The State of Energization of the Membrane of *Escherichia coli* as Affected by Physiological Conditions and Colicin K†

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ABSTRACT: The bacterial protein colicin K, when added to sensitive *Escherichia coli* in the presence of 3,3′-dihexyloxacarbocyanine, causes a doubling in fluorescence of the probe. Glucose and oxygen cause a decreased fluorescence while anoxia and cyanide cause a rise in fluorescence. These results in conjunction with the work of other laboratories suggest that colicin K causes a depolarization of the transmembrane electrical potential. Fluorescence in the absence of colicin K was relatively independent of KCl, NaCl, and $MgCl_2$ concentrations below 0.1 M. Although colicin K caused rapid efflux of the K^+ analogue $^{86}Rb^+$, the fluorescence rise was only partially blocked by 0.13 M KCl. The level of fluorescence caused by the action of colicin K was inversely proportional to the logarithm of the concentration of $MgCl_2$ over the range of 2 μM to 4 mM. This suggests that a Nernst electrochemical potential for an anion can counteract a membrane depolarization caused by colicin. After colicin K action, the fluorescence of the carbocyanine could be further increased by anoxia or cyanide. The distribution of the weak base dimethyloxazolidinedione indicated that the pH in the interior of aerobic *E. coli* supplied with lactate was alkaline by 0.1 unit and unaffected by colicin. These results suggest that colicin K does not completely depolarize the membrane potential and does not interfere with the component of membrane energization generated by electron transport. Colicin K does not act as a cationophore. The partial depolarization of the membrane may account for the inhibition of active solute transport caused by colicin K.

The protein antibiotic colicin K is capable of interacting with sensitive *Escherichia coli* with kinetics and concentration dependence consistent with a single molecule per cell being sufficient to cause death as measured by inability to form colonies. Associated with cell death caused by colicin K are a number of physiological changes (see Luria, 1973) including an uncoupling of respiration-linked solute transport across the membrane (Fields and Luria, 1969a). Respiration (Fields and Luria, 1969b) and α-methyl glucoside accumulation (Fields and Luria, 1969a) continue after colicin action. Cells treated with colicin K eventually lose their internal potassium (Nomura and Maeda, 1965; Wendt, 1970) and magnesium (Lusk and Nelson, 1972). ATP levels drop as a result of colicin K action on normal cells (Fields and Luria, 1969b), but in a mutant (uncA) lacking the Ca^{2+},Mg^{2+}-activated ATPase activity, colicin K causes ATP levels to double while proline and gluamine transports are still inhibited (Plate et al., 1974). In *E. coli* certain transport systems are energized directly from phosphate bond energy (e.g., glutamine transport), while others are coupled specifically to an energized state of the membrane generated through the Ca^{2+}, Mg^{2+}-ATPase or through electron transport (e.g., proline transport) (Berger, 1973).

It has been suggested that the primary target for colicin K action is either the energized state of the membrane generated by electron transport and by the Ca^{2+},Mg^{2+}-ATPase (Plate et al., 1974), or the site at which electron transport couples to active solute transport (Luria, 1973). This report is an attempt to determine the effects of colicin K on various forms of the energized state of the bacterial membrane. The membrane potential is continuously monitored by the fluorescent probe CC_6[1] (Sims et al., 1974; Hoffman and Laris, 1974; Tedeschi, 1974; Laris and Pershadsingh, 1974; Kashket and Wilson, 1974). The proton gradient is estimated from the distribution of the weak base DMO (Kashket and Wong, 1969; Kashket and Wilson, 1974; Waddell and Bates, 1969). The possible contribution of other ions to the membrane potential is also examined.

Harold (1972) has reviewed the forms of membrane energization that may be responsible for active transport in bacteria. According to the chemiosmotic hypothesis (Mitchell, 1966), substrate oxidation produces a protonmotive force composed of an electrical potential ($\Delta\psi$) and a chemical potential (ΔpH), at 37 °C: $\Delta p = \Delta\psi - 62\Delta pH$. Griniuviene et al. (1974) have estimated that *E. coli* have a membrane potential of 140 mV, inside negative. It is not clear whether active transport in *E. coli* is energized by a proton gradient directly (West and Mitchell, 1972) or by an electrical potential (Hirata et al., 1974) or by a mechanochemical coupling (Young et al., 1971; Kaback, 1972; Boyer, 1974).

Experimental Procedure

Materials. CC_6 was kindly provided in crystalline form by Dr. Alan S. Waggoner, Amherst College (Sims et al., 1974). A solution of 1 mg/ml ethanol was prepared and stored protected from light at −10 °C. Trypsin (two-times crystallized) was from Worthington Biochemical Corp. (Freehold, N.J.). Valinomycin (Sigma Chemical Co., St. Louis, Mo.) was made 1 mg/ml ethanol and stored at −10 °C; 85% DL-lactate (Mallinckrodt Chemical Works, St. Louis, Mo.) was brought to pH 7.0 with 50% NaOH, dilut-

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[1] Abbreviations: CC_6, 3,3′-dihexyloxacarbocyanine; DMO, 5,5-dimethyl-2,4-oxazolidinedione.

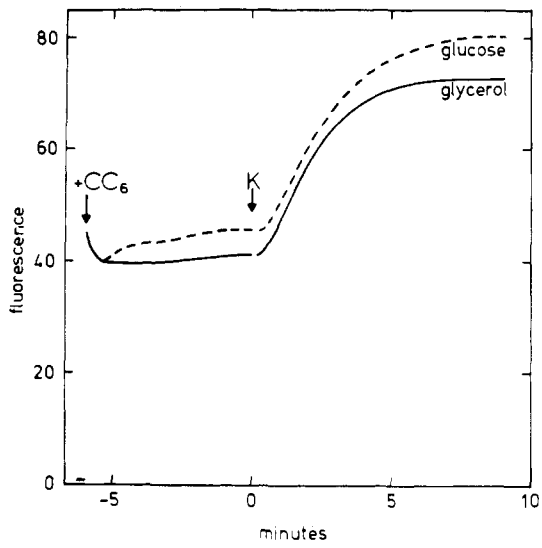

FIGURE 1: Colicin K induced CC_6 fluorescence in *E. coli* G-6. Cells grown with either glucose (- - -) or glycerol (—) were harvested at 5 × 10^8 cells/ml, washed with an original volume of 10 mM Tris-Cl, pH 7.3 at room temperature, resuspended in one-tenth the original volume of 10 mM Tris containing the original carbon source, and stored at 16 °C. Within 30 min 0.15 ml cells were diluted tenfold in the cuvette with medium of the same composition at 37 °C followed shortly by 2 μl of CC_6 solution. At 0 time colicin K was added at a multiplicity of 4.2 (20 μl of a 1:100 dilution in 10 mM Tris).

ed to 40% and autoclaved. $^{86}RbCl$, $KH_2{}^{32}PO_4$, [^{14}C]DMO (5,5-dimethyloxazolidine-2,4-dione-*2*-^{14}C, 11 mCi/mmol), ^3H_2O, [^{14}C]sorbitol (200 mCi/mmol), and D-1-[^{3}H]sorbitol (6.8 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). D-Sorbitol (Difco) was used to dilute the specific activity of radiolabeled sorbitol. 10 mM Tris base (Sigma) was brought to pH 7.3 with HCl resulting in 8.3 mM Cl^-. All other chemicals were reagent grade.

Cells. Escherichia coli strain G-6 (Hfr, *his*$^-$) is a derivative of K-12 (Plate et al., 1974) (University of Texas at Houston stock collection No. 6). Cells were grown by reciprocal shaking at 37 °C in 38 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 0.2 mM $MgSO_4$ (M9A medium), 100 μg/ml L-histidine, 3 μM thiamine, and either 0.4% glucose, glycerol, or sodium DL-lactate as indicated to mid-log phase (5 × 10^8 cells/ml, 100 Klett with a green filter).

Colicin purification and plating of bacteria for survival have been described (Brewer, 1974). The amount of cell death caused by colicin (survival ratio S/S_0) was expressed in terms of a multiplicity (m) of killing units per cell ($S/S_0 = e^{-m}$).

Fluorescence measurements were performed as previously described with forced oxygenation (Brewer, 1974). CC_6 fluorescence was excited at 470 nm (5-nm slit) and measured at 502 nm (6-nm slit) in the ratio mode to compensate for source fluctuations. Scatter due to bacteria alone was 2% of the pre-colicin signal and was not significantly affected by any of the treatments used here. Fluorescence is quantitated in arbitrary linear units.

Tris–EDTA Valinomycin Treatment. Procedures were adapted from those of Leive (1968), Pavlasova and Harold (1969) and West and Mitchell (1972). The common features of these procedures are washing harvested cells two times with an equal volume of 0.12 M Tris-Cl, pH 8.0, and resuspending in one-tenth volume of the same buffer. After warming to 37 °C, Na-EDTA, pH 7 is added to 0.2 mM. Control cells were incubated without the addition of EDTA.

Method 1. Cells grown to mid-log phase on lactate are treated as above. After 2 min at 37 °C, the suspension is made 1 mM in $MgSO_4$, centrifuged, and assayed for fluorescence at 37 °C in 20 mM Tris-Cl, pH 7.4, 0.2 mM $MgSO_4$, 150 mM NaCl, 0.4% lactate. Valinomycin (1 μM) and later KCl (0.15 M) were added after steady-state fluorescence levels were obtained (several minutes).

Method 2. Cells grown to mid-log phase on lactate are treated as above. After 2 min at 37 °C, the suspension is centrifuged and washed in 250 mM sucrose. The cells are resuspended and assayed in 20 mM Tris-Cl, pH 7.4, 0.2 mM $MgSO_4$, 250 mM sucrose, 0.4% lactate. Valinomycin (1 μM) and, several minutes later, KCl (0.03 M) were added after steady-state fluorescence levels were obtained.

Intracellular pH. Cells grown with lactate to 5 × 10^8 cells/ml were harvested and resuspended in growth medium at a concentration of 1.2 × 10^9 cells/ml in a 50-ml Erlenmeyer flask on a gyratory shaker at 37 °C. To 9.43 ml of cells were added 0.33 ml of [^{14}C]DMO (final concentration 0.67 μCi/ml, 61 μM) and 0.33 ml of [^{3}H]sorbitol (0.33 μCi/ml, 67 μM). In order to determine the water space, in a parallel incubation, 0.33 ml of [^{14}C]sorbitol (0.67 μCi/ml, 67 μM) and 0.33 ml of ^3H_2O (2.5 μCi/ml) were added in place of the other radioactive species. After 5 min in each of two separate experiments, two aliquots were filtered without washing and counted without drying in 33% Triton scintillation fluid. Colicin K was then added at multiplicities of 2 or 4 and three more aliquots were filtered. Corrections were made for channel efficiency and spillover and the internal pH was calculated (Waddell and Butler, 1959):

$$pH_i = pK_a' + \log\left[\frac{C_i V_t}{C_e V_i}(10^{(pH_e - pK_a')} + 1) + 10^{(pH_e - pK_a')}\right]$$

where $pK_a' = 6.13$ is the ionization constant for DMO at 37 °C; pH_e is the external pH 7.00 as measured on a Radiometer pH meter with a combination electrode; C_i is the intracellular concentration of DMO; C_e is the extracellular concentration of DMO; V_t is the total volume of water remaining with the filter; V_i is the intracellular water volume. DMO was not toxic at the concentration used as shown by subsequent dilution and plating.

Results

Time Course of Fluorescence. When mid-log-phase *Escherichia coli* are oxygenated in the presence of glycerol at 37 °C, addition of the fluorescent dye CC_6 results in substantial fluorescence, which reaches a steady-state level after several minutes (Figure 1). The addition of colicin K causes, after a short lag, a rise in the system fluorescence. The relative magnitude of the rise is sensitive to the relative amount of cells and dye, kept constant in experiments reported here. Cells grown and tested with glucose as the carbon source exhibit a similar response to colicin K (Figure 1). The following experiments were conducted to test whether the colicin-caused rise in fluorescence was due to a membrane depolarization.

Role of Carbon Source. When glucose-grown cells are washed free of the carbon source and assayed for CC_6 fluorescence in the absence of glucose, a gradual rise in fluorescence is observed; a steady-state level of fluorescence was not attained within the indicated time period (Figure 2). The addition of gluocse causes a fast drop in fluorescence to a level close to that found in cells incubated with glucose from the start. The addition of colicin K produces as usual a

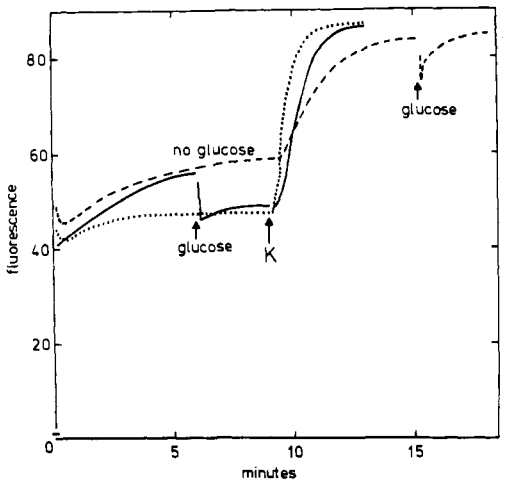

FIGURE 2: Effect of glucose and colicin K on CC_6 fluorescence. Cells grown with glucose were prepared as described in Figure 1 (···), or resuspended without glucose (- - -, —). At 0 time 2 μl of CC_6 solution was added. Glucose (5 μl of a 40% solution yielding 0.13%) was added as indicated. Colicin K was added at 9 min at a multiplicity of 8 (- - -, —) or 100 (···).

rise in fluorescence after a short lag. In the absence of glucose, however, the lag is longer (30 vs. 15 s) and the rise is slower. In either case, the steady-state of fluorescence after colicin is essentially the same. In cells treated with colicin K in the absence of glucose, subsequent addition of the sugar produces only a transient drop in fluorescence (Figure 2). Thus the addition of a carbon source to energy-deprived cells results in a drop in fluorescence, consistent with an increased polarization of the membrane potential.

Effects of Anoxia. It may be expected that respiration serves to energize the membrane and that anoxia would cause this energized state to be dissipated without re-energization. If a form of the energized state of the membrane were an electrical potential that can be monitored by CC_6 fluorescence, then one might observe changes in fluorescence associated with the respiratory state of the cells. It has been shown previously that colicin K does not inhibit respiration (Fields and Luria, 1969a). Oxygen is required, however, for colicin to act in the presence of nonfermentable substrates (Brewer, 1974; Jetten and Jetten, 1975). Table I shows that exhaustion of oxygen from the medium by cells respiring glycerol causes the fluorescence to rise substantially. The magnitude of the rise caused by anoxia is nearly equal to the change observed when respiring cells are treated with colicin. Exhaustion of oxygen from the medium by respiring cells that have been treated with colicin K produces a further rise in system fluorescence. To determine the fluorescence that corresponds to zero membrane potential, a small amount of toluene was added that makes the membrane permeable to small molecules. The high fluorescence observed suggests that the membrane potential is brought to near zero by colicin treatment followed by anoxia.

Addition of potassium cyanide, another respiratory inhibitor, causes a fast rise in fluorescence is respiring cells or cells treated with colicin K (Table II). As expected (Plate, 1973), cells treated with KCN do not respond subsequently to colicin K unless an overwhelming dose of colicin is employed. These results suggest two sources of the membrane potential: one energized by electron transport and another as yet unknown source that can be destroyed by colicin K.

Table I: Effects of Colicin K and Anoxia on CC_6 Fluorescence.[a]

Treatment	Steady-State Fluorescence	Fluorescence Change
Control ($+O_2$)	55	
Anoxic	86	31
Colicin K ($+O_2$)	81	26
Anoxic	108	53 (27)
Toluene (0.5%)	117	62

[a] Cells grown with glycerol were prepared as described in Figure 1. Anoxia was achieved by discontinuing the forced oxygen supply. Colicin K was added at a multiplicity 17. The number in parentheses is the difference from colicin treatment caused by anoxia.

Table II: Effects of Colicin K and KCN on CC_6 Fluorescence.[a]

Treatment	Steady-State Fluorescence	Fluorescence Change[d]
1. Control	49	
+ KCN (2 mM)	62	13
+ KCN, then colicin K[b]	62	13 (0)
+ KCN, high colicin K[c]	82	33 (20)
2. Control	51	
+ colicin K[b]	71	20
+ colicin K,[b] then KCN	96	45 (25)

[a] Cells grown with lactate to 5×10^8 cells/ml were transferred directly to the cuvette at 37 °C. The first addition was made 4 min after the addition of 2 μl of CC_6 solution. The second addition was made 6 min later. [b] ($m = 4$). [c] ($m = 200$). [d] Numbers in parentheses are differences from the previous treatment.

The combination of anoxia and colicin reduces the membrane potential to virtually zero.

Relation of CC_6 Fluorescence to Transmembrane Potential. Valinomycin can be used to establish electrochemical equilibrium of potassium ions across a membrane, making the transmembrane potential dependent on the ratio of potassium ions on either side of the membrane. Studies using valinomycin with lipid vesicles or red blood cells (Sims et al, 1974; Hoffman and Laris, 1974) or *Streptococcus* cells (Laris and Pershadsingh, 1974; Kashket and Wilson, 1974) have shown that CC_6 fluorescence is an indicator of the transmembrane potential, higher fluorescence representing a depolarization and lower fluorescence a hyperpolarization. Because of the presence in *E. coli* of an outer lipopolysaccharide membrane, valinomycin action must be studied after a pretreatment with EDTA plus Tris to destroy the permeability barrier to the drug (Pavlasova and Harold, 1969). This treatment by itself, however, raises the CC_6 system fluorescence to a higher level (200%, 125% of control cells for Methods 1 and 2, respectively), comparable to that seen with untreated cells to which colicin had been added. No additional change in fluorescence is observed upon introduction of valinomycin or KCl. These findings preclude calibration of fluorescence with the Nernst potential for potassium.

Trypsin Rescue. It has been observed that cells that have adsorbed colicin K but have not yet been damaged can be rescued from inhibition of certain transport processes and from death by treatment with trypsin (Plate and Luria, 1972). To determine whether the physiological effects of colicin might be linked to the energy state of the membrane, trypsin was added at various times during the course of CC_6

fluorescence development after addition of colicin K. Conceivably, there might be a primary event in the action of colicin K that is not subject to trypsin rescue while all succeeding events are rescuable. If one assumes that trypsin takes 20 s to mix and act in the cuvette, the percentage of cells unable to divide when plated (cells "killed" by colicin) turns out to be directly proportional to the percentage change in CC_6 fluorescence (data not shown). Trypsin addition causes an initial sharp rise in fluorescence followed by a gradual decline approaching the level of pre-colicin fluorescence. Control experiments in the absence of colicin or in the absence of cells exhibit the same phenomenon, implying that trypsin itself affects fluorescence. This precludes interpretation of cellular membrane potentials. In other experiments with amounts of colicin K above two killing units per cell (survival $< e^{-2}$), a maximum CC_6 fluorescence change was reached independent of the amount of colicin added (data not shown), indicating that the rise in fluorescence is an all-or-none event. These data establish that the changes reflected in the rise in CC_6 fluorescence are another expression of the irreversible action of colicin K on sensitive *E. coli*.

Rubidium Studies. Colicin could cause a membrane depolarization by an influx of cations or an efflux of anions. Anion efflux is suspected because (1) the CC_6 fluorescence rise is observed in the absence of extracellular permeable cations (Figure 1); (2) cells treated with colicin K lose their internal potassium (Wendt, 1970); and (3) colicin-treated cells lose organic anions (Fields and Luria, 1969b). Nevertheless, it is important to confirm the colicin-caused potassium efflux under conditions in which the fluorescence effect is seen, to show that a substantial amount of potassium is released from the cell and to compare the kinetics of efflux with the course of fluorescence development. The radioisotope $^{86}Rb^+$ was employed as an analogue of potassium. In the fluorescence cuvette, cells are preloaded to steady state with $^{86}Rb^+$ in the absence of Na^+. The addition of colicin K causes a marked[2] efflux of cellular $^{86}Rb^+$ with kinetics similar to the rise in CC_6 fluorescence (Figure 3). Obviously influx of Rb^+-K^+ is not responsible for the rise in fluorescence caused by colicin K.

Ion Effects on CC_6 Fluorescence. To examine further possible roles of ion fluxes in fluorescence development caused by colicin K, fluorescence was measured in the presence of NaCl, KCl, or $MgCl_2$. By itself the observed K^+ efflux would be expected to hyperpolarize the membrane and the extracellular addition of high concentrations of K^+ should block K^+ efflux, contributing to a faster or more extensive depolarization. The fluorescence of CC_6 and cells before colicin addition was found to be independent of the external concentration of the chloride salts of potassium (or sodium or magnesium) over the range 3×10^{-2} to 10^{-4} M (10^{-2} to 10^{-6} M for $MgCl_2$) (Figure 4A). Figure 4B shows that CC_6 fluorescence stimulated by colicin is inhibited in proportion to the log of the KCl concentration over the range 4 to 50 mM. Complete inhibition is not observed. Below 4 mM KCl, no inhibition of fluorescence is found. A similar complex relationship is found for sodium chloride in the medium. These unexpected results suggest that NaCl or KCl can partially restore (repolarize) the membrane potential. Thus membrane depolarization caused by colicin K can not be due to a cation channel permitting K^+ or Na^+ influx. $MgCl_2$ in the medium at 4 mM is able to inhibit completely the colicin-induced rise in fluorescence (Figure 4). Higher concentrations of $MgCl_2$ result in a gradual fall in fluorescence below the pre-colicin level (not shown). Lower concentrations are less inhibitory. A maximum fluorescence response to colicin K is found for $MgCl_2$ below 2 μM. The relationship of fluorescence stimulation to bulk ion concentration is shifted almost 3 log units toward lower concentrations for the divalent cation than for the monovalent species. The rate of rise in fluorescence was somewhat variable but generally stimulated two-to-threefold by $5 <$ NaCl < 50 mM, $0.2 <$ KCl < 5 mM or $MgCl_2 < 2$ μM (not shown). Combinations of these ions failed to exhibit syner-

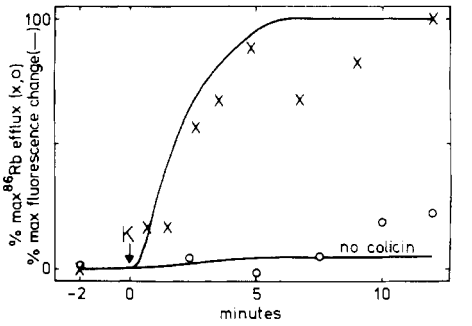

FIGURE 3: Relation of CC_6 fluorescence to ^{86}Rb efflux caused by colicin K. Cells grown with glycerol were prepared as described in Figure 1. At −12 min, 2 μl of CC_6 solution and 5 μl of $^{86}RbCl$ (2.5 μCi/ml, 8.3 μM final concentration) were added. Aliquots (50 μl, 2.5×10^6 cells) taken as indicated (X, O) were diluted 20-fold into Tris-glycerol medium containing 100 μM $^{85}RbCl$ at 37 °C on top of a 0.45-μm pore filter. Vacuum was applied, followed by washing with 2 ml of the same medium. Filters were dried and counted in 33% Triton scintillation fluid. The initial loading of the cells to equilibrium with ^{86}Rb (32 000 cpm/aliquot) is not shown. 100% maximum efflux was 16 000 cpm/aliquot. Fluorescence was followed simultaneously (—). Boiled colicin K (lower curve) or active colicin K (upper curve, multiplicity of 5) was added at 0 time.

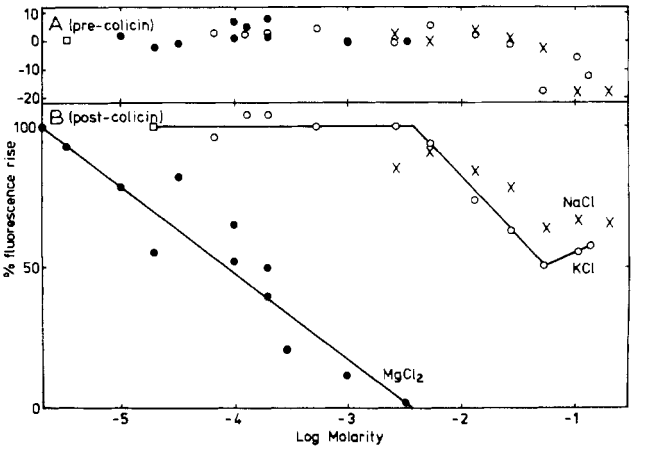

FIGURE 4: Effect of salts on CC_6 fluorescence changes caused by colicin K. Cells grown with glycerol were prepared as described in Figure 1. Cells were diluted tenfold into the cuvette at 37 °C containing 10 mM Tris-Cl, glycerol, and the indicated salt concentration, followed by the addition of 2 μg of CC_6 solution. (A) Fluorescence before colicin as percent of change caused by colicin in the absence of salts. (B) Maximum fluorescence after colicin K (multiplicity of 8) as percent of change caused by colicin in the absence of salts. KCl (O); NaCl (X); $MgCl_2$ (●); no addition of salts, arbitrary concentration plotted (□).

[2] The intracellular concentration of Rb^+ can be calculated using the Rb^+ specific activity, concentration, and observed cpm and assuming an intracellular water space of 8×10^{-16} l./cell (calculated from the sorbitol excluded volume, see Experimental Procedure). Pre-colicin $[Rb^+_{in}]$ = 140 mM; post-colicin $[Rb^+_{in}]$ = 70 mM. These numbers can not be used to estimate membrane potentials without first validating a number of assumptions.

gistic stimulation of the rate or inhibition of the steady state; no rates faster than those obtained with the optimal concentration of a single species and no steady-state levels of fluorescence different from those of KCl or NaCl alone were found. The negative slopes in Figure 4 imply that the membrane potential after colicin action is an electrochemical potential for an anion.

The observation that $MgCl_2$ is able to block the rise in CC_6 fluorescence caused by colicin K may imply that in some unknown manner $MgCl_2$ prevents the dissipation of the membrane potential. Alternatively, colicin may not act in the presence of 4 mM $MgCl_2$. If this were true, then trypsin treatment might rescue these cells from death. Unfortunately, when the cells were tested for viability inconsistent results were obtained (unpublished observations). However, measurements of susceptibility to proton permeability, induced by carbonyl cyanide-*m*-chlorophenylhydrazone, indicate that colicin K has acted under these conditions (Weiss, 1975).

Phosphate Transport. E. coli cells actively transport the phosphate anion to an internal concentration of approximately 10 mM (Medveczky and Rosenberg, 1971). In mitochondria, Lehninger (1974) reports a role of phosphate anion in antiport with other anions. It may be interesting therefore to determine the effect of colicin K on phosphate transport in *E. coli*. Figure 5 shows the biphasic uptake of $^{32}P_i$ by *E. coli* similar to that reported by Medveczky and Rosenberg (1971). Addition of colicin K at 5 min after ^{32}P addition increased only slightly the rate of ^{32}P uptake. The failure to observe inhibition by colicin suggests that phosphate transport is not dependent on the membrane potential.[3] Failure to observe substantial stimulation of phosphate uptake implies that anion efflux is not primarily a counterport with phosphate and that on the time scale monitored, the known release of phosphorylated intermediates of glycolysis (Fields and Luria, 1969b) is not limited by the rate of phosphate transport.

pH Changes. Sensitive *E. coli* do not change their proton permeability after treatment with colicin E1 (Feingold, 1970) or colicin K (Weiss, 1975). The possibility that colicin K was causing a depolarization of the membrane potential due to a loss of the transmembrane proton gradient remained to be examined. The weak acid DMO was employed as a radioactive tracer to measure differing degrees of ionization caused by a putative proton gradient (Waddell and Bates, 1969; Kashket and Wong, 1969). When *E. coli* cells are incubated at 37 °C in medium with lactate as carbon source, they appear to have an interior alkaline by 0.1 pH (pH 7.09 ± 0.02). Over a 10-min period, the intracellular pH was not significantly changed by treatment of the cells with colicin K under the described conditions (pH 7.12 ± 0.02). Thus, it appears that colicin K causes a membrane depolarization without altering the proton gradient across the membrane.

Discussion

The rise in CC_6 fluorescence caused by colicin K appears to monitor another irreversible feature of the "all or none" event in the action of this protein on sensitive bacteria (for others, see Luria, 1973). The interpretation that CC_6 is acting as a probe of colicin-caused depolarization of the transmembrane potential is consistent with other studies involving lipid vesicles and red blood cells (Sims et al., 1974; Hoffman and Laris, 1974), mitochondria (Tedeschi, 1974), and *Streptococcus* (Laris and Pershadsingh, 1974; Kashket and Wilson, 1974). As shown herein, a rise in CC_6 fluorescence is associated with treatments known to interfere with energization of the membrane: lack of carbon source, anoxia, and cyanide. Conversely, substances known to energize the membrane such as carbon source or oxygen result in a rapid decrease in system CC_6 fluorescence in cells formerly deprived of these chemicals.

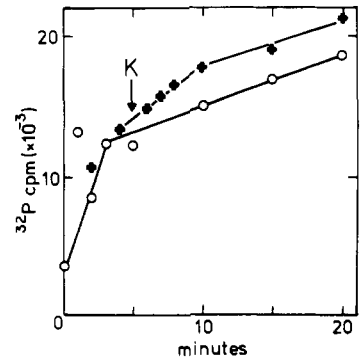

FIGURE 5: Effect of colicin K on phosphate transport. Cells grown with glycerol were prepared as described in Figure 1. At −3 min cells were diluted tenfold into 37 °C Tris-glycerol medium and preincubated at 37 °C. At 0 time $KH_2{}^{32}PO_4$ was added (2.5 μCi/ml, 50 μM). At the times shown, a 1.0-ml aliquot was filtered (0.45-μm pore size) and washed with 2 ml of 37 °C Tris-glycerol, 1 mM KP_i, pH 7.3. Filters were dried and counted in 33% Triton scintillation fluid. Either no colicin (O) or colicin K at a multiplicity of 3.5 (+) was added at 5 min.

The alternative explanation for the rise in fluorescence that must be kept in mind is that CC_6 may not be acting as a reliable probe of the membrane potential but that after the action of colicin K new environments for CC_6 may occur that are independent of membrane potential. This possibility is strengthened by some similarities in the fluorescence response to colicin of chlorotetracycline (Brewer, 1974), 8-anilino-1-naphthalenesulfonate, and *N*-phenyl-1-naphthylamine (Cramer et al., 1973; Phillips and Cramer 1973; and Helgerson et al., 1974). The fluorescence of *N*-phenyl-1-naphthylamine at least, which could not be responding directly to membrane potential, suggests that colicin E1 causes a change in the state of the membrane lipids. However, this change could be a secondary effect of membrane depolarization on membrane conformation.

It is of considerable interest that after colicin action anoxia or CN^- can further depolarize the membrane to a level near zero. Assuming that CC_6 fluorescence is a reliable probe of the membrane potential, these results imply that oxygen-dependent processes (i.e., electron transport) are one major source of the membrane potential and that there is another source that can be blocked by colicin K. The relationship of these two sources of the membrane potential to energization of substrate transport needs to be examined. For example, proline transport is known to be both dependent on oxygen (Klein and Boyer, 1972) and inhibited by colicin K (Fields and Luria, 1969a; Plate et al., 1974). Are the effects of colicin K and anoxia additive in inhibition of proline transport? In this regard, it is interesting that, in *E. coli* partially deenergized by CN^-, lactose is still actively transported albeit to a lesser extent (Lancaster et al., 1975). It appears that colicin K may uncouple active transport by directly interfering with the energized state of the membrane in the form of an electrical potential.

[3] Phosphate uptake is energized by ATP (Harold and Rosenberg, personal communication).

Ions. Schultz and Solomon (1961) have reported that log phase *E. coli* maintain a constant intracellular concentration of K^+ (211 mM) over a range of 5–120 mM in external K^+, while the intracellular concentration of Na^+ equals one-half the extracellular Na^+ concentration. The ratio of extracellular to intracellular chloride was found to be about 3 (Schultz et al., 1962). These measurements were made on cell pellets, which would be anaerobic. Potassium or rubidium are known to be actively transported in *E. coli,* with energization dependent on respiration (Klein and Boyer, 1972). According to the chemiosmotic model (Mitchell, 1966), substrate oxidation results in proton extrusion. Harold et al. (1970) found by the DMO method that glycolyzing *Streptococcus faecalis* cells have an interior alkaline by 0.5–1.0 pH units. The DMO method has been used with *E. coli* (Kashket and Wong, 1969) and *Streptococcus lactis* (Kashket and Wilson, 1974). The limitations on the measurement of intracellular pH by the DMO method have been discussed by Waddel and Bates (1969). The manipulations involved most likely render the bacteria anaerobic. In this report, the interior of *E. coli* was found to be alkaline by only 0.1 pH unit corresponding to an electromotive potential of 6 mV. The finding that colicin K does not alter the proton gradient is consistent with the findings of Feingold (1970) and Weiss (1975) that colicin E1 does not alter proton permeability.

Prior to colicin treatment, the absence of an effect of exogenous salts on fluorescence is probably due to *E. coli* actively adjusting its ion content to yield a fixed membrane potential that is relatively independent of the exogenous K^+, Na^+, Mg^{2+}, and Cl^- concentrations. The complex relationship of the CC_6 fluorescence response to colicin in the presence of NaCl, KCl, and particularly $MgCl_2$ can not yet be explained. However, after colicin the negative slopes suggest that the membrane potential is dependent on an electrochemical equilibrium of an anion. It may be relevant that colicin causes the efflux of pyruvate and certain phosphorylated intermediates of glycolysis (Fields and Luria, 1969b), all anions.

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