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Effect of colicins Ia and E1 on ion permeability of liposomes

(aqueous channels/voltage independence/gramicidin/membrane vesicles)

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ABSTRACT **Colicins Ia and E1 are shown to inhibit the formation and bring about the collapse of a potassium diffusion potential imposed across the membrane of liposomes prepared from soybean or *Escherichia coli* phospholipids. Such depolarization results from a colicin-induced increase in membrane ion permeability. Colicins E2 and E3 do not depolarize such membranes. In addition to the colicin Ia-induced rapid efflux of preloaded rubidium, sodium, phosphate, or choline from liposomes, a slower efflux of preloaded sucrose or glucose 6-phosphate occurs. However, treated liposomes do not leak inulin or dextran, demonstrating that the effects of E1 and Ia are not due to a general disruption of membrane structure. The fact that colicin-induced ion efflux is observed in the complete absence of a membrane potential shows that the action of these colicins on liposomes is not voltage dependent. These results provide strong evidence that the depolarization of *E. coli* cells by colicins Ia and E1 results from a colicin-induced increase in membrane permeability to ions. It is proposed that this is brought about by the direct interaction of the colicin molecules with the bacterial cytoplasmic membrane.**

Colicins E1, Ia, and K inhibit energy metabolism in sensitive *Escherichia coli* cells (1–3). The effects of these colicins have recently been shown to be derived from their ability to disrupt the energized state of the bacterial cytoplasmic membrane by inducing membrane depolarization (4–6).

We have reported that colicin Ia-treated cells, as well as membrane vesicles prepared from such cells, are unable to generate a membrane potential (6). We have further shown that membrane vesicles prepared from untreated cells can be depolarized by colicin under conditions in which colicin Ia molecules are mechanically incorporated into membrane structure by subjecting a mixture of colicin and vesicles to a single freeze-thaw cycle (7). By this technique, it has been possible to demonstrate that membrane vesicles prepared from sensitive, resistant (do not adsorb Ia), and tolerant (adsorb Ia, but insensitive to its action) cells are equally sensitive to Ia action. It was concluded that colicin Ia-induced membrane depolarization results from the direct interaction of the colicin molecule with the cytoplasmic membrane. In whole cells it is likely that access of colicin molecules to the cytoplasmic membrane is mediated by the colicin Ia outer membrane receptor. Tolerant mutants are considered to be blocked in the mechanism whereby the colicin is translocated to its inner membrane target.

The above results raise the intriguing possibility that the colicin Ia molecule itself has ionophore-like properties and might function as an ion carrier or channel former. If this is the case, it is expected that the colicin might be effective in artificial membrane systems. Indeed, Schein *et al.* (8) have carried out a series of important and elegant experiments which convincingly demonstrated that colicins E1, Ia, and K increase the conductivity of planar phospholipid bilayer membranes prepared from soybean phospholipids. Furthermore, these authors made the remarkable discovery that these colicins induced the formation of voltage-dependent aqueous channels in this artificial membrane system. These authors proposed that *in vivo* these colicins function by forming voltage-dependent channels in the bacterial cytophasmic membrane.

In the present study, we have examined the effect of colicin Ia and several other colicins on the ion permeability of liposomes. We report that colicins Ia and E1 (but not E2 or E3) depolarize an artificially generated membrane potential in such liposomes by inducing the transmembrane flow of ions. During this study, Kayalar and Luria (9) have reported that liposomes treated with colicin K become leaky to ions and low molecular weight molecules.

MATERIALS AND METHODS

Formation of Liposomes. The method used was modified from the procedure of Kasahara and Hinkle (10). Forty-five milligrams of acetone-washed soybean or *E. coli* phospholipids in 0.1 M potassium or sodium phosphate (pH specified) was flushed with nitrogen gas and dispersed by sonication at 25–30°C for 10 min in a bath-type sonicator. Aliquots (500 μl) of this dispersion were next frozen in ethanol/dry ice, thawed at room temperature, and sonicated for 15 sec. Magnesium sulfate was next added to a final concentration of 0.5 mM unless otherwise specified. This procedure leads to the formation of liposomes having an internal space of 1.2–2.0 μl/mg of phospholipids by the filtration method reported by Eisenbach *et al.* (11), with [^{14}C]sucrose. For formation of liposomes containing radioactive ions, the relevant radioactive ion was added to the preparation prior to the freeze-thaw step and the second sonication.

Determination of Colicin-Induced pH Change. Liposomes (100 mg of phospholipids per ml) were prepared in the presence of 0.1 M potassium [^{32}P]phosphate (pH 7.5) (7×10^3 cpm/μl) and dialyzed against 0.1 M choline phosphate (pH 5.0) at 10°C. The choline phosphate outer solution was changed several times until the pH change and radioactivity in the dialysate were negligible. A portion of the liposomes was suspended in 0.1 M choline phosphate (pH 5.0) to give a final concentration of about 67 mg of phospholipids per ml. The pH changes were monitored continuously by a pH meter (Radiometer) connected to a recorder.

Materials. [^{3}H]Triphenylmethylphosphonium ($TPMP^+$) bromide and nigericin were from H. R. Kaback. Other radioactive materials were obtained from New England Nuclear or Amersham/Searle. Soybean lipid (L-α-phosphatidylcholine, type II-S), valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and gramicidin were purchased from Sigma.

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Abbreviations: $TPMP^+$, triphenylmethylphosphonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Colicin Ia was purified as reported (12). Purified colicin E1 was obtained from W. A. Cramer and purified colicins E2 and E3 from D. R. Helinski. The appropriate action of all colicins on whole *E. coli* cells was verified. Crude *E. coli* phospholipids were obtained from John Cronan, Jr., and used after acetone precipitation. Bovine serum albumin and RNase A were purchases from Sigma. DNase I was a product of Worthington.

RESULTS

Effect of colicins Ia and E1 on an artificially imposed membrane potential

The effect of colicin on liposomes was examined under conditions in which the generation of a membrane potential could be observed. Such a potential can be induced by the addition of the potassium ionophore, valinomycin, to liposomes prepared so as to have a transmembrane potassium diffusion gradient. The formation of the membrane potential can be monitored by a determination of the distribution of the lipophilic cation, $TPMP^+$. As can be seen in Fig. 1*A*, although dilution of liposomes containing internal potassium phosphate into choline phosphate so as to generate a potassium concentration gradient does not result in the uptake of $TPMP^+$, subsequent addition of valinomycin leads to accumulation of this cation, indicating the generation of a membrane potential. The magnitude of this membrane potential was calculated to be −114 mV (inside negative). The generation of this potential is dependent on the presence of a potassium concentration gradient across the membrane; liposomes diluted into potassium phosphate instead of choline phosphate do not take up $TPMP^+$ in response to valinomycin addition. Addition of colicin Ia immediately after dilution into choline phosphate drastically decreases the subsequent level of $TPMP^+$ taken up after valinomycin addition.

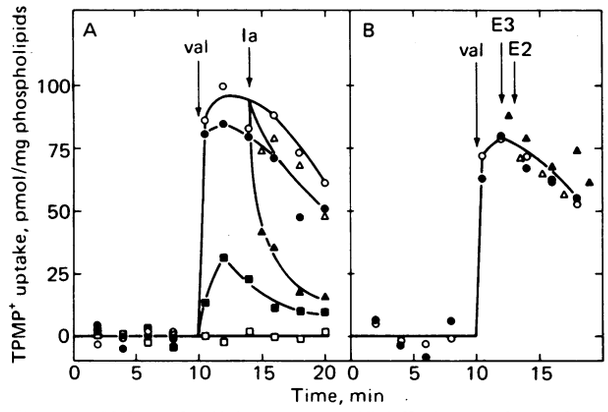

FIG. 1. Effect of colicins on an artificially imposed membrane potential. Aliquots (2 μl) of liposomes prepared in the presence of 100 mM potassium phosphate (pH 5.4) were diluted into 200 μl of either 100 mM choline phosphate (pH 5.4) or 100 mM potassium phosphate (pH 5.4) each containing 0.5 mM magnesium sulfate and [^{3}H]$TPMP^+$ (final concencration 1 μM; specific acitivity 4.36 Ci/mmol). Incubation was continued for 10 min at 25°C prior to addition of valinomycin (final concentration 5.4 μM). At the indicated times, incubation was terminated by addition of 2 ml of 100 mM choline phosphate (pH 5.4). Each total sample was next filtered through Millipore Celotate filters (0.2 μm pore size) and washed with dilution buffer. Colicins were added either immediately after the initial dilution of liposomes or after addition of valinomycin (val). (*A*) Except for the curve in which liposomes were diluted into potassium phosphate buffer (□), all other curves represent liposomes diluted into choline phosphate buffer. ○, No addition. Ia (5 μg/ml, final concentration) added at 0 (■) or at 14 (▲) min; Ia inactivated for 5 min in boiling water and added at 0 (●) or at 14 (△) min. (*B*) ○, No addition. E3 (final concentration 5.2 μg/ml) added at 0 (●) or at 12 (▲) min; E2 (final concentration 7.5 μg/ml) added at 13 min (△).

Furthermore, addition of the colicin after the generation of a membrane potential leads to its rapid dissipation. On the other hand, heat-inactivated colicin Ia has little effect on the ability of such liposomes to generate a membrane potential whether added prior to or after its generation.

Whereas colicins Ia and E1 disrupt the energized membrane state in treated cells, colicins E2 and E3 specifically induce DNA breakdown and ribosome inactivation, respectively (13, 14). Therefore, if the effect of colicin Ia on liposomes observed in Fig. 1*A* reflects *in vivo* colicin action, one would expect similar results with colicin E1, but not with E2 or E3. In fact, the action of colicins E1 was identical to that seen with colicin Ia (data not shown), whereas colicin E2 or E3 had no effect on the ability of such liposomes to generate a membrane potential (Fig. 1*B*). Addition of E2 or E3 at a concentration that is 20-fold higher than the minimum concentration required to observe the action of colicin Ia or E1 on liposomes had no effect.

Effect of colicins Ia and E1 on ion permeability

In order to determine the mechanism of the colicin-induced depolarization of liposomes, we examined the colicin-induced movement of the major ions of this artificial membrane system under the conditions used for the experiments described in Fig. 1. For this purpose, liposomes were loaded with individual radioactive ions, and the ability of such liposomes to retain such ions after dilution into choline phosphate buffer was determined.

Efflux of Rubidium-86. Because of its longer half-life, $^{86}Rb^+$ was used instead of radioactive potassium. In contrast to untreated vesicles, which are essentially impermeable to $^{86}Rb^+$ (Fig. 2*A*), addition of colicin Ia immediately after dilution of liposomes into choline phosphate buffer leads to immediate $^{86}Rb^+$ efflux. By 10 min, Ia-treated liposomes had lost 75% of their $^{86}Rb^+$ content. The amount of internal $^{86}Rb^+$ remaining at this time closely correlates with the reduced level of valinomycin-dependent $TPMP^+$ accumulation (70% inhibition) observed in colicin-treated liposomes (see Fig. 1*A*). Addition of valinomycin to Ia-treated liposomes leads to a loss of Rb^+ that is greater than that observed for liposomes treated with valinomycin alone. Addition of active colicin Ia to liposomes after valinomycin enhances the extent of Rb^+ efflux. Heat-inactivated colicin has little effect whether added immediately after dilution into choline phosphate or after addition of valinomycin. The action of colicin Ia in this system is completely inhibited by antibody against Ia. Addition of lysozyme, RNase, or DNase (all at 100 μg/ml) to liposomes does not lead to Rb^+ efflux. Although both colicin Ia and valinomycin induce $^{86}Rb^+$ efflux, colicin treatment, unlike treatment with the K^+ ionophore, does not lead to the generation of a membrane potential (see Fig. 1*A*). This situation is analogous to whole-cell studies demonstrating that the colicin induces efflux of intracellular potassium yet collapses the electrical potential of the cytoplasmic membrane (6, 15). Thus, the colicin must induce not only the efflux of rubidium (or potassium), but also the movement of other ions in order to compensate for electrical imbalance across the membrane.

Efflux of Phosphate. Phosphate efflux was examined by using [^{32}P]phosphate-containing liposomes diluted into nonradioactive choline phosphate. As can be seen in Fig. 2*B*, addition of colicin Ia to such liposomes leads to immediate [^{32}P]phosphate efflux. In contrast, untreated liposomes retain essentially all their [^{32}P]phosphate during the period of incubation even after addition of valinomycin. Addition of the colicin to valinomycin-treated liposomes leads to immediate efflux of liposomal [^{32}P]phosphate. Heat-inactivated Ia has no effect when added either before or after generation of the membrane potential by valinomycin addition.

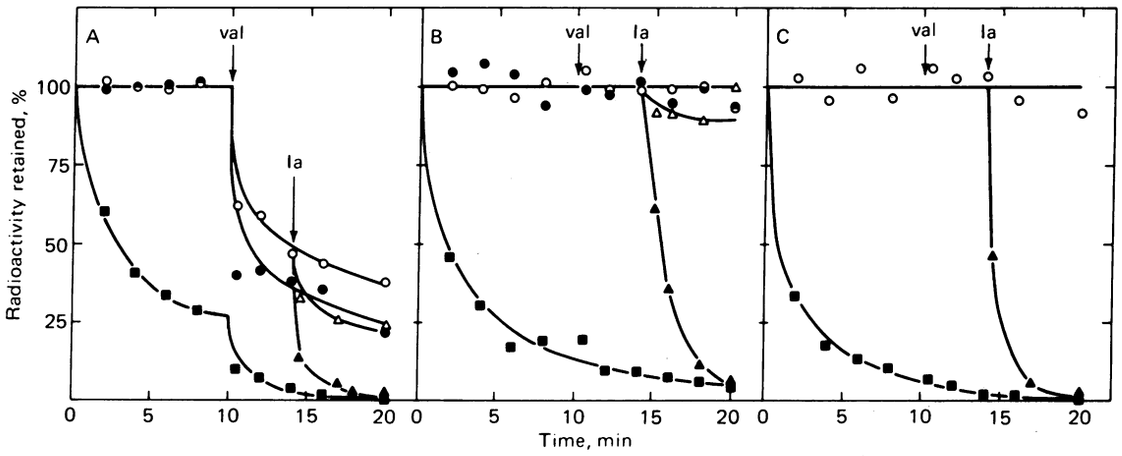

FIG. 2. Colicin Ia-dependent ion efflux. Aliquots (500 μl) of liposomes (45 mg of phospholipid per ml) were subjected to a single freeze-thaw cycle in the presence of ^{86}RbCl (final concentration 5 mM, 2.1 × 10^5 cpm/μl) (*A*), [^{32}P]phosphate (final concentration 100 mM, 5.7 × 10^4 cpm/μl) (*B*), or [^{14}C]choline chloride (final concentration 0.5 mM, 4.1 × 10^4 cpm/μl) (*C*) and sonicated for 15 sec. Aliquots (2 μl) of liposomes were diluted into 100 mM choline phosphate, pH 5.4/0.5 mM magnesium sulfate and incubated at 25°C. Valinomycin (5.4 μM final concentration) was added at 10 min (val). Where indicated, colicin Ia was added at a final concentration of 5 μg/ml. (*A*) $^{86}Rb^+$ efflux. ○, No addition. Ia added at 0 (■) or at 14 (▲) min; heat-treated Ia added at 0 (●) or at 14 (△) min. (*B*) [^{32}P]Phosphate efflux. ○, No additions. Ia added at 0 (■) or at 14 (▲) min; heat-treated Ia added at 0 (●) or at 14 (△) min. (*C*) [^{14}C]Choline efflux. ○, No additions. Ia added at 0 (■) or at 14 (▲) min. The raw cpm obtained were corrected for background by subtracting the limiting amount of radioactivity remaining after prolonged incubation of liposomes with colicin Ia. The backgrounds represented 3.8% (Rb^+), 13.5% (phosphate), and 10% (choline) of the total amount of cpm present in the filtered sampled aliquot. Data are expressed in percent control, which represents liposomes incubated in the absence of colicin. Control values were 3 × 10^4, 6.4 × 10^3, and 7 × 10^3 cpm for $^{86}Rb^+$, [^{32}P]phosphate, and [^{14}C]choline, respectively.

Movement of Choline. As can be seen in Fig. 2*C*, colicin Ia induces the immediate efflux of choline from liposomes to which [^{14}C]choline had been added. As expected, such efflux does not occur upon valinomycin treatment. Because, under the conditions used for this experiment, the concentration of choline is higher outside (about 100 mM) than inside (0.5 mM) the liposomes, the observed efflux of [^{14}C]choline likely represents dilution by exchange with the large excess of external nonradioactive choline which is able to freely move across the membrane after the disruption of liposome permeability by the colicin.

In addition to the colicin-induced efflux of $^{86}Rb^+$, [^{14}C]-choline, [^{32}P]phosphate, and ^{22}Na (data not shown), Ia-treated liposomes also become permeable to sucrose and glucose 6-phosphate. However, in this case, the rates of efflux are at least 1/30th those observed with rubidium. Colicin-dependent increase in liposome permeability to these molecules is not derived from a general disruption of membrane structure. This conclusion is based on the finding that the colicin does not induce efflux of [^{14}C]inulin (M_r 5000) or of [^{3}H]dextran (M_r 20,000).

Effect of colicins E1, E2, and E3 on retention of rubidium by liposomes

Liposomes containing $^{86}Rb^+$ were diluted into choline phosphate containing various amounts of colicins Ia, E1, E2, and E3, and the amount of $^{86}Rb^+$ retained was determined after incubation for 5 min at 25°C. As can be seen in Fig. 3, whereas colicins Ia and E1 elicit efflux, liposomes treated with colicin E2 or E3 retain the cation at all concentrations shown and at colicin levels that are 10-fold higher than those used in this particular experiment (data not shown).

Colicin-induced ion efflux is not voltage dependent

The colicin-induced ion efflux observed in this system proceeds under conditions in which no membrane potential exists across the liposomal membrane. For example, the effluxes observed in Figs. 2 and 3 proceed in the absence of valinomycin and, thus, under conditions of no membrane potential (see Fig. 1). Further evidence for a lack of voltage dependence comes from several observations. (*i*) Colicin-induced efflux occurs under conditions in which the ionic conditions are identical inside and outside the liposomes. (*ii*) The imposition of a membrane potential (valinomycin-dependent K^+ efflux) does not alter the rate of colicin-induced ion efflux. (*iii*) CCCP has no effect on the rate of colicin-induced efflux whether occurring in the presence or absence of a membrane potential. (*iv*) The membrane-permeable anion SCN^- has no effect on the rate of colicin-induced ion efflux whether added to the inside, outside, or both sides of the membrane.

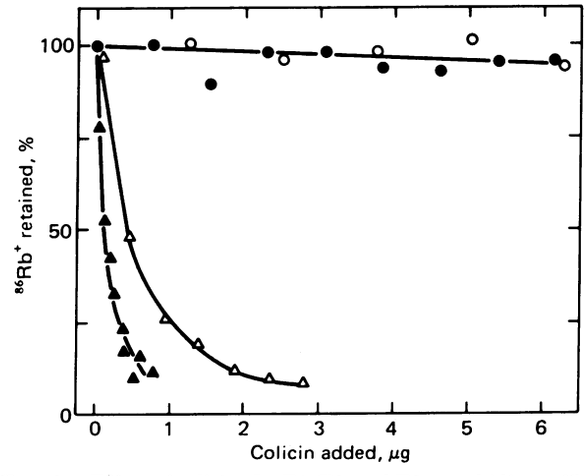

FIG. 3. Effect of colicins Ia, E1, E2, and E3 on rubidium efflux. Liposomes containing $^{86}Rb^+$ (final concentration 5 mM, 1.1 × 10^5 cpm/μl) were as described for Fig. 2. Aliquots (2 μl) were diluted into 100 mM choline phosphate, pH 5.0/0.5 mM magnesium sulfate containing various amounts of colicin. The amount of ^{86}Rb retained by these liposomes was determined after a 5-min incubation at 25°C as described in the legend for Fig. 2. Colicins: ▲, Ia; △, E1; ●, E2; ○, E3. The data were treated and are expressed as for Fig. 2. The control value was 3 × 10^4 cpm; the background was 7%.

Table 1. Colicin Ia-dependent pH change in liposomes

Addition	pH change, units	Loss of ^{32}P, % of untreated
Choline chloride	0.02	0
CCCP (10 μM)	0.18	0
CCCP + valinomycin (7 μM)	0.63	0
Nigericin (0.2 μM)	0.60	0
Colicin Ia (4.5 μg/mg phospholipid)	0.25	36

Liposomes prepared in 0.1 M potassium [^{32}P]phosphate (pH 7.5) were dialyzed against 0.1 M choline phosphate (pH 5.0) and the pH external to the liposomes was monitored continuously at 25°C. Additons were made after 7.5 min of preincubation during which no pH changes were observed. The pH changes were determined at 10 min after addition of reagents. At the end of each experiment, a portion (2 μl) of the liposome suspension was taken for the determination of [^{32}P]phosphate retention as described in the legend for Fig. 2.

Effect of colicin Ia on liposome permeability to protons

From the above results, it is obvious that the colicin-induced increase in liposome permeability is rather nonspecific. It was, thus, expected that such membranes would be permeable to protons after colicin treatment. To test this possibility, we resuspended liposomes containing 0.1 M choline phosphate (pH 7.5) in 0.1 M choline phosphate (pH 5.0) and monitored the external pH. As shown in Table 1, the subsequent addition of choline chloride caused little change in the external pH. In contrast, the proton ionophore CCCP led to a small increase in external pH which was stimulated by the addition of valinomycin. Under these conditions, the CCCP-induced proton influx is electrogenic and restricted by its own movement. Stimulation by valinomycin is, probably, due to the relief of this restriction by potassium efflux. The K^+/H^+ ionophore, nigericin, causes rapid and complete equilibrium of pH across the membrane. Addition of colicin Ia (in choline chloride) led to an increase in the external pH, presumably due to proton influx. [^{32}P]Phosphate efflux was also determined under these same conditions and found to occur only when Ia was added. These results suggest that colicin Ia does induce an increase in proton permeability.

In contrast to the results seen here, we have previously observed an increase in the transmembrane ΔpH in colicin Ia-treated whole cells (6) or in membrane vesicles prepared from *E. coli* (7). This led us to conclude that in these cases membrane permeability to protons does not change after colicin treatment.

Table 2. Membrane potential and ΔpH in gramicidin-treated *E. coli* membrane vesicles

Gramicidin	Assay pH	$\Delta\psi$, mV	ΔpH, unit mV	$\Delta\bar{\mu}H^+$, mV
−	5.5	−64	2.01 −118	−182
+	5.5	−15	2.42 −143	−158
−	6.6	−76	1.20 − 71	−147
+	6.6	−28	1.79 −105	−133

Membrane vesicles were prepared from *E. coli* K-12 strain JK 140 by the method of Kaback (17) and resuspended in 50 mM potassium phosphate (pH 5.5 or 6.6) containing 10 mM magnesium sulfate to give a final concentration of 4.8 mg of membrane protein per ml. Aliquots (800 μl) of membrane vesicles were incubated for 5 min with or without gramicidin A (final concentration 0.2 μg/ml) at room temperature prior to the start of experiment. The membrane potential ($\Delta\psi$) and ΔpH were calculated as described (7) by flow dialysis at an initial pH of 5.5 or 6.6. Vesicles were energized by the addition of the artificial electron donor system, 20 mM ascorbate plus 0.1 mM phenazine methosulfate. The protonmotive force, $\Delta\bar{\mu}H^+$, was calculated as $\Delta\psi - 58.8\ \Delta pH$ according to Mitchell (18).

In an attempt to resolve the discrepancy, we have examined the ability of whole-cell-derived membrane vesicles to generate a transmembrane ΔpH after treatment with gramicidin A, which facilitates the diffusion of monovalent cations across lipid bilayer membranes by forming channels (16). The results presented in Table 2 show that gramicidin-treated vesicles exhibit a reduced capacity for generation of $\Delta\psi$, yet manifest an increased ΔpH. This is identical to the situation seen with vesicles treated with either colicin Ia or valinomycin (7). One possible explanation for these results is that although gramicidin makes vesicle membrane permeable to ions, including protons, the induced proton flux is too small to collapse ΔpH due to the low concentration of protons in the system (less that 3.2 μM). One can make a similar argument for colicins Ia and E1.

DISCUSSION

The observed depolarization and ion efflux were induced only by colicins E1 and Ia, which inhibit energy metabolism in whole cells. Colicins E2 and E3 were totally inactive under all conditions tested. Specificity of action was further demonstrated by the finding that antibody against Ia specifically inhibited colicin Ia-dependent $^{86}Rb^+$ efflux.

Although various proteins increase the permeability of liposomes to Na^+ (19) and glucose (20), the reported concentrations of proteins used in these studies were several orders of magnitude higher and the rates of efflux much slower than those observed in colicin-dependent ion efflux. For example, whereas 0.4 mM lysozyme caused the efflux of 40% of the total internal $^{22}Na^+$ content in 1 hr (19), we observed 90% efflux of $^{86}Rb^+$ within 15 sec after addition of 30 nM colicin Ia to a similar amount of lipsomes. Indeed, in our system addition of lysozyme (7 μM), DNase (3 μM), or RNase (7 μM) did not lead to detectable loss of $^{86}Rb^+$.

Colicin Ia conducts ion movements rather nonselectively, inducing the efflux of rubidium, sodium, phosphate, and choline. However, the colicin does not cause a general disruption of membrane structure; treated liposomes remain impermeable to dextran and inulin. The observed slow leakage of glucose 6-phosphate in Ia-treated liposomes is in contrast to our finding that colicin Ia-treated whole cells manifest enhanced glucose 6-phosphate transport as a result of the colicin-induced increase in ΔpH (6). It is possible that if leakage of glucose 6-phosphate and protons (see below) does occur in treated whole cells, it is small enough to be masked by the increased rate of glucose 6-phosphate uptake resulting from an increase in ΔpH as a result of the collapse in the membrane potential.

A discrepancy exists between the colicin-induced proton permeability seen in liposomes and the colicin-induced increase in ΔpH observed in whole cells and vesicles. In view of our finding that gramicidin collapses the membrane potential yet increases the ΔpH in membrane vesicles (see also ref. 21), we feel it most likely that the colicin does, indeed, promote proton permeability in whole cells and vesicles, but that the flux of protons is very small compared to the rate of flux of other ions in the system (such as K^+) which are present in much higher concentration. Schein *et al.* (8) have speculated that the colicin K-induced proton flux across the membrane of whole cells would be too small to collapse ΔpH because of the low concentration of protons compared to the large intracellular buffering capacity.

Although Schein *et al.* (8) have reported that colicins K, E1, and Ia act by forming voltage-dependent channels in planar phospholipid bilayer membranes formed from soybean phospholipids, no voltage dependence was observed in the system reported in this paper. Furthermore, using a liposome system similar to ours, Kayalar and Luria (9) have reported that the

colicin K-induced alteration in membrane permeability does not require a membrane potential. The basis for discrepancy between the results obtained in liposomes and the planar membrane system remains to be elucidated.

Because the experiments reported here were carried out with highly purified colicins Ia and E1, it is concluded that colicin molecules *per se* can induce membrane leakiness without any contribution from membrane protein components. The results presented here add further support to our previously described (7) notion of the mode of action of colicin Ia, which involved colicin Ia-induced membrane depolarization via direct interaction between colicin molecules and the bacterial cytoplasmic membrane.

The molecular details whereby colicins Ia and E1 alter membrane permeability remain to be clarified. Although it is possible that these molecules span the cytoplasmic membrane, forming aqueous "channels" alone, it is also possible that these colicins indirectly affect membrane permeability by altering the state of membrane phospholipids. For colicin action on whole cells, membrane proteins might make some contribution to the overall process of colicin-induced membrane depolarization.

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