JOURNAL OF BACTERIOLOGY, Oct. 1972, p. 148-160
Vol. 112, No. 1

Effects of Colicins E1 and K on Permeability to Magnesium and Cobaltous Ions

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Received for publication 22 May 1972

The energy-dependent exchange of intracellular Mg^{2+} with extracellular Mg^{2+} or Co^{2+} is inhibited by colicin E1 and, less strongly, by colicin K. Treatment with either colicin causes a net loss of intracellular Mg^{2+}. This loss begins immediately in cells treated with colicin E1, but in colicin K-treated cells the onset of Mg^{2+} loss is delayed 1 to 10 min, depending upon the temperature and the multiplicity of colicin K. Both colicins differ from chemical inhibitors of energy-yielding metabolism; energy poisons block transport of Mg^{2+} and Co^{2+}, but both colicins increase passive permeability to Mg^{2+} and Co^{2+}. Inhibitors of energy-yielding metabolism (and of Mg^{2+} exchange) block the initiation of Mg^{2+} loss by either colicin, but do not stop colicin-promoted efflux once it has begun. Colicin E1 added before colicin K prevents the more rapid Mg^{2+} efflux characteristic of colicin K-treated cells. Quantitative comparisons of the effects of colicins E1 and K upon permeability to Mg^{2+} and Co^{2+} lead us to conclude that the two colicins are not identical in their mode of action.

It is perhaps surprising that two oppositely charged proteins—colicin E1 being cationic (25) and colicin K anionic (14) at neutral *p*H—which adsorb to different receptors in the cell surface (21, 24) should have such similar effects on many cellular processes. Yet, both colicins inhibit synthesis of deoxyribonucleic acid, ribonucleic acid, and protein (21); both inhibit transport of amino acids (16), β-galactosides (16, 21), and potassium (7, 8, 13, 28); neither inhibits transport of α-methylglucoside (9) or mediated diffusion of *o*-nitrophenylgalactoside into the cell (9); neither inhibits respiration (10); and metabolism of glucose is affected similarly by both colicins (10). Although both colicins lower cellular adenosine triphosphate levels (8, 9, 13), normal levels can be artifically maintained without overcoming the effects of colicin E1 on transport and macromolecular synthesis (8). Because the colicins also inhibit transport in membrane vesicles (2), a direct effect on the membrane has frequently been postulated.

Mg^{2+} transport affords the opportunity of distinguishing between an action of colicins on the membrane that selectively damages the permeability barrier and an uncoupling of metabolic energy from transport. Whereas cells that have been treated with an energy poison release intracellular pools of amino acids and galactosides, they retain intracellular Mg^{2+}, and exchange of Mg^{2+} across the membrane is inhibited (17, 26, 27). If a colicin acts as an uncoupler, Mg^{2+} exchange will be inhibited in colicin-treated cells; if the colicin weakens the permeability barrier, exchange of Mg^{2+} may not be inhibited and the colicin-treated cell may be unable to retain Mg^{2+}. Either mode of action could lead to release of accumulated amino acids or galactosides.

Transport of Co^{2+} may also be used to distinguish between uncoupling of energy from transport and damage to the permeability barrier. Co^{2+} is a competitive inhibitor of Mg^{2+} transport and a substrate for one Mg^{2+} transport system (19, 20). Normal transport of Co^{2+} is inhibited by uncouplers of energy metabolism and presumably will be inhibited by colicins that uncouple energy metabolism. Mutants lacking Co^{2+} transport will be made permeable to Co^{2+} by colicins that damage the permeability barrier.

By using transport of Mg^{2+} and Co^{2+}, we have found differences between the action of colicins E1 and K. Both colicins inhibit the normal transport systems and increase passive

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permeability. Colicin E1, however, inhibits transport more strongly than colicin K and damages the permeability barrier less. Both colicins cause efflux of Mg^{2+}, but with different kinetics. The most striking evidence for differences between the colicins is that prior treatment with colicin E1 prevents the action of colicin K on the cell.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* strain LA324 (F^-, *lacI*, *pro*, *thi*, *str*) was from S. E. Luria's collection. Strain DN53 was derived from LA324 after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) and selection for resistance to Co^{2+} in the absence of Mg^{2+}; it does not transport Co^{2+} (20). In previous communications, LA324 and DN53 were called A324 and cor-53, respectively (17, 19, 20). *tolA* and *tolC* derivatives (known as *tol* II and *tol* VIII in reference 18) were isolated from LA324 without mutagenesis by selection with colicin E1 and screening for detergent and dye sensitivity (18).

Bacteria were grown at 37 C with shaking in medium N (19) containing 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.4, 0.5% sodium DL-lactate as carbon source, 0.1 mg of L-proline/ml, 2 mg of thiamine/liter, and $MgSO_4$ or $^{28}MgCl_2$ at 0.04 to 0.1 mM.

Measurement of transport. Mg^{2+} exchange and Co^{2+} uptake were assayed as previously described (17, 19), except that sodium DL-lactate (0.5%) served as energy source. Cells labeled with $^{28}Mg^{2+}$ could be stored on ice in buffer N (medium N without thiamine, proline, lactate, or Mg^{2+}) for at least 2 days without losing their ability to retain or exchange Mg^{2+}, to take up Co^{2+}, or to be killed by colicins. Cells were used within 1 day of harvest.

Preparation of colicins. Colicin E1 was prepared from strain Y20(E1) grown in 1 liter of LB broth supplemented with 0.4% glucose and induced with mitomycin C (1 mg/liter) according to Schwartz and Helinski (25). The colicin after extraction with buffered 1 M NaCl was dialyzed against 1 liter of water and then two 2-liter volumes of 0.15 M NaCl containing 10 mM potassium phosphate, *p*H 7.0, to remove contaminating Mg^{2+}.

Colicin K was prepared according to Fields and Luria (9). It was diluted sufficiently in buffer N so that contaminating Mg^{2+} in transport assays was negligible. Both colicins were stored frozen in small samples. The colicins were titered by mixing diluted samples with cells of strain LA324 and plating the surviving cells on LB agar. A multiplicity (m) of killing units per cell gives a proportion of survivors equal to e^{-m}. The colicin E1 preparation contained 10^{11} killing units/ml and the colicin K preparation 10^{14} killing units/ml.

Radioactive materials. $^{28}Mg^{2+}$ was purchased from Brookhaven National Laboratory, Upton, New York. $^{60}CoCl_2$ and [^{14}C]thiomethylgalactoside were purchased from New England Nuclear, Boston, Mass.

RESULTS

Colicin K causes efflux of Mg^{2+}. When bacteria containing $^{28}Mg^{2+}$ are suspended in Mg^{2+}-free medium, only a small amount of $^{28}Mg^{2+}$ is lost from the cells (17, 19, 26). The cells used in Fig. 1 illustrate the maximum acceptable leakage of $^{28}Mg^{2+}$. If $^{24}Mg^{2+}$ is present in the medium, exchange with cellular $^{28}Mg^{2+}$ takes place and the radioactive isotope appears in filtrates of the cell suspension. (We use the term "exchange" to mean equal fluxes of Mg^{2+} into and out of the cell. No molecular mechanism for equating entry and exit is suggested. The observation that the cellular Mg^{2+} content does not change under the conditions of measuring exchange implies that entry equals efflux [27]. Entry of $^{28}Mg^{2+}$ therefore occurs by exchange with intracellular $^{24}Mg^{2+}$. Exit of $^{28}Mg^{2+}$ from cells not treated with colicins depends on extracellular $^{24}Mg^{2+}$ and occurs by exchange. Colicin-treated cells suffer a

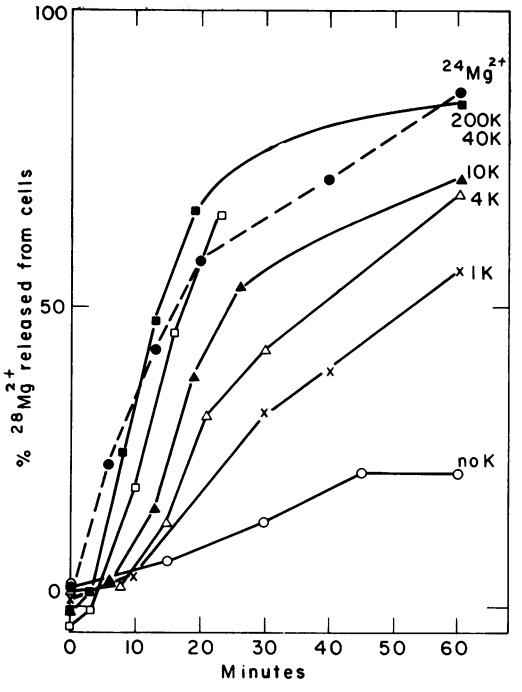

FIG. 1. *Efflux of $^{28}Mg^{2+}$ at different multiplicities of colicin K and exchange with $^{24}Mg^{2+}$. Cells of strain LA324 were grown with $^{28}Mg^{2+}$ and resuspended in buffer N + lactate at 10^8 cells/ml at 25 C. Either colicin K or 1 mM $^{24}MgSO_4$ was added at zero time. Samples were filtered through Millipore filters (pore size 0.45 μm) and radioactivity in the filtrates was determined (17, 19). Colicin multiplicities were calculated from survival at 30 min. Symbols: ●, $^{24}Mg^{2+}$; ○, no additions; colicin K at a multiplicity of: ■, 200; □, 40; ▲, 10; △, 4; ×, 1.*

net loss of Mg^{2+}, demonstrable as exit of $^{28}Mg^{2+}$ in the absence of extracellular $^{24}Mg^{2+}$. In addition to the net outward flux, entry of $^{28}Mg^{2+}$ can occur by exchange, and exit of intracellular $^{28}Mg^{2+}$ can be stimulated by extracellular $^{24}Mg^{2+}$, presumably also by exchange.) When colicin K is added to sensitive bacteria containing $^{28}Mg^{2+}$, net efflux of the $^{28}Mg^{2+}$ occurs (Fig. 1). Net efflux may be distinguished from Mg^{2+} exchange because efflux does not require $^{24}Mg^{2+}$ in the medium. Colicin K-promoted efflux does require extracellular ions; it occurs in buffer N or 0.1 M Tris-hydrochloride, *p*H 7.4, but not in isotonic sucrose (data not shown). Boiled colicin preparations do not promote $^{28}Mg^{2+}$ efflux, which demonstrates that $^{24}Mg^{2+}$ contaminating the colicin preparations is not responsible for the loss of $^{28}Mg^{2+}$ from cells treated with active colicin K (*unpublished data*). K-promoted Mg^{2+} efflux begins only after a lag period that is longer at lower colicin multiplicity, although adsorption of the colicin is essentially complete within the first 1 or 2 min (data not shown). The cells are killed with single-hit kinetics, yet adsorption of more than one killing unit increases both the rate at which the cell becomes unable to retain Mg^{2+} and the rate of Mg^{2+} efflux.

Both the length of the lag period and the rate of Mg^{2+} efflux are affected by temperature (Fig. 2). Lower temperatures prolong the lag and, at or below 12 C, even high multiplicities of colicin K fail to cause efflux of Mg^{2+} within 40 min (Fig. 2a). In contrast, at temperatures of 8 and 12 C, Mg^{2+} exchange takes place in control cells (Fig. 2b). The longer times required for the same amount of colicin to initiate efflux at lower temperatures seem to imply that the colicin causes some temperature-dependent process that ultimately makes the cell unable to retain Mg^{2+}. The rate of this process at any given temperature can be increased by increasing the multiplicity of colicin K, even beyond the single hit necessary to kill the cell. Perhaps the maximum amount of colicin K that can act on a cell, determined by the number of colicin receptors, is insufficient to make the cell unable to retain Mg^{2+} within 40 min at 12 C.

Extent of colicin K-promoted efflux of Mg^{2+}. The amount of Mg^{2+} remaining in K-treated cells reaches a steady level after 60 min. If by this time each dead cell had lost all its initial Mg^{2+}, the proportion of the initial cellular Mg^{2+} in a population after treatment with colicin K would be equal to the proportion of surviving cells. Instead, the proportion of cellular Mg^{2+} after 60 to 80 min of treatment with low multiplicities of colicin K is larger than the proportion of survivors, as if each dead cell has lost half its Mg^{2+} (Fig. 3). At multiplicities higher than four, however, much more than 50% of the initial cellular Mg^{2+} is lost (Fig. 1, 2a).

Action of colicin K before initiation of Mg^{2+} efflux. Colicin K inhibits galactoside transport before any efflux of Mg^{2+} occurs (Fig. 4). The bacteria, labeled with $^{28}Mg^{2+}$ by growth in medium containing $^{28}Mg^{2+}$, were loaded with thiomethyl-β-galactoside (TMG) for 20 min before the addition of colicin K.

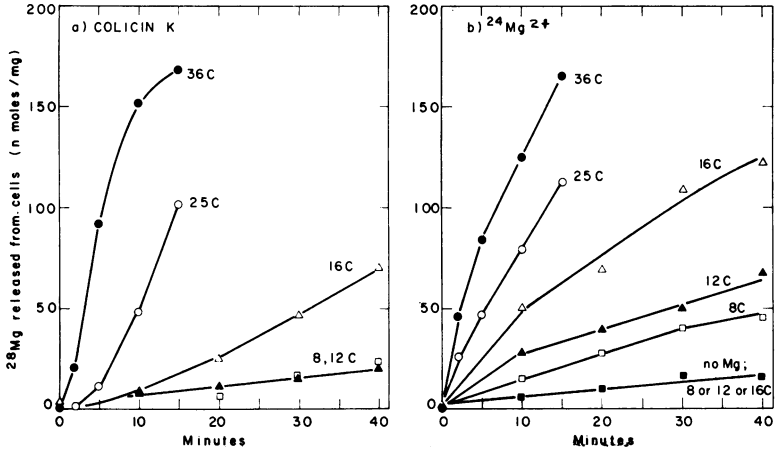

FIG. 2. *Colicin K-promoted Mg^{2+} efflux and Mg^{2+} exchange at different temperatures. Performed as in Fig. 1, except that the cell suspensions were incubated at 36, 27, 16, 12, or 8 C. a) Colicin K, multiplicity 200; b) 1 mM $^{24}Mg^{2+}$.*

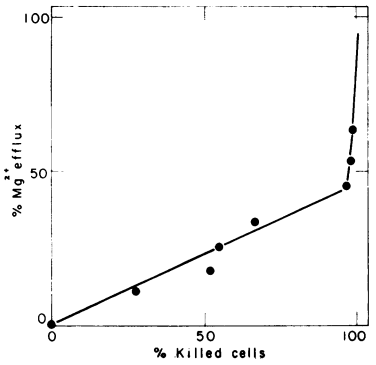

FIG. 3. *Total amount of Mg^{2+} efflux. Cells of strain LA324 were treated with low multiplicities of colicin K at 25 C and survival was measured at 30 min. Efflux of $^{28}Mg^{2+}$ was measured also between 60 and 80 min, and did not increase during this period. The figure shows data from two separate experiments.*

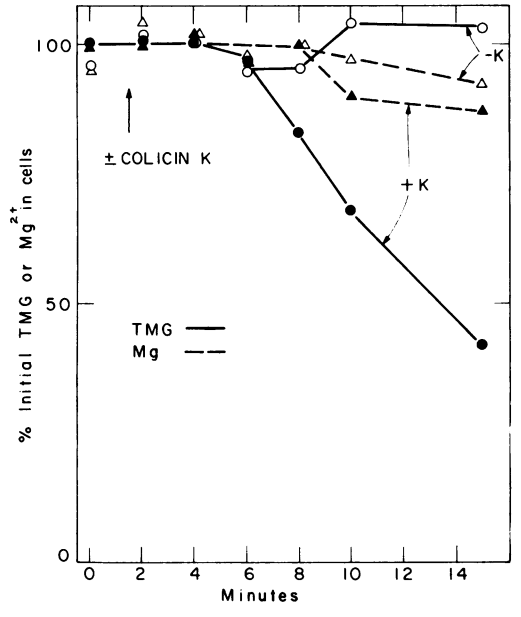

FIG. 4. *Efflux of thiomethylgalactoside (TMG) and Mg^{2+} from cells treated with colicin K. Cells of strain LA324 grown with $^{28}Mg^{2+}$ were suspended in buffer N + lactate at 4 × 10^8/ml at 25 C. [^{14}C] TMG, 2.4 mCi/mmole, was added at a final concentration of 1 mM, and the mixture was aerated. After 20 min, zero-time samples were filtered; colicin K (m = 2.9, measured after 10 min) was added 1.5 min thereafter to one sample. Samples were washed on the filters with three 1-ml portions of buffer N + 1 mM Mg^{2+} at 25 C. Appropriate liquid scintillation channels could distinguish between ^{14}C and ^{28}Mg; the samples were also counted after 10 days, when the ^{28}Mg had decayed to negligible levels.*

Extensive efflux of TMG had occurred within 15 min after the addition of colicin. During this time no significant efflux of $^{28}Mg^{2+}$ occurred. Loss of Mg^{2+} is clearly not a primary effect of colicin K and cannot be responsible for the inhibition of galactoside transport.

Action of colicin K on Mg^{2+} exchange before and after initiation of Mg^{2+} efflux. During the first phase of treatment with colicin K, entry and efflux of Mg^{2+} must occur at equal rates, because the cellular Mg^{2+} content does not change. In the second phase, the rate of efflux must exceed the rate of entry of Mg^{2+} in order to produce the observed net loss. The experiments in Table 1 were designed to study the flux of $^{28}Mg^{2+}$ into the cells in both phases of colicin action. The flux of $^{28}Mg^{2+}$ into control cells occurs by exchange, since there is no net change in cellular Mg^{2+}. The flux of $^{28}Mg^{2+}$ into colicin-treated cells may represent a net increase in addition to exchange, particularly when the cells are exposed to the higher concentrations of $^{28}Mg^{2+}$. We have not meas-

TABLE 1. *Rate of entry of $^{28}Mg^{2+}$ in strain LA324 treated with colicin K*[a]

Conditions	Rate of entry of $^{28}Mg^{2+}$ (nmoles per mg per min)		
	No colicin	After colicin K treatment	
		2 to 6 min	15 to 19 min
Expt 1			
0.012 mM $^{28}Mg^{2+}$	4	2	1.9
1.2 mM $^{28}Mg^{2+}$	7.6	38	32
Expt 2			
0.1 mM $^{28}Mg^{2+}$		18	9
0.1 mM $^{28}Mg^{2+}$ + 5 × 10^{-5} M FCCP		0	5

[a] Equivalent cultures of strain LA324 were grown with $^{24}Mg^{2+}$ or $^{28}Mg^{2+}$, and treated with colicin K (m = 5). Colicin K-promoted loss of $^{28}Mg^{2+}$ from the labeled cells was assumed to be equal to the loss of $^{24}Mg^{2+}$ from unlabeled cells treated with the same multiplicity of colicin K. The unlabeled cells were exposed to $^{28}Mg^{2+}$ at the indicated concentrations, and the rate of entry of $^{28}Mg^{2+}$ was corrected for lowering of the specific activity by excreted $^{24}Mg^{2+}$. No efflux had occurred 6 min after addition of colicin K; 65% of the initial cellular Mg^{2+} remained at 15 min. In experiment 2, *p*-trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) was added with the $^{28}Mg^{2+}$.

ured the Mg^{2+} content of cells treated with colicin K in 1 mM Mg^{2+}.

During the first phase of colicin K action, the flux of 0.012 mM $^{28}Mg^{2+}$ into the colicin-treated cells in inhibited, but the flux of 1.2 mM $^{28}Mg^{2+}$ is stimulated (Table 1). Apparently the affinity of the colicin-treated cell for external Mg^{2+} is lower, but the maximal rate of entry of $^{28}Mg^{2+}$ is increased. The flux of $^{28}Mg^{2+}$ during this period is completely inhibitable by the uncoupler *p*-trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (Table 1, experiment 2).

During the second phase of colicin K action, the rate and concentration dependence of $^{28}Mg^{2+}$ entry do not change. Initiation of efflux of Mg^{2+} therefore does not coincide with a dramatic decrease in the ability of Mg^{2+} to enter the cell, particularly from the low extracellular concentrations of Mg^{2+} in which colicin-promoted efflux is measured. The flux of $^{28}Mg^{2+}$ during the second phase, however, becomes much less sensitive to FCCP.

Inhibitors of colicin K. If the bacteria are first treated with uncouplers or inhibitors of oxidative phosphorylation, colicin K does not cause efflux of Mg^{2+} (Fig. 5). The colicin does adsorb to the cells treated with inhibitors, for they become unable to form colonies. Cyanide and FCCP completely block colicin K-promoted efflux of Mg^{2+}, and completely block exchange of Mg^{2+} (Fig. 5a, b). Azide, which is less effective in blocking Mg^{2+} exchange, is only partially effective in blocking colicin K (Fig. 5c). Omission of a source of metabolizable carbon (lactate) inhibited Mg^{2+} exchange 75% without altering the Mg^{2+} efflux promoted by colicin K (Fig. 5d). The inhibitors may act by depriving the cell of energy necessary for the initial action of colicin K, or by altering the conformation of the membrane in some way that prevents the colicin from acting.

Figure 5 demonstrates that the addition of FCCP, cyanide, or azide during measurement of Mg^{2+} exchange blocks that exchange. In contrast, if the inhibitors are added during colicin K-promoted efflux, they do not stop the efflux of Mg^{2+}. In the first phase of colicin action, whereas FCCP is capable of stopping Mg^{2+} exchange, FCCP can also prevent colicin K-promoted Mg^{2+} efflux. At a multiplicity of colicin K sufficient to give 3.6% survival after 2 min adsorption and to initiate efflux of Mg^{2+} after 10 min, the addition of FCCP at 2 min or at 5 min prevents the loss of Mg^{2+} for at least 30 min. If FCCP is added 10 min after the colicin, just as Mg^{2+} efflux begins, efflux continues at the same rate as in cells treated with colicin K alone. The FCCP-sensitive step in colicin action therefore is prerequisite for efflux, but the process of efflux is not sensitive to FCCP.

Other agents that will prevent but cannot stop colicin K-promoted Mg^{2+} efflux are the sulfhydryl reagent N-ethyl maleimide (NEM) (1 mM) and incubation at 0 C (*unpublished data*). Both NEM and cold prevent exchange of Mg^{2+} (17).

Effects of colicin K on transport of Co^{2+}. Co^{2+} is a competitive inhibitor of Mg^{2+} transport, and is transported by an energy-dependent system (19). Because intracellular Co^{2+} is poisonous, Co^{2+}-resistant mutants can be selected which fail to transport Co^{2+} (19, 20). One such mutant, DN53, has been used to examine the effects of colicin K on the permeability barrier to Co^{2+}. Permeability to Co^{2+} in a mutant lacking the normal transport system for Co^{2+} serves as an indicator of nonspecific damage to the permeability barrier, whereas Co^{2+} uptake in the parental strain is mediated by an energy-dependent transport system and will be sensitive to an uncoupling action of colicin K.

Table 2 shows that colicin K, unlike the uncoupler FCCP, does not inhibit total Co^{2+} uptake in the parental strain LA324. Instead, colicin K causes the mutant DN53 to take up Co^{2+}, as might be expected if the permeability barrier had been damaged. Both strains take up Co^{2+} at the same rate after treatment with colicin K, although DN53 lacks the normal transport system for Co^{2+}. If colicin K causes the same increase in passive permeability in strain LA324, and the rate of Co^{2+} entry through the colicin K-promoted route and the normal system are additive, then the normal system must be inhibited in colicin K-treated cells of LA324.

If both entry of Co^{2+} and net loss of Mg^{2+} occur because of the same damage to the membrane, both processes should begin at the same time after addition of colicin K. The experiment shown in Fig. 6 attempts to correlate initiation of Co^{2+} uptake in DN53 with initiation of Mg^{2+} efflux, but the two processes do not always begin simultaneously. Co^{2+} uptake begins 6 or 7 min after addition of colicin K, whereas Mg^{2+} efflux may begin at 6 or 12 min, depending on the multiplicity of colicin. It is possible that less damage is needed to produce detectable entry of Co^{2+} than to produce net loss of Mg^{2+}. Measurable entry of Co^{2+} requires only that Co^{2+} crosses the membrane whereas net loss of Mg^{2+} requires both

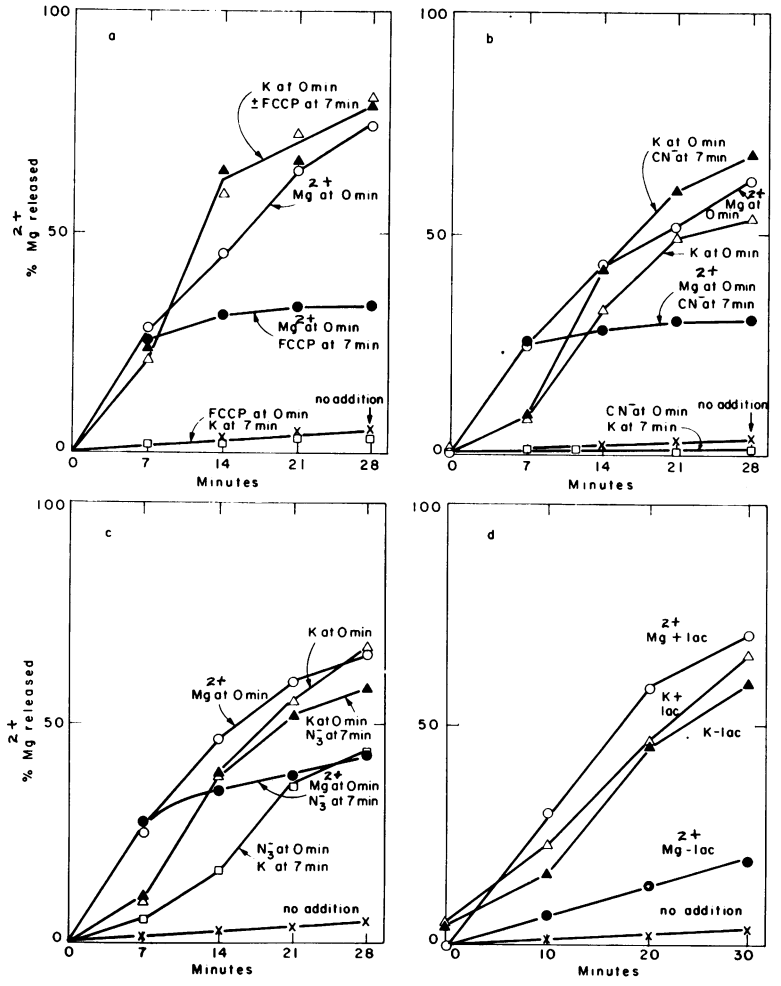

FIG. 5. *Effect of inhibitors of Mg^{2+} exchange on colicin K-promoted Mg^{2+} efflux. Strain LA324 grown with $^{28}Mg^{2+}$ was resuspended in buffer N + lactate (except that lactate was omitted from some incubations in part d). Colicin K (m = 5 to 10), $^{24}Mg^{2+}$ (0.1 mM) or inhibitors were added at the indicated times and the appearance of $^{28}Mg^{2+}$ in the filtrate was measured as in Fig. 1. Inhibitors: (a) FCCP, 2×10^{-5} M; (b) KCN, 5 mM; (c) NaN_3, 10 mM.*

movement across the membrane in the opposite direction and failure to be recaptured by the transport system. In any case, colicin-promoted Co^{2+} entry does not correlate with the initial phase of altered permeability to Mg^{2+} which occurs during the first 6 min of colicin treatment (Table 1).

If colicin-promoted Co^{2+} entry as revealed in strain DN53 does not begin until after the first phase of colicin action, then Co^{2+} entry in strain LA324 during the first phase should be mediated by the normal transport system. No net inhibition of Co^{2+} uptake during the first phase has been observed (*unpublished data*). Thus normal Co^{2+} uptake must become inhibited only as the colicin-promoted uptake begins.

Several lines of evidence suggest that the colicin K-promoted uptake of Co^{2+} is not mediated by the normal Co^{2+} transport sysem. It takes place in the mutant DN53 in which the normal system does not function. Strain LA324 has a K'_T for Co^{2+} of 0.4 mM (19) and DN53 treated with colicin K has an apparent K'_T of 5 mM (*unpublished data*). Co^{2+} uptake normally is competitively inhibited by Mg^{2+} (19); 50 μM Mg^{2+} completely inhibits uptake of 0.1 mM Co^{2+} by LA324 but is without effect on Co^{2+} uptake by K-treated cells of either strain. Finally, in either strain, the colicin-promoted

TABLE 2. *Effect of colicin K on permeability to Co^{2+}* [a]

Colicin K	Rate of entry of $^{60}Co^{2+}$ (nmoles per mg per min)			
	Strain LA324		Strain DN53	
	-FCCP	+FCCP	-FCCP	+FCCP
-	5.9	0.3	0.5	0.1
+	6.2	2.3	6.2	2.3

[a] The cells were treated with (+) or without (-) colicin K (m = 100) for 15 min at 24 C and ± *p*-trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (5×10^{-5} M) for 5 min before the addition of 0.1 mM $^{60}CoCl_2$, 6.3×10^5 counts per min per μmole.

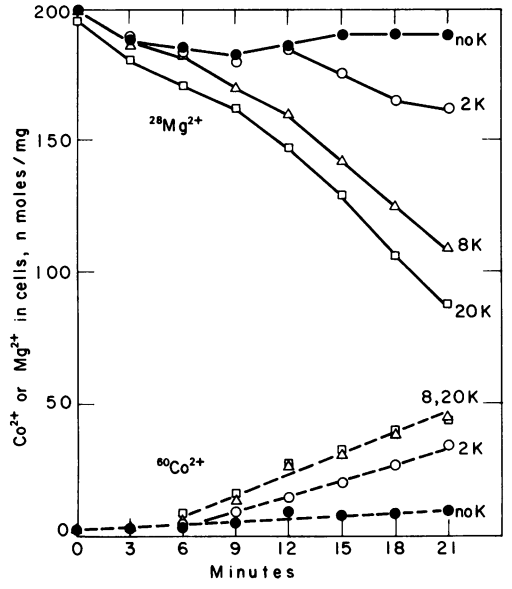

FIG. 6. *Entry of Co^{2+} and efflux of Mg^{2+} from colicin K-treated cells of strain DN53. Strain DN53 was grown with $^{28}Mg^{2+}$ and resuspended at 25 C in buffer N + lactate + 0.12 mM $^{60}Co^{2+}$, 6.7×10^5 counts per min per μmole, + different amounts of colicin K. Survival was measured after 6 min. Samples were filtered, washed with buffer N + 1 mM Mg^{2+}, and radioactivity in the cells was determined. $^{60}Co^{2+}$ was counted after the $^{28}Mg^{2+}$ had decayed and $^{28}Mg^{2+}$ was determined as the difference between the counts on the day of the experiment and the counts from $^{60}Co^{2+}$. Solid lines, $^{28}Mg^{2+}$; dashed lines, $^{60}Co^{2+}$.*

uptake of Co^{2+} is less sensitive to FCCP than is normal Co^{2+} uptake (Table 2). Most properties of colicin K-promoted Co^{2+} uptake are consistent with the hypothesis that Co^{2+} enters the cell because the colicin has damaged the permeability barrier.

Colicin E1 inhibits $^{28}Mg^{2+}$ exchange into cells. Colicin E1 inhibits exchange of $^{28}Mg^{2+}$ into cells much more than equivalent killing multiplicities of colicin K. Cells treated with colicin E1 and then exposed to 0.01 mM $^{28}Mg^{2+}$ exhibit a greatly reduced rate of exchange (Fig. 7). The inhibition is not due to efflux of $^{24}Mg^{2+}$ from the cells and consequent dilution of the specific activity of extracellular $^{28}Mg^{2+}$, because the total amount of $^{24}Mg^{2+}$ in the cells is only 20% of the amount of $^{28}Mg^{2+}$ in the medium. The slight inhibition of exchange by boiled colicin E1 may be due to the presence of a small amount of $^{24}Mg^{2+}$ in the colicin preparation; if there were 0.08 mM $^{24}Mg^{2+}$ contaminating the preparation, it would be sufficient to produce the observed inhibition by dilution of the specific activity of the $^{28}Mg^{2+}$ in the assay. Alternatively, anions in the preparation may be capable of binding Mg^{2+}, lowering the effective concentration and hence the rate of exchange.

Mg^{2+} exchange in control cells is a saturable process (19, 26). In E1-treated cells, however,

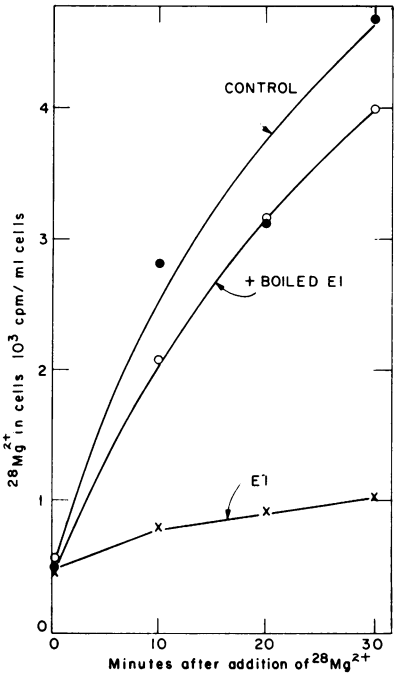

FIG. 7. *Colicin E1 inhibits exchange of $^{28}Mg^{2+}$ into cells. Cells of strain LA324 at 4×10^7/ml in buffer N + lactate were treated with colicin E1 (multiplicity 3.2) or an equal volume of boiled colicin E1 preparation for 15 min at 25 C before the addition of 0.01 mM $^{28}Mg^{2+}$, 1.2×10^7 counts per min per μmole, and washed with cold buffer N + 1 mM $^{24}Mg^{2+}$.*

the rate of exchange of $^{28}Mg^{2+}$ is proportional to the concentration of extracellular Mg^{2+} up to at least 1 mM (Fig. 8). At 1 mM extracellular Mg^{2+}, exchange is as rapid in colicin E1-treated cells as in control cells. In failing to prevent exchange of 1 mM Mg^{2+}, colicin E1 differs from chemical energy poisons. For example, the uncoupler FCCP inhibits exchange of 1 mM Mg^{2+} as well as 0.01 mM (Fig. 8). At the lower concentration of Mg^{2+}, however, colicin E1 brings about an inhibition of Mg^{2+} exchange similar to that observed in chemically poisoned cells and much more severe than the 50% inhibition of exchange of 0.01 mM Mg^{2+} in cells treated with colicin K (Table 1). The rate of exchange of $^{28}Mg^{2+}$ in E1-treated cells differs from the rate in colicin K-treated cells at higher Mg^{2+} concentration as well. At 0.1 mM extracellular Mg^{2+}, exchange in cells treated with E1 is inhibited (Fig. 8), but in cells treated with colicin K it is stimulated (Table 1).

The linear relationship between the rate of Mg^{2+} exchange and extracellular Mg^{2+} concentration suggests that colicin E1-treated cells allow exchange through some low-affinity route analogous to that of colicin K-treated cells. The observed exchange rates are lower in colicin E1-treated cells possibly because of more complete inhibition of normal Mg^{2+} exchange or less damage to the permeability barrier by this colicin.

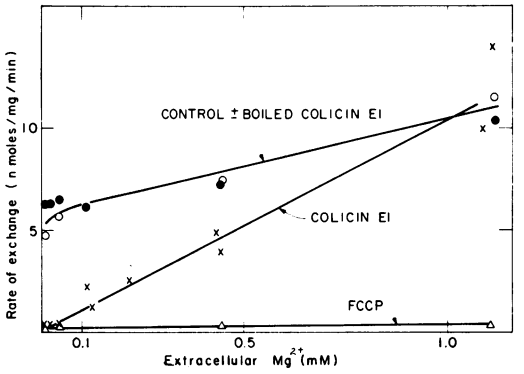

FIG. 8. *Initial rate of exchange of $^{28}Mg^{2+}$ into cells as a function of $^{28}Mg^{2+}$ concentration. Colicin E1 (m = 6), boiled colicin or p-trifluoromethoxy carbonylcyanide phenylhydrazone (5×10^{-5} M) was added to cells of strain LA324 at 2×10^{8}/ml in buffer N + lactate at 24 C. After 5 min $^{28}Mg^{2+}$, 5.7×10^{5} counts per min per μmole, was added to give the indicated final concentrations. Samples of the cell suspension were filtered and washed at 1, 2, 3, and 4 min after the addition of $^{28}Mg^{2+}$ and the rate of entry of the isotope was determined. The data for E1-treated cells are from two separate experiments. Open circles, boiled colicin E1.*

Colicin E1 inhibits exchange of $^{28}Mg^{2+}$ out of cells. After bacteria have been grown in medium containing $^{28}Mg^{2+}$, exchange may be measured as the Mg^{2+}-dependent release of $^{28}Mg^{2+}$ from the cells. If the same process of Mg^{2+} exchange mediates the flux of $^{28}Mg^{2+}$ both into and out of the cell, inhibitors of entry of $^{28}Mg^{2+}$ must inhibit exchange of cellular $^{28}Mg^{2+}$ with extracellular $^{24}Mg^{2+}$. As expected, colicin E1 inhibits exchange of 0.1 mM extracellular $^{24}Mg^{2+}$, measured as exit of $^{28}Mg^{2+}$, to an extent similar to the inhibition of exchange of 0.1 mM $^{28}Mg^{2+}$ with unlabeled cells. High concentrations of extracellular Mg^{2+} overcome the inhibition, whether exchange is measured as entry (Fig. 8) or exit (Fig. 9) of $^{28}Mg^{2+}$.

The inhibition of exchange of 0.1 mM Mg^{2+} in cells treated with colicin E1 depends upon temperature (Fig. 10). Although exchange is almost completely inhibited at 23 C, at 37 C the colicin has almost no effect on the rate of exchange. Increased damage to the permeability barrier at 37 C could mask inhibition of the normal transport system.

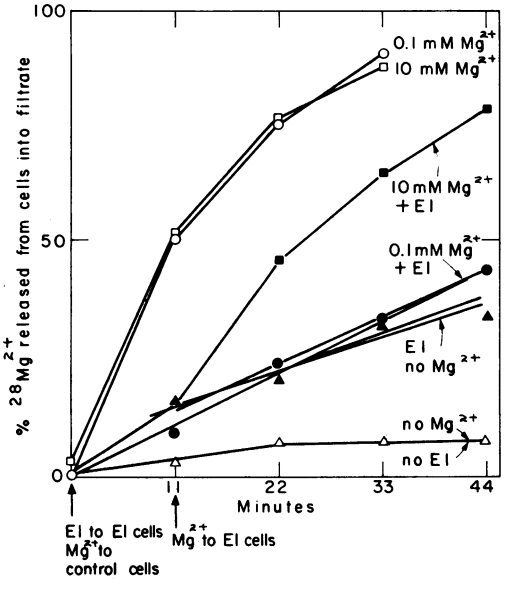

FIG. 9. *Exchange of $^{28}Mg^{2+}$ out of cells. Strain LA324 was grown with $^{28}Mg^{2+}$ in medium N, harvested, and resuspended in buffer N + lactate at a cell density of 6×10^{7}/ml at 24 C. Control cells were incubated for 11 min in this medium before the addition of 0.1 or 10 mM $^{24}MgSO_4$. Colicin E1 (m = 5) was added at 0 min to three samples of cells; at 11 min, two of the samples received 0.1 or 10 mM $^{24}MgSO_4$. At intervals thereafter, 1-ml samples were filtered, the filtrates collected, and the radioactivity in the filtrates determined.*

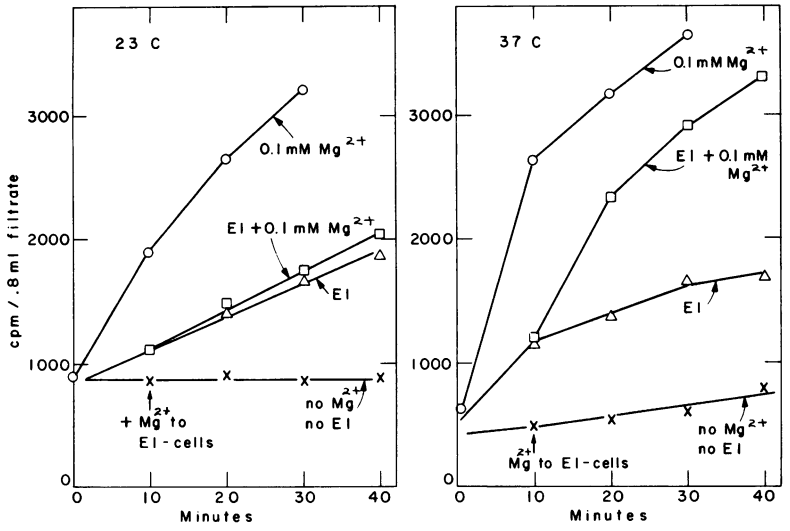

FIG. 10. *Exchange of $^{28}Mg^{2+}$ out of cells at 23 C and 37 C. Strain LA324 was grown with $^{28}Mg^{2+}$ and treated with colicin E1 as in Fig. 9, except that exchange was measured at 23 C and at 37 C. The initial $^{28}Mg^{2+}$ was 4,500 counts per min per 0.8 ml of the incubation mixture.*

Colicin E1 causes efflux of Mg^{2+}. Figures 8 and 9 show that cells treated with colicin E1 excrete Mg^{2+} at significantly higher rates than control cells. The rate of efflux of Mg^{2+} from colicin E1-treated cells averaged 1.9 nmoles per mg of protein per min in several experiments performed at 25 ± 2 C, a 10-fold increase over the average rate of efflux from untreated cells. The observed efflux of $^{28}Mg^{2+}$ cannot be due to exchange with the 2 to 10 μM extracellular Mg^{2+} derived from the E1 preparation, because at these low concentrations exchange is completely inhibited (Fig. 8). Thus, exit of $^{28}Mg^{2+}$ must reflect a net loss of cellular Mg^{2+}. The kinetics of the loss of Mg^{2+} caused by colicin E1 differ from the kinetics of colicin K-promoted efflux in two respects. At similar multiplicity and temperature, colicin E1 initiates Mg^{2+} efflux essentially immediately, in contrast to the lag before colicin K initiates Mg^{2+} efflux. Secondly, the rate of colicin E1-promoted Mg^{2+} efflux is 25% of the maximum rate of Mg^{2+} exchange; colicin K-promoted efflux is as rapid as Mg^{2+} exchange.

Inhibitors of colicin E1 action. The efflux of Mg^{2+} caused by colicin E1 is blocked by the same compounds that prevent the action of colicin K. If the cells are first treated with FCCP or KCN, colicin E1 fails to bring about efflux of Mg^{2+} (Fig. 11a and b). The inhibitors do not prevent adsorption of the colicin, as shown by measurement of survival after dilution and plating. Adding the inhibitors during exchange of intracellular $^{28}Mg^{2+}$ stops the exchange almost immediately. In contrast, adding the inhibitors after colicin E1 does not stop Mg^{2+} efflux, which is actually stimulated by FCCP and KCN. Cells treated with colicin E1 are known to respond to FCCP by allowing entry of hydrogen ions (8); KCN, the salt of a weak acid, may also increase proton permeability. The increased rate of Mg^{2+} efflux could result from the need to balance the positive charge of the entering protons.

Azide, although it is a good inhibitor of Mg^{2+} exchange, has little effect on the ability of colicin E1 to promote Mg^{2+} efflux. Similarly, starvation for a source of energy (lactate) inhibits Mg^{2+} exchange without reducing the rate of efflux from E1-treated cells. It is not yet clear whether the difference between the inhibitory effect of FCCP or KCN and lack of inhibition of colicin action by azide or starvation is due to quantitative differences in energy charge or to qualitative differences in membrane conformation. It may be significant that both colicins E1 and K respond similarly to each of the inhibitory conditions.

Colicin E1 prevents the action of colicin K. If cells are treated with colicin E1 prior to the addition of colicin K, the slow loss of Mg^{2+} characteristic of E1-treated cells is maintained (Fig. 12). Colicin K fails to increase the rate of efflux to that normally observed in K-treated cells. The rapid loss of Mg^{2+} characteristic of colicin K-treated cells continues if colicin E1 is added later, during the colicin K-promoted efflux. The action of colicin E1 on control cells

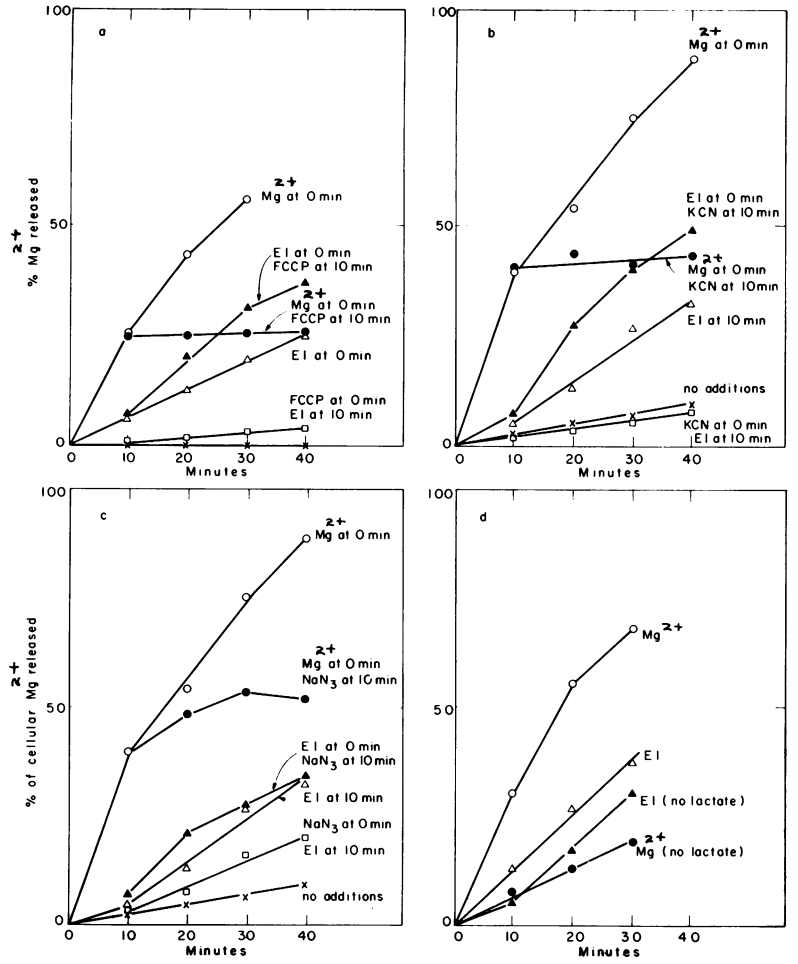

FIG. 11. *Effect of inhibitors and starvation on efflux of Mg^{2+} from cells treated with colicin E1. Strain LA324 was grown with $^{28}Mg^{2+}$, harvested, and resuspended at 24 C in buffer N ± lactate. Inhibitors were added 10 min after the addition of 0.1 mM $^{24}MgSO_4$ to demonstrate inhibition of Mg^{2+} exchange. The same inhibitors were added 10 min before colicin E1 or 10 min after colicin E1 (m = 4). (a) p-Trifluoromethoxy carbonylcyanide phenylhydrazone, 5×10^{-5} M; (b) KCN, 10 mM; (c) NaN_3, 10 mM; (d) no lactate.*

is rapid enough to inhibit Mg^{2+} exchange almost immediately; had colicin E1 acted equally rapidly to inhibit colicin K-promoted efflux of Mg^{2+}, the inhibition would have been observed. The fact that colicin E1-treated cells cannot respond to colicin K provides evidence that the two colicins have different effects on the cell.

Colicin E1 inhibits uptake of Co^{2+}. The effects of colicin E1 on uptake of Co^{2+} are consistent with the interpretation that the colicin inhibits energy metabolism and also increases permeability to divalent cations. Table 3 shows that colicin E1 inhibits the energy-dependent (FCCP-sensitive) uptake of Co^{2+} by the wild-type strain LA324. The inhibition by the same multiplicity of colicin was much more dramatic at 24 C than at 37 C, as was the inhibition of Mg^{2+} exchange (Fig. 10).

The cobalt-resistant mutant DN53 exhibits greatly reduced ability to take up cobalt (Table 3). Colicin E1 increased the rate of uptake of Co^{2+} by the mutant to the rate observed in the colicin-treated wild type, a rate which is a larger proportion of the control rate at 37 C than it is at 24 C (Table 3). The increased permeability of colicin E1-treated DN53 to Co^{2+} at 37 C must result from damage to the permeability barrier rather than decreased inhibition of the normal transport

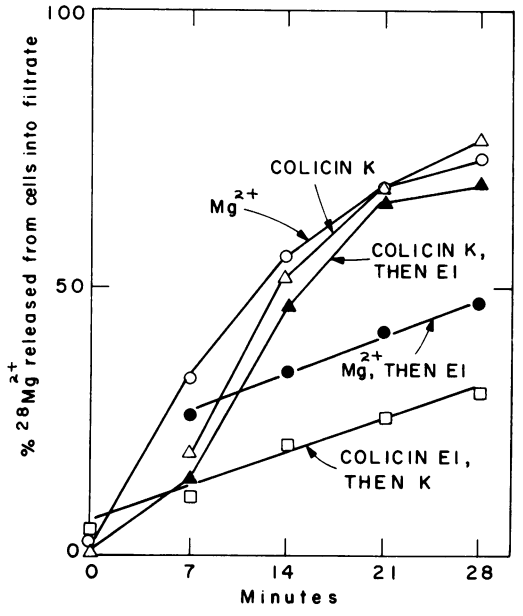

FIG. 12. *Colicin E1 blocks the action of colicin K. Cells of LA324 grown with $^{28}Mg^{2+}$ were harvested and resuspended in buffer N + lactate at 24 C. At 0 min, $^{24}Mg^{2+}$ (0.1 mM) was added to two samples of cells (○, ●); at 7 min, one of these (●) received colicin E1 (m = 5). Two samples (△, ▲) were treated with colicin K (m = 5) at 0 min; one of these (▲) received colicin E1 at 7 min. Another sample (□) received colicin E1 at 0 min and colicin K at 7 min. Appearance of $^{28}Mg^{2+}$ in filtrates of samples of the incubation mixtures was followed.*

system. By analogy, increased permeability of E1-treated LA324 to Co^{2+} and Mg^{2+} at 37 C could result from the same damage rather than from a decreased inhibition of transport.

Co^{2+} uptake in cells treated with colicin E1 is less sensitive to FCCP than is the normal transport process (Table 3). The reduced sensitivity to FCCP occurs in the presence or absence of the normal Co^{2+} transport system, and is qualitatively similar to the FCCP-insensitivity of uptake of Co^{2+} in colicin K-treated cells (Table 2). If permeability of DN53 to Co^{2+} reflects membrane damage, colicin E1 may simply cause less of the same type of damage as colicin K.

Genetic controls. Mutants that are tolerant to colicins adsorb them but are not killed. One *tolA* mutant (tolerant to colicins E1, E2, E3, and K) and one *tolC* mutant (tolerant to colicin E1 only) derived from strain LA324 were tested. Colicin E1 did not inhibit Mg^{2+} exchange in either mutant (data not shown). Colicin K promoted Mg^{2+} efflux in the *tolC* mutant but not the *tolA* mutant.

The *tolC* strain after treatment with E1 was still susceptible to the action of colicin K. Adsorption of colicin E1, therefore, is not sufficient to block the action of colicin K; action of colicin E1 on the cell is required.

DISCUSSION

Two kinds of colicin action. Two kinds of action are necessary to produce the effects of colicin E1 or colicin K on cellular permeability to Mg^{2+} and Co^{2+}. The first may be thought of as an uncoupling of energy from transport, manifested as an inhibition of normal Mg^{2+} and Co^{2+} transport in colicin-treated cells. The inhibition by colicin E1 is plainly evident in lower total rates of Mg^{2+} exchange and Co^{2+} uptake. Colicin K, however, inhibits Mg^{2+} exchange only at very low Mg^{2+} concentrations, and inhibits Co^{2+} uptake only after a lag period during which TMG accumulation is inhibited and the kinetics of Mg^{2+} exchange are altered. Both colicins lower the affinity of the cell for external Mg^{2+} while increasing or not affecting the rate of Mg^{2+} exchange at high concentrations. In this respect they differ from chemical uncouplers of metabolic energy, which inhibit exchange even at the higher Mg^{2+} concentrations.

The second type of action is distinctly different from that of chemical uncouplers, and may explain the resistance of exchange of higher concentrations of Mg^{2+} to the inhibitory power of the colicins. Both colicins cause an increase in permeability to Mg^{2+} and Co^{2+} that appears to be due to damage to the permeability barrier. The colicin-promoted permeability is particularly obvious in the

TABLE 3. *Effect of colicin E1 on permeability to Co^{2+}* [a]

Temp (C)	Colicin E1	Rate of entry of $^{60}Co^{2+}$ (nmoles per mg per min)			
		Strain LA324		Strain DN53	
		-FCCP	+FCCP	-FCCP	+FCCP
24	-	5.5	0.17	0.48	0.07
24	+	1.4	0.48	1.4	0.69
37	-	5.5	0.35	0.69	0.41
37	+	2.8	1.7	3.1	1.4

[a] The bacteria were treated with (+) or without (-) colicin E1 (m = 4.6) at 24 or 37 C for 5 min before the addition of *p*-trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (2×10^{-5} M). $^{60}CoCl_2$, 0.1 mM, 6.3×10^5 counts per min per μmole, was added 10 min after the colicin, and the rate of uptake during the next 15 min was determined.

mutant DN53, which is normally impermeable to Co^{2+}. Exchange of Co^{2+} or Mg^{2+} in colicin-treated cells is insensitive to several inhibitors of normal transport. Its dependence on Mg^{2+} or Co^{2+} concentration indicates that the permeation mechanism has lost affinity for the ions and that competition between Mg^{2+} and Co^{2+} for a limited number of transport sites can no longer limit their rate of entry. Possibly either chemical or conformational changes in the membrane could change its permeability in these ways.

Efflux of Mg^{2+}. Both colicins cause efflux of cellular Mg^{2+}, perhaps as a direct result of inhibiting exchange of low concentrations of Mg^{2+}. When cells are suspended in buffer containing very low concentrations of Mg^{2+} (0.01 mM), the total cellular Mg^{2+} stays constant, implying that the rates of exit and entry are equal. If colicin E1 is added, entry of Mg^{2+} from the low extracellular concentration is severely inhibited. If the intracellular concentration is higher (near 1 mM [11]), exit may not be inhibited, as exchange of 1 mM Mg^{2+} is not. The result of a decrease in entry without a decrease in exit must be net efflux of Mg^{2+} from the colicin E1-treated cells. Chemical uncouplers, which inhibit exchange of 1 mM as well as 0.01 mM Mg^{2+}, inhibit exit of the cellular Mg^{2+} as well as entry, and no net loss of Mg^{2+} is observed.

If colicin K is added instead of colicin E1, Mg^{2+} efflux begins only after a lag but then proceeds more rapidly than from colicin E1-treated cells. The contrast may be understood in terms of the different effects of the colicins on Mg^{2+} exchange. Colicin K inhibits entry of Mg^{2+} much less than colicin E1, perhaps not enough to cause net loss of Mg^{2+}. After a lag period, however, the permeability barrier has been damaged and the cells become unable to retain Mg^{2+}. Loss of Mg^{2+} from cells that are highly permeable may be analogous to defective retention of potassium in a mutant with increased rates of potassium exchange (15).

The dependence of Mg^{2+} efflux from colicin K-treated cells on multiplicity and temperature bears a striking resemblance to that of potassium efflux reported by Dandeu et al. (7) and by Wendt (28). Once initiated, potassium efflux is more rapid than Mg^{2+} efflux, but stronger binding of Mg^{2+} by intracellular macromolecules may account for the difference in rate. It seems possible that the same sort of damage to the membrane could be responsible for efflux of both cations.

Differences between colicins E1 and K. In addition to the different effects of the two colicins on exchange and retention of Mg^{2+}, the ability of colicin E1 to block colicin K-promoted Mg^{2+} efflux strongly suggests that the two colicins act differently. Yet the essential difference between them may not lie in the presence or absence of rapid Mg^{2+} efflux after an initial lag. Possibly colicin E1 fails to promote such efflux because it blocks some potentially colicin K-like action of its own. The essential difference between the actions of colicins E1 and K then would be found in some earlier event, which in the case of colicin E1 prevents subsequent rapid efflux of Mg^{2+}. The difference might be related to the stronger inhibition of Mg^{2+} exchange in colicin E1-treated cells. A faster uncoupler-like action of colicin E1 could explain both the inhibition of Mg^{2+} exchange and blockage of a later rapid efflux of Mg^{2+}, since uncouplers have both these effects.

Inhibitors of colicin action. Inhibitors of energy metabolism seem generally able to block colicin action. Cavard et al. (4) have reported that dinitrophenol prevents the lysis of cells by colicin K. Colicin E2 is unable to act upon cells in the presence of dinitrophenol (12, 23), cyanide, or FCCP (L. Saxe, *personal communication*). Neither in these cases nor when inhibitors block colicin-promoted Mg^{2+} efflux can one distinguish between a requirement for metabolic energy and a requirement for a state of the membrane present only in unpoisoned cells. Conformational changes in the membrane have been invoked to explain the enhancement of fluorescence of cell-bound dye upon addition of FCCP (5), and may take place whenever the cell lacks energy.

Mechanism of increased permeability. How do the colicins make a normally impermeable membrane leaky to divalent cations? Gross lysis of colicin K-treated cells has been reported (3, 4), but does not occur in minimal medium (6). The observation that entry of Co^{2+} is stimulated in the same cell population that undergoes loss of Mg^{2+} argues against lysis being the cause of Mg^{2+} efflux. In addition, colicin-treated cells are not made permeable to *o*-nitrophenylgalactoside and can still accumulate phosphorylated sugars (9). The reported increase in lysophosphatidylethanolamine in colicin-treated cells (3) might make them more permeable to divalent cations; the lag before colicin K-promoted efflux might be the time required for production of sufficient lysophosphatidylethanolamine. Alternatively, a conformational change in the membranes of colicin-treated cells might increase permeability. The increased quantum

yield of the fluorescent dye 1-anilino-8-naphthalene sulfonic acid (ANS) bound to colicin E1-treated cells has been interpreted as reflecting a conformational change (5). Whether either the hydrolysis of phosphatidylethanolamine or the change in the environment of ANS is at all directly related to colicin action and, if so, how either event is related to the effects of colicins on transport and permeability remain to be established.

ACKNOWLEDGMENTS

We thank E. P. Kennedy, S. E. Luria, and C. A. Plate for helpful discussions and H. J. Unsold for preparing colicin K. This work was supported by Public Health Service grants NB-02946 from the National Institute of Neurological Diseases and Stroke, GM-13952 from the National Institute of General Medical Sciences to E. P. Kennedy, and grant AI-03038 from the National Institute of Allergy and Infectious Disease and grant AB30575x from the National Science Foundation to S. E. Luria. J.E.L. was a postdoctoral fellow of the National Institutes of Health and D.L.N. was a postdoctoral fellow of the National Cystic Fibrosis Research Foundation. We would also like to thank Barbara Emrick for typing the manuscript.

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