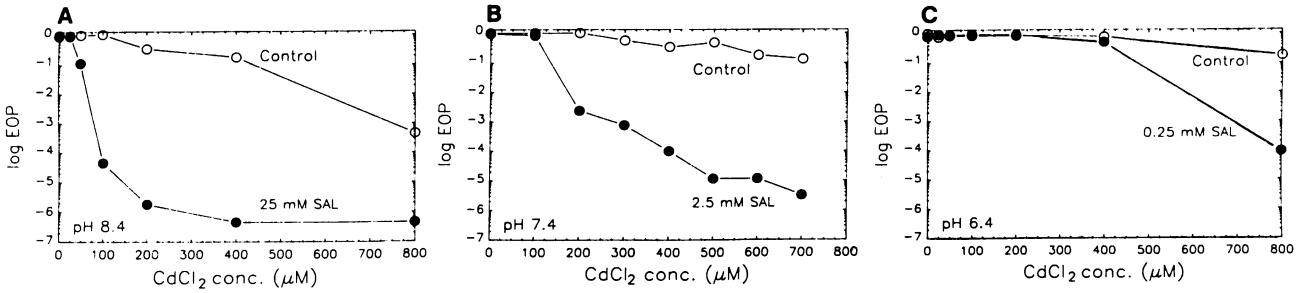

FIG. 1. Effects of salicylate on the EOP of strain N99 as a function of $CdCl_2$ concentration. Plates were buffered at pH 8.4 (A), 7.4 (B), or 6.4 (C) and contained the indicated concentrations of sodium salicylate (SAL). Control, No sodium salicylate.

test chemical. Control experiments showed that up to 4% ethanol (added with certain test compounds) had no effect on the EOP, either in the presence or in the absence of $CdCl_2$.

Agar double-diffusion tests. These qualitative and rapid tests were performed as described previously (1, 6), with slight variations.

Cadmium uptake. Cadmium uptake was measured by the filter assay of Laddaga and Silver (14), except that cells were grown at 37°C in LB medium (supplemented as indicated) to an A_{600} of 0.7 (0.2 mg [dry wt] per ml), concentrated 2.5 times in fresh LB broth (supplemented as indicated), and aerated at 37°C for 3 min before the addition of $^{109}Cd^{2+}$.

RESULTS

Synergism between $CdCl_2$ and salicylate. Agar double-diffusion tests with several strains of *E. coli* indicated strong synergistic inhibitory effects between $CdCl_2$ and salicylate, acetyl salicylate, and benzoate but not between $CdCl_2$ and acetate (data not shown). Quantitative plating experiments with strain N99 (Fig. 1B) showed that at pH 7.4 with 700 μM $CdCl_2$ alone, about 10% of the cells formed colonies. However, in the presence of 2.5 mM sodium salicylate (by itself nonlethal) plus 200 μM $CdCl_2$ (by itself nonlethal), there was a 400-fold decrease in the EOP. At 700 μM $CdCl_2$, the decrease in the EOP due to the presence of 2.5 mM salicylate was 5×10^5-fold. The effective concentrations of salicylate at pH 7.4 with 200 or 400 μM $CdCl_2$ are shown in Fig 2B. Clearly, 2.5 to 10 mM salicylate potentiated the effects of $CdCl_2$. Thus, salicylate and $CdCl_2$ are strongly synergistic with respect to inhibition of *E. coli* colony formation.

Synergism as a function of pH_{ext}. At high pH_{ext}, the membrane potential of *E. coli* is high (4, 10). If the entry of, and therefore the susceptibility to, $CdCl_2$ depends on the membrane potential, it should increase at high pH_{ext}. The reverse should be true at low pH_{ext}, at which the membrane potential is low. Figures 1 and 2 show the effects of $CdCl_2$ and salicylate on EOP as a function of pH_{ext}. Increasing the pH_{ext} from 7.4 (Fig. 1B) to 8.4 (Fig. 1A) considerably increased the susceptibility to $CdCl_2$ alone. However, the susceptibility to $CdCl_2$ was barely lower at pH_{ext} 6.4 (Fig. 1C) than at pH_{ext} 7.4. Nevertheless, at each pH_{ext}, certain nonlethal concentrations of salicylate potentiated the toxicity of $CdCl_2$.

The concentrations of salicylate required for synergy increased with increasing pH_{ext}. If the concentration of salicylate in the cell is responsible for the synergy and if salicylate behaves as an ideal membrane-permeating weak acid, then the concentration of salicylate needed at pH_{ext} 8.4 should be 10-fold greater than that needed at pH 7.4 and 100-fold greater than that at pH 6.4 (20). Making such estimates was complicated by the need to take into account the influence of the pH_{ext} on susceptibility to $CdCl_2$ alone. Nevertheless, from the data in Fig. 2 (and other data not shown), it is estimated that only about fourfold more salicylate in the medium was required at pH 8.4 (and 100 μM $CdCl_2$) than at pH 7.4 (and 200 μM $CdCl_2$) and that about 2.5 times the salicylate concentration required at pH_{ext} 6.4 (and 500 μM $CdCl_2$) was required at pH_{ext} 7.4. Thus, an increase in the pH_{ext} of 2 U required an increase in effective salicylate concentration of only about 10-fold and not the 100-fold increase expected for an ideal membrane-permeating weak acid.

Synergism with related compounds. The results of experiments with several other compounds in combination with $CdCl_2$ (Table 1) indicate that the structure of the weak acids affects the synergism with Cd^{2+}. Cinnamate and acetyl salicylate had clear synergistic effects and benzoate had slightly weaker effects. (The nonacidic analgesic, acetaminophen, was not at all effective.) However, two of the weak acids tested, acetate and *p*-hydroxy benzoate, were

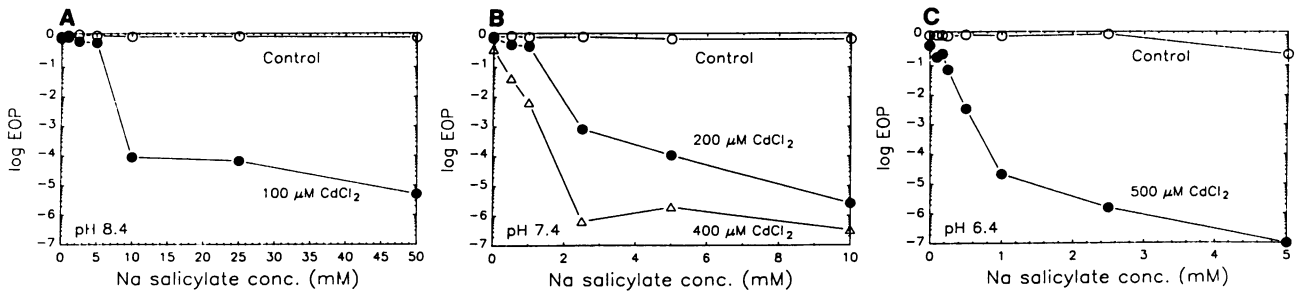

FIG. 2. Effects of $CdCl_2$ on the EOP of strain N99 as a function of salicylate concentration. (A) pH 8.4; (B) pH 7.4; and (C) pH 6.4. Control, No $CdCl_2$.

TABLE 1. Effects of various compounds and $CdCl_2$ on the EOP of strain N99[a]

Expt and compound (concn [mM]) [pK_a][b]	EOP[c]	
	No $CdCl_2$	200 μM $CdCl_2$
1		
None	1.0	1.01
Acetate (25) [4.76]	0.96	1.17
Acetate (50)	0.99	1.08
Acetaminophen (2.5)	1.01	0.97
Acetaminophen (5.0)	0.97	1.20
Acetyl salicylate (2.5) [3.50]	1.00	0.0049
Acetyl salicylate (5.0)	1.03	<0.0020
Benzoate (2.5) [4.20]	1.14	0.14
Benzoate (5.0)	1.04	0.024
Cinnamate (2.5) [4.44]	1.14	<0.0020
Cinnamate (5.0)	1.11	<0.0020
p-Hydroxy benzoate (2.5) [4.58]	1.13	0.25
p-Hydroxy benzoate (5.0)	1.16	0.27
2		
None	1.0	0.94
Acetaminophen (10)	0.79	1.01
Acetate (100)	0.89	0.86
p-Hydroxy benzoate (10)	0.88	0.78
Salicylate (5) [2.97]	0.96	0.00002

[a] A fresh culture of N99 grown overnight in LB, pH 7.4, at 37°C was diluted and plated on LB plates with the indicated supplements at pH 7.4.
[b] Data from reference 9.
[c] The titers of the culture determined from the control plates were 2.0×10^9 and 2.4×10^9 in the two experiments.

not synergistically inhibitory with $CdCl_2$, even at fairly high concentrations. Since acetate is more effective at low pH_{ext} than at neutral pH_{ext}, both as a chemotactic repellent (20, 24) and as a potentiator of growth inhibition with kanamycin (1), the effect of acetate was compared at pH_{ext}s of 6.4, 7.4, and 8.4, (with 20, 100, and 400 mM acetate, respectively). No synergy of acetate with Cd^{2+} was found at any of these pHs (data not shown).

Synergism of salicyl alcohol and Cd^{2+}. To test whether a carboxyl group is required for the synergistic activity of salicylate, the effect of salicyl alcohol (*o*-hydroxy-benzyl alcohol or saligenin) was examined. Figure 3 shows that in the presence of 20 mM salicyl alcohol (by itself not lethal), the effect of 200 μM $CdCl_2$ was increased about 10^3-fold at pH 7.4. In a separate experiment (not shown), with 400 μM $CdCl_2$ alone, the EOP was 0.5. When 15 or 25 mM salicyl alcohol was also present, the EOP was reduced to 10^{-3} or 10^{-4}, respectively. Thus, an acidic structure is not necessary to potentiate susceptibility to Cd^{2+}.

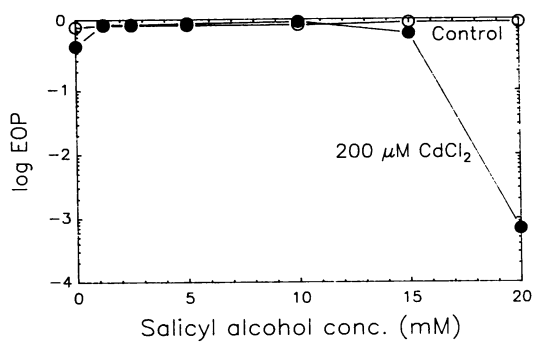

FIG. 3. EOP of strain N99 at pH 7.4 with $CdCl_2$ as a function of salicyl alcohol concentration. Control, No $CdCl_2$.

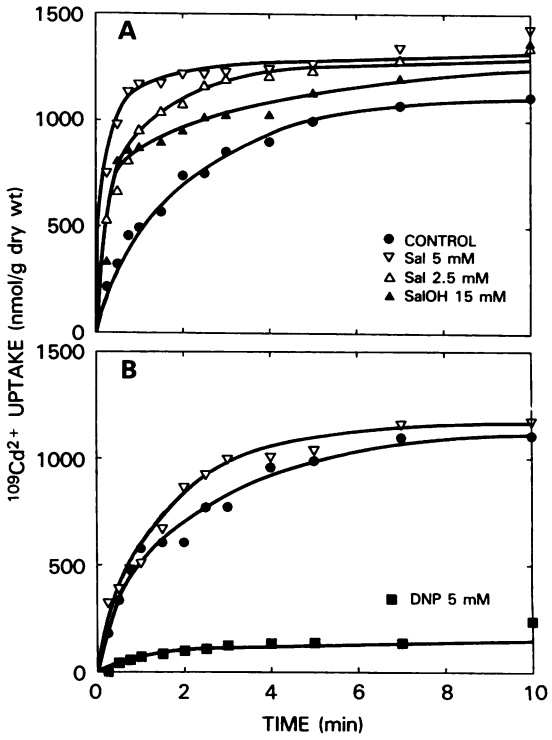

FIG. 4. Effect of salicylate and other compounds on $^{109}Cd^{2+}$ uptake by strain N99. (A) Growth, preincubation, and assay of the cells were carried out in LB broth, pH 7.4 (control), or in LB broth supplemented with either salicyl alcohol (SalOH) or sodium salicylate (Sal). (B) A culture grown in LB broth, pH 7.4, was sedimented and resuspended in broth or in broth supplemented with either sodium salicylate or 2,4-dinitrophenol (DNP). Assays were initiated by the addition of 10 μM $^{109}Cd^{2+}$, and samples were removed at the times indicated and processed.

Effects of salicylate and salicyl alcohol on $^{109}Cd^{2+}$ uptake. The possibility that enhanced Cd^{2+} uptake by salicylate and salicyl alcohol was responsible for the synergism was tested with $^{109}Cd^{2+}$ (Fig. 4). The initial rates of accumulation of $^{109}Cd^{2+}$ were faster and the final extents were greater for cells grown and assayed in the presence of 5 mM salicylate than for control cells (Fig. 4A). Decreasing the concentration of salicylate to 2.5 mM or using 15 mM salicyl alcohol also stimulated uptake but to a lesser extent than 5 mM salicylate (Fig. 4A). Cells grown in 5 mM salicylate also showed increased rates of uptake when assayed in the presence of 0.1 or 1 μM $^{109}Cd^{2+}$. Cells grown and assayed in 50 mM acetate, however, showed reduced uptake in the presence of 0.1 μM $^{109}Cd^{2+}$ but normal uptake in the presence of 10 μM $^{109}Cd^{2+}$ (data not shown). Thus, the increased accumulation of $^{109}Cd^{2+}$ found in cells grown in either salicylate or salicyl alcohol correlates with the increased susceptibility of such cells to Cd^{2+}.

The effect of salicylate on $^{109}Cd^{2+}$ accumulation was less when the cells were first exposed to the salicylate only 3 min before the exposure to $^{109}Cd^{2+}$ (Fig. 4B). Hence, maximum stimulation of uptake seems to require a substantial period of exposure to salicylate. Nevertheless, uncoupling oxidative phosphorylation by adding the protonophore 2,4-dinitrophenol to the cells 3 min before exposure to $^{109}Cd^{2+}$ virtually abolished the uptake (Fig. 4B). This is consistent with an energy requirement for uptake, as observed by Laddaga and Silver (14).

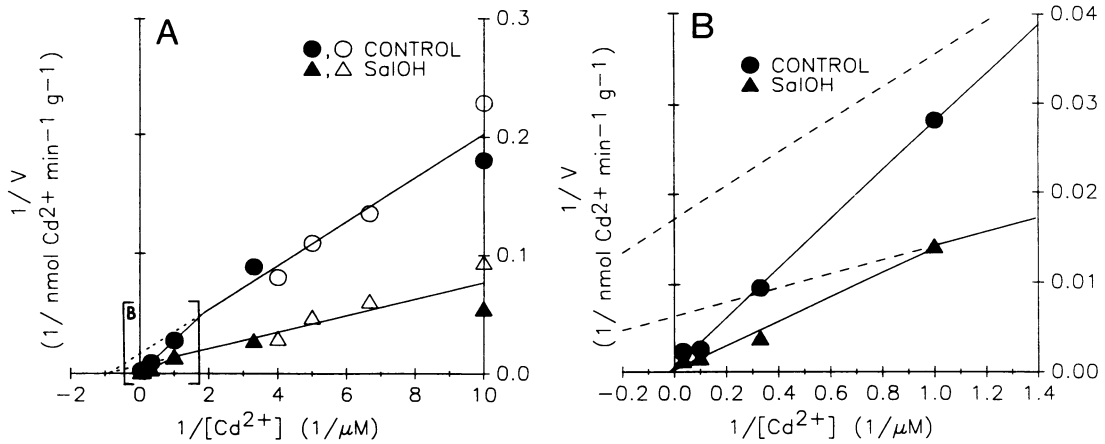

FIG. 5. Lineweaver-Burk plot of $^{109}Cd^{2+}$ uptake for cells grown in the absence (control) or presence of 20 mM salicyl alcohol (SalOH). (A) Open and closed symbols represent data from two experiments. (B) The indicated portion of panel A expanded to show the data at high $^{109}Cd^{2+}$ concentrations. The dotted lines are carried over from panel A so that the slopes at the different $^{109}Cd^{2+}$ concentrations can be compared.

A more comprehensive analysis of uptake was carried out with cells grown in LB broth containing 20 mM salicyl alcohol. Salicyl alcohol was used instead of salicylate to avoid the possibility of complicating weak-acid effects. The results of two sets of experiments are presented as Lineweaver-Burk plots in Fig. 5. For the control cells, high-affinity (Fig. 5A) and low-affinity (Fig. 5B) uptake systems could be distinguished, with calculated K_ms of 1.0 and 52 μM Cd^{2+} and V_{max}s of 0.059 and 1.5 μmol of Cd^{2+} per min per g (dry weight) of cells, respectively. For cells grown in 20 mM salicyl alcohol, the V_{max}s of both uptake systems were increased by about 2.5-fold while the K_ms remained the same (Fig. 5). Thus, the effect of salicyl alcohol was to increase the activity of both uptake systems.

DISCUSSION

Since the accumulation of Cd^{2+} by *E. coli* occurs via an active transport process (10) (Fig. 4B), it was anticipated that accumulation would depend on the membrane potential. Therefore, salicylate and other weak acids which increase the membrane potential of *E. coli* were expected to increase the susceptibility of the bacteria to Cd^{2+}. Indeed, salicylate and $CdCl_2$ were synergistically inhibitory for *E. coli* (Fig. 1 and 2). Furthermore, the cells were found to be more susceptible to Cd^{2+} at pH 8.4 than at 7.4 (Fig. 1A and B). This may be expected if Cd^{2+} uptake is responsive to membrane potential which is about −140 mV at pH_{ext} 8.4 and about −130 mV at 7.4 (4). However, at pH 6.4 the membrane potential (−80 mV) is much lower than at pH 7.4, yet the susceptibility to Cd^{2+} by control cells was only slightly lower (compare Fig. 1C and B). Thus, Cd^{2+} lethality was not directly correlated with membrane potential.

Higher concentrations of salicylate were required for the synergistic effects with $CdCl_2$ at the higher pH_{ext}s. For a given concentration of a membrane-permeating weak acid, the lipophilic protonated form and the dissociated anion inside the cell are expected to decrease 10-fold for each unit increase in pH_{ext} provided that the cell maintains a constant internal pH (pH_{int}) (20). Thus, since 1 mM salicylate was sufficient to decrease the EOP 10^5-fold at pH_{ext} 6.4, we would expect 10 mM to be necessary at pH_{ext} 7.4 and 100 mM to be necessary at pH_{ext} 8.4. However, as little as 2.5 mM salicylate was effective at pH_{ext} 7.4 and 10 mM was effective at pH_{ext} 8.4.

The potency of related compounds tested (Table 1) indicated an inverse relation between the pK_a and the effectiveness of the acid (in order of effectiveness, salicylate [pK_a, 3.0] > acetyl salicylate [pK_a, 3.5] > benzoate [pK_a, 4.2]). However, the higher pK_as of acetate (4.8) and of *p*-hydroxy benzoate (4.6) did not explain their lack of synergy with Cd^{2+} (Table 1). First, even at pH 6.5, *p*-hydroxy benzoate and acetate are about 98% dissociated. Second, in experiments using similar concentrations of acetate and the same or related bacterial strains, acetate was effective in sensitizing *E. coli* to aminoglycosides (1) and in inducing resistance to chloramphenicol (21). Furthermore, cinnamate, with a pK_a of 4.4, was much more effective than benzoate (pK_a, 4.2 [Table 1]). These data suggest that it is the structure of the effective compounds and not necessarily their acidity that is important. Strong evidence for this conclusion comes from the observation that salicyl alcohol is synergistic with Cd^{2+} (Fig. 3). Since this is a nonacidic molecule, it is unlikely that it directly affects the membrane potential of the cells. Therefore, it appears that the benzyl structure itself is the critical factor.

We recently reported that acetate, benzoate, salicylate, acetyl salicylate, and salicyl alcohol potentiate the lethal effects of kanamycin (1). Two mechanisms were proposed. One, indicated by the effectiveness of acetate at low pH_{ext}, was to raise the membrane potential. Here, however, we found that acetate did not increase Cd^{2+} susceptibility (Table 1) or Cd^{2+} accumulation (data not shown). Thus, it is possible that any effect of acetate to increase uptake by increasing membrane potential is masked by a second effect to inhibit Cd^{2+} accumulation.

The other mechanism is unknown but is related to the benzyl structure, since growth in salicyl alcohol potentiated kanamycin susceptibility (1). Since benzyl derivatives are potent effectors of diverse functions in *E. coli*, ranging from chemotaxis to transcription (12, 13, 20–23) any of a number of mechanisms might be implicated. However, the similarities between the effects of salicylate and salicyl alcohol on susceptibility to Cd^{2+} and their effects on susceptibility to kanamycin suggest that a common mechanism is involved.

The kinetic analysis of Cd^{2+} uptake reported here indicates the existence of high- and low-affinity uptake systems (Fig. 5). Laddaga and Silver (14) had previously observed a single uptake system (K_m, 2.1 μM Cd^{2+}; V_{max}, 0.83 μmol/

min/g) for cells grown in a triethanolamine minimal medium; for cells grown in tryptone broth, uptake did not follow Michaelis-Menten kinetics. The uptake system found in their minimal medium appears to be similar to our high-affinity system, with a K_m only twice that described here. While the V_{max} that they measured was about 14-fold higher, it is not unreasonable to expect that a higher V_{max} would be found for cells grown in minimal medium, since such cells are 30 times more sensitive to Cd^{2+} than cells grown in broth (14, 19). Our results show that the V_{max}s of the high-affinity system and of the newly observed low-affinity system are both increased by about 2.5-fold in cells grown and assayed in the presence of 20 mM salicyl alcohol (Fig. 5) or 5 mM salicylate (Fig. 4A). The correlated increases in uptake and toxicity of Cd^{2+} due to growth in salicylate or salicyl alcohol suggest that the increased uptake is responsible for the increased toxicity.

Effects similar to those observed here have been observed for cells expressing several *tet* (resistance) genes at high levels. In such cells, susceptibility to Cd^{2+} (7), kanamycin (8), gentamicin (15), and fusaric acid (2) is increased, as is the uptake of potassium (5, 8) and gentamicin (16). With the exception of fusaric acid, all of these substances are positively charged. Griffith and coworkers have shown that these collateral effects of *tet* seem to depend on the presence of certain portions of the Tet protein in the inner membrane of the cells and not on the presence of tetracycline or of the complete protein capable of tetracycline efflux (8). Since the membrane potential was not significantly increased by the presence of the Tet protein (16), it was proposed that Tet might increase uptake either by enhancing the activity of specific carriers or by somehow reducing the membrane potential requirement. Although we have not observed increased sensitivity to fusaric acid in salicylate-grown cells (unpublished results), the effects of salicylates and of Tet proteins on uptake of and susceptibility to positively charged antimicrobial agents are remarkably similar. Therefore, we propose that a common factor (such as the anionic transporter proposed by Bryan and Kwan [3] for aminoglycoside uptake) for uptake of various cations may be enhanced by growth in salicylates or by the presence of the Tet protein.

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