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Changes in E. Coli Cell Envelope Structure Caused by Uncouplers of Active Transport and Colicin E1

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It is of interest to inquire whether agents that uncouple or deenergize membranes cause concomitant structural changes. The agents considered here are the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone and the bacteriocidal protein colicin El, agents for which there is some precedent for believing that they interact with membranes.

In intact E. coli ML 308-225 cells the inhibition of [^{14}C]-proline active transport by FCCP increases with uncoupler concentration from ~ 20% at 2 μM to ~100% at 5 μM. The increase in the rotational relaxation time (ρ) of the cell-bound fluorescent probe N-phenyl-1-naphthylamine (PhNap)[1] and 8-anilino-1-naphthalenesulfonate (ANS) under these conditions shows the same dependence on FCCP concentration. For cells treated with EDTA to remove part of the outer lipopolysaccharide layer, inhibition of proline transport and the increase in ρ value of ANS show the same dependence on FCCP concentration with saturation at 0.3 μM. EDTA treatment causes a large increase in the binding and rotational relaxation time of PhNap, the latter quantity approaching a value obtained with purified inner membrane. Similar effects are produced in untreated cells by 5 μM FCCP. It is concluded that a) EDTA treatment removes a permeability barrier to FCCP and PhNap in the outer membrane; b) uncoupling by FCCP removes a similar permeability barrier to PhNap; c) binding of amphiphilic ANS, assumed to be located in the outer membrane, is hardly changed by these treatments; d) deenergization of the inner membrane by FCCP thus causes a structural change in the outer membrane as measured by the permeability change to hydrophobic PhNap and the increase in ρ values of the amphiphilic ANS; e) The binding sites reached by PhNap within the permeability barrier at or near the inner membrane are changed by FCCP from their initial state. This is inferred from an increase in PhNap quantum yield extrapolated to infinite cell concentration, and from removal by FCCP of an apparent phase transition sensed by the PhNap rotational relaxation time. Thus, uncoupling and deenergization by FCCP appears to cause structural change both in the outer membrane and inside the permeability barrier of the outer membrane.

Transmission of the colicin El response in the envelope of intact and EDTA-treated cells can also be monitored by an increase in ANS and PhNap fluorescence intensity, a smaller fractional increase in dye binding, and a large increase in probe rotational relaxation time. The fluorescence changes of ANS again imply structural effects in the outer membrane caused by colicin. The binding and fluorescence changes of PhNap caused by colicin El acting on intact cells again imply an effect of deenergization on the permeability barrier of the outer membrane. Fluorescence changes

[1] Abbreviations: FCCP – carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ANS – 8-anilino-1-naphthalenesulfonate; PhNap, N-phenyl-1-naphthylamine; EDTA – ethylenediaminetetraacetate.

with PhNap in intact and EDTA-treated cells show that the dye binding sites are altered in the presence of colicin El. It is also shown that the PhNap intensity change can be blocked by low concentrations of vitamin B_{12}, which competes for the colicin El receptor.

Some properties are presented of the probe chlorotetracycline, which has been proposed by others to be an indicator of magnesium. The probe appears to reside in an environment somewhat similar to that of ANS, but the colicin-induced changes in its fluorescence parameters appear to be small under our conditions.

Key words: colicin E1, membrane deenergization, cell envelope structural change, fluorescence probe

INTRODUCTION

The E. coli cell envelope is a complex, multilayered structure consisting of an outer membrane, a peptidoglycan layer, a periplasmic gap, and a cytoplasmic membrane (1). Biochemical techniques and electron microscopy have led to a model for the static structure of the cell envelope (2, 3). However, very little is known about dynamic structural changes in vivo. A plausible way to bring about such changes is to change the state of energization of the inner membrane. Two kinds of agents that might be used to bring about such changes are proton translocating uncouplers and colicins interfering with energy metabolism.

The ring-substituted derivatives of carbonyl cyanide phenylhydrazone (CCP) are examples of uncouplers of oxidative phosphorylation (4) and agents that dissipate the membrane high energy state. The mechanism of action of these agents is most simply explained in terms of their well-documented ability to increase proton conductance across model membrane systems (5,6) and to inhibit energy-linked proton movement in energy-transducing membranes (e.g., Ref. 7). It is also well documented that these compounds react with membrane protein (8,9) as well as albumin (10, 11). It is also known that these compounds bind to mitochondria (12,13) and lipid-depleted mitochondria (14). With the latter data serving as precedent, one may expect that membranes may not be completely passive in the presence of these uncouplers.

It is of interest to compare the mechanism of action of chemical uncouplers such as the CCP compounds in bacterial membranes with that of the class of colicins that appears to deenergize membrane function in a single-hit fashion. Colicin El is a protein, of molecular weight 56,000 (15), that attaches to a receptor protein located in the outer membrane of the sensitive cell (16). After this attachment, the killing event leads to a decrease in the intracellular ATP and potassium levels (17), inhibition of active transport (18), altered membrane permeability properties (19), and loss of viability. In some of the original literature on such colicins it was also suggested that they act by causing structural changes in the membrane (20,21). At the present time, in the context of the chemiosmotic hypothesis (22), one would tend to infer that colicins that interfere with cellular energy level most likely do so by depolarizing the cellular membrane potential.

In order to experimentally observe in vivo structural changes, noncovalently bound fluorescent probes are used. The fluorescence from these probes provides information concerning their chemical (23,24) and physical (25) environment. Small changes in the physical behavior of the probe can be measured and correlated with biochemical and

physiological events measured under the same conditions. Fluorescent probes provide a means of detecting structural changes as they occur in vivo, but the interpretation of the physical behavior of the cell-bound probe depends on knowledge of the location within the cell envelope and on the assumption that the probe is faithfully reporting on its environment. The experiments presented here indicate that deenergization of the E. coli inner membrane leads to structural changes in the cell envelope.

METHODS AND MATERIALS

Strains, media, and growth conditions. Escherichia coli ML 308-225 was grown in medium A containing (grams/liter): $(NH_4)_2SO_4$, 1.0; sodium citrate-$2H_2O$, 0.6; $MgSO_4$, 0.1; K_2HPO_4, 7.0; KH_2PO_4, 3.0; succinate (adjusted to pH 7.0 with KOH), 10.0; pH 7.0. E. coli strain B/1,5 was grown at 37° or at 20° as indicated in the figure legends, on M9 medium containing (g/liter): NH_4Cl, 1.0; $MgSO_4$, 0.13; NaH_2PO_4, 3.0; Na_2HPO_4, 6.0; glucose, 1.0; pH 7.0. All cultures were grown to a titer of $\sim 5 \times 10^8$ cells/ml, washed twice in M9 medium without carbon source, and resuspended in M9 without carbon source to the original titer.

Preparation of EDTA-treated ML 308-225 cells. ML 308-225 whole cells were treated with 0.1 mM K-EDTA (pH 7.0) for 1.5 min at room temperature essentially as described by Leive (26). The fluorescence intensity and polarization of 2 μM PhNap were measured in all EDTA-treated cell preparations before use in transport experiments. EDTA treatment results in a twofold to threefold increase in the intensity and a twofold increase in the polarization of 2 μM PhNap in the EDTA-treated cells relative to control cells.

Fluorescence polarization and lifetime measurements. Fluorescence polarization was measured with the instrument previously described (27), or a subnanosecond fluorometer (SLM Instruments, Urbana, Illinois) operating in the static polarization mode described below. Excitation for polarization and lifetime measurements was at 360 nm and emission was defined with a 2-mm-thick $NaNO_2$ liquid filter and a Corning 3-74 filter. Fluorescence lifetimes were measured by phase shift and modulation change (28) with incident light modulated at a frequency of 10 MHz. An individual lifetime measurement took approximately 2 min to complete. Values of lifetime, polarization, and rotational relaxation time utilized or plotted in Fig. 8 and Tables III–V were calculated with an on-line computer from the raw data.

Assay of [^{14}C]-proline transport. A 1.0 ml sample containing cells (titer $\sim 5 \times 10^8$, $\sim$ 0.07 mg total protein/ml) was incubated at room temperature with stirring. After 8 min in the presence of FCCP, D-lactate was added to a final concentration of 10 mM and the incubation continued for 5 min. Sample aliquots of 0.2 ml were then added to test tubes containing 10 μl of [^{14}C]-proline (specific activity 260 mCi/mmole, final concentration 10 μM). Transport was terminated by diluting the samples with 2 ml of 0.1 M LiCl and filtering the diluted sample through a millipore filter. The test tube and filter were washed once with 2 ml of 0.1 M LiCl. Activity was counted using a Nuclear-Chicago Gas Flow Counter operating at 10% efficiency. A control sample was prepared by first diluting the cells, adding the [^{14}C]-proline, and filtering immediately. The counts obtained from this sample were subtracted from all sample counts to correct for nonspecific binding. Aliquots of each sample were added to four separate test tubes and transport was terminated at 0.5, 1.5, 3.0 and 5.0 min.

Measurement of the probe binding to cells. The binding of the probes was measured by centrifugation, essentially as described previously (29). However, the amount of probe bound to the cell pellet and free in solution was determined by making the respective supernatant samples 3% in Triton X-100 and measuring the fluorescence intensity. In the presence of 3% Triton X-100 both ANS and PhNap are highly fluorescent with emission peaks at 490 nm and 430 nm, respectively. The relative fluorescence levels of the supernatant samples were read on the scanning fluorometer described previously (30). A standard curve of fluorescence intensity versus probe concentration in 3% Triton X-100 showed that 1 μM ANS and 0.5 μM PhNap could be accurately detected. Readings were corrected for the experimentally determined decrease in intensity due to light absorption by FCCP. Control experiments showed that no fluorescent material was released from the cells under these conditions. After correcting for the dilutions involved in making the supernatants 3% in Triton X-100, the probe bound (%) was obtained from the ratio of the intensity of probe in the pellet to the intensity of probe in pellet plus supernatant.

RESULTS

Studies on FCCP

The rotational relaxation time (ρ) of a fluorescence probe is a quantity calculated according to the Perrin equation from the 3 measured quantities; the polarization (p), the limiting polarization in a rigid solvent (p_0), and fluorescence lifetime (τ). ρ is a fluorescence parameter of interest since in a spherical approximation it is proportional to the average viscosity or microviscosity of the environment.* FCCP added to intact cells of the strain ML 308–225 causes an increase in the rotational relaxation time of the probes PhNap and ANS (Fig. 1). Each data point in Fig. 1 is derived from an individual polarization and lifetime measurement; the latter data have been presented elsewhere (31). The major changes in rotational relaxation time occur in the range 2–5 μM, and the data in Fig. 2 show that this is just the range in which proline transport is inhibited for cells incubated under conditions almost identical to those of Fig. 1.

It is of interest that the uncoupler FCCP does not seem to move freely through the outer membrane of the cell. It is known that the lipopolysaccharide of the outer membrane presents a barrier to hydrophilic molecules as large as pentalysine (32) and also more hydrophobic molecules such as actinomycin (26). Treatment of cells with EDTA, according to the method of Leive (26), is known to result in removal of 30–50% of the lipopolysaccharide from the outer membrane and removal of a permeability barrier to actinomycin D. EDTA treatment does not inhibit proline transport and allows ~ 100% inhibition of proline transport by only 0.3 μM FCCP (Fig. 3), approximately 20-fold less FCCP than required for this degree of inhibition in untreated cells.

EDTA treatment also appears to remove a permeability barrier to the probe PhNap since its rotational relaxation time in the absence of FCCP increases to approximately 10.9 nsec in EDTA-treated cells (Fig. 4) from 2.6 nsec (Fig. 1), and this approaches the value of 16.8 nsec obtained in purified inner membrane vesicles (31). Removal of the permeability barrier by EDTA results in an approximately threefold increase in PhNap

*$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$; $\rho = \frac{3\eta V}{RT}$, where η is the viscosity, V is the effective volume of the probe molecule, R is the gas constant, and T is the absolute temperature.

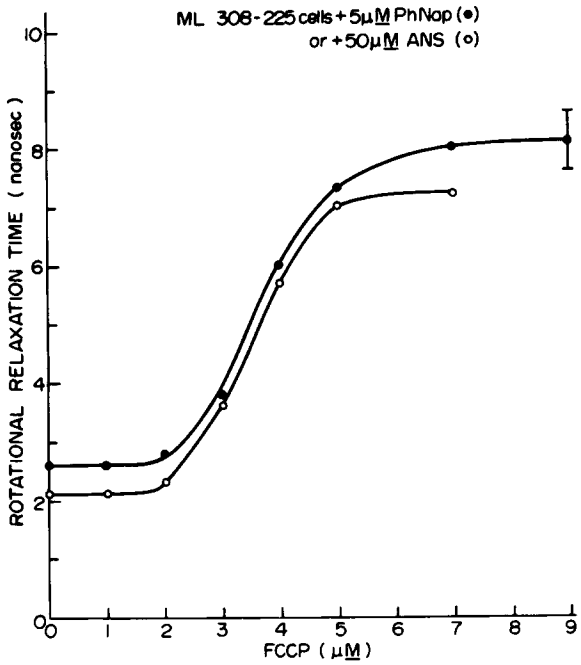

Fig. 1. FCCP-induced increase in the rotational relaxation time (ρ) of 50 μM ANS or 5 μM PhNap in the presence of strain ML 308-225 calculated from the Perrin equation using the fluorescence polarization values and the average lifetime values presented in Ref. 31. An increase in ρ indicates an increase in the microviscosity of the environment of the probe.

binding (Table I). The binding is 6.6% in the absence of EDTA and 20% in its presence. EDTA treatment does not seem to affect the binding and fluorescence properties of the amphiphilic molecule ANS (Table I and Fig. 4). There is a small increase in the rotational relaxation time of ANS, from 2.1 nsec to 3.2 nsec in EDTA-treated cells in the absence of FCCP. The latter value of the rotational relaxation time compares with a value of 24.1 nsec obtained with inner membrane vesicles.

The effect of FCCP on the fluorescence parameters of the two probes in EDTA-treated cells shows that the behavior of ANS is very similar to that in untreated cells except that the concentration range in which FCCP exerts its effect on fluorescence is decreased approximately 20-fold to the range in which it inhibits transport. FCCP appears to have only a slight effect on the rotational relaxation time of the PhNap probe in EDTA-treated cells, which, as noted above, is initially at a much higher level than in untreated cells. Thus, the change in fluorescence parameters of PhNap caused by FCCP may be very similar to that caused by EDTA treatment, and may be simply explained by deenergization leading to increased dye uptake (33) into or toward the inner membrane. The environment of the PhNap bound in the presence of FCCP is different from the environment of this probe in the absence of FCCP. This is shown by two experiments:

If the concentration of probe in the cell suspension is held constant and the fluorescence intensity measured as a function of increasing cell titer, then a double reciprocal plot – $(\text{intensity})^{-1}$ versus $(\text{cell concentration})^{-1}$ – extrapolated to infinite cell concentration will give a measure of the intensity when all the probe is bound to the cells. This

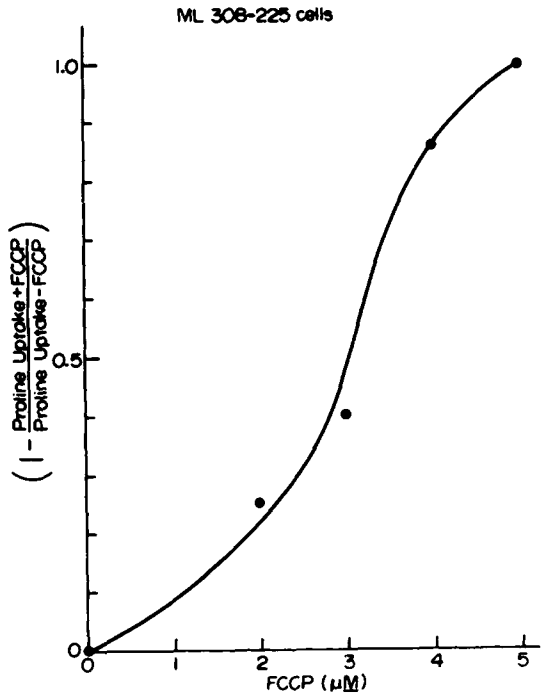

Fig. 2. Inhibition of proline transport in ML 308–225 whole cells versus FCCP concentrations. Cells were washed twice and resuspended in M9 medium without carbon source. Cells were incubated with the indicated concentration of FCCP for 8 min. Then 10 mM D-lactate was added and the incubation continued for 5 min. The [^{14}C]-proline transport assay was performed as described in Methods, the proline uptake in 30 sec being measured. Inhibition of uptake measured at longer times (1.5, 3, and 5 min) gave similar results. The cell titer was 7.7 × 10^8. Temperature, 25°C. Initial control rate of proline transport, 7.0 nmoles/min-mg protein.

experiment was performed at two fixed PhNap concentrations (3 μM and 5 μM) before and after the addition of 5 μM FCCP to whole cells (Fig. 5). Extrapolation of the plots to the I^{-1} axis intercept shows that 5 μM FCCP causes an increase of a factor of 2.8 and 2.7 in the intensity with 5 μM and 3 μM PhNap bound to the cells, respectively. The increase in intensity at infinite cell concentration indicates an increase in the quantum yield of bound probe (34).

Secondly, in the presence of FCCP the apparent phase transition defined by the temperature dependence of the rotational relaxation time of PhNap (Fig. 6) is removed. The discontinuity in rotational relaxation time of PhNap bound to cells without FCCP is shown. It has been documented that the rotational relaxation time of PhNap is proportional to the viscosity of glycerol-water mixtures over a range of 15–220 cP (31). The probe rotational relaxation time also reports accurately the apparent phase transition of fatty acid auxotrophs supplemented with different unsaturated fatty acids, as well as phospholipid dispersions derived from these cells (31).

Thus, the uncoupler FCCP appears to cause uptake of the PhNap probe and movement of the dye toward the inner membrane and a different environment for the probe. The disappearance of the apparent phase transition in the presence of FCCP implies that

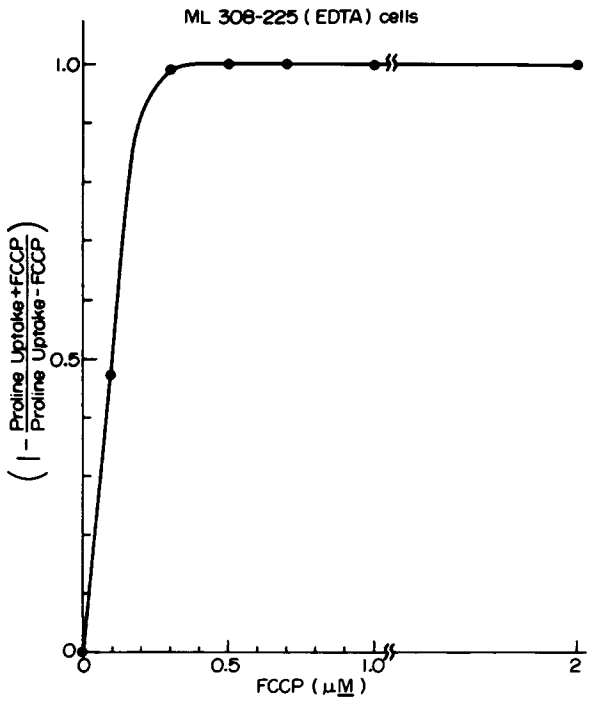

Fig. 3. Inhibition of [^{14}C]-proline transport in EDTA-treated ML 308-225 cells versus FCCP concentration. The experimental procedure is as described in Fig. 2 and Methods. The cell titer was 6.2×10^8. Control rate of proline transport, 5.4 nmoles/min-mg protein.

in addition to PhNap movement from the outside medium into the cell, and through the permeability barrier toward the inner membrane, part of the envelope within the permeability barrier has been altered in structure by the incubation with FCCP. This inference follows from Figs. 5 and 6 and the fact that both the inner and outer membranes are expected to show the phase transition (35). The existence of structural changes in the cell envelope outside the inner membrane, caused by FCCP presumably acting to deenergize the inner membrane, is also clearly documented by the changes in the fluorescence parameters of ANS, which are not complicated by changes in dye binding.

Studies on Other Uncouplers

The uncoupler 5-chloro-3-t-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) has also been shown to increase the proton conductance of model lipid membranes to an extent somewhat greater than that caused by FCCP (5). S-13 uncouples oxidative and photophosphorylation at concentrations comparable to those of FCCP (13). However, S-13 has no inhibitory effect on proline transport in whole cells of strain ML 308-225 at a concentration of 5 μM (31). It also does not change the fluorescence properties of bound ANS. This appears to be one more example of an outer membrane barrier to hydrophobic molecules, since EDTA treatment results in 0.3 μM S-13 causing 70% inhibition of proline transport (31). S-13 activity at this low concentration in EDTA-treated cells is also shown by an increase in the fluorescence intensity of bound ANS (31).

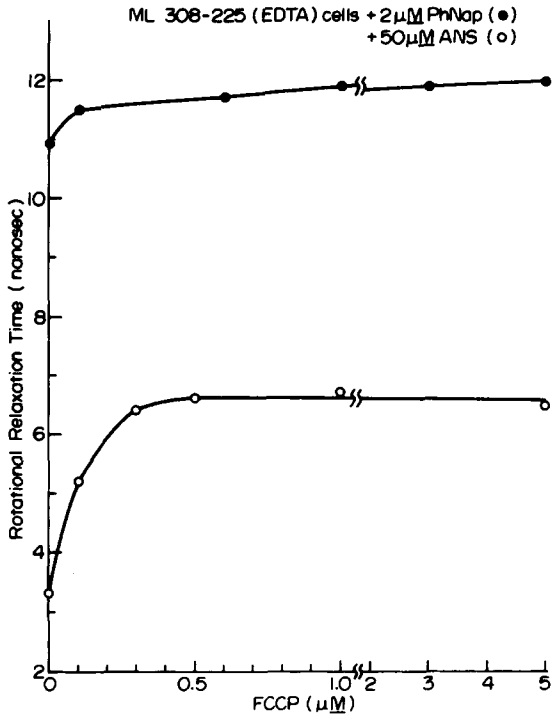

Fig. 4. The rotational relaxation time (ρ) of 50 μM ANS or 2 μM PhNap bound to EDTA-treated ML 308-225 cells versus final FCCP concentration. Values were calculated using the Perrin equation using fluorescence polarization and lifetimes from Ref. 31.

TABLE I. FCCP-Induced Changes in the Binding of ANS (50 μM) and PhNap (5 μM and 2 μM) to ML 308-225 Whole Cells and EDTA-Treated Cells

Sample	ANS bound (%)[a]	PhNap bound (%)[a]
Whole cells	2.8 ± 0.4	6.6 ± 1.6
Whole cells + 5 μM FCCP	3.2 ± 0.2	19.5 ± 1.1
EDTA-treated cells	3.3	20
EDTA-treated cells + 0.3 μM FCCP	3.1	–[b]

[a]Standard deviation based on four separate experiments.
[b]It is assumed that PhNap binding does not change upon addition of FCCP to EDTA-treated cells because there is no change in fluorescence intensity.

Studies on Colicin El

Previously published fluorescence probe experiments on colicin El document the existence of colicin-induced structural changes in the cell envelope (27,29,30,36). These results can be summarized as follows. a) Colicin El causes an increase in the fluorescence intensity and a small blue shift in the emission maxima of ANS and PhNap bound to the cell envelope (29,30). b) There is no fluorescence change when colicin El is added to

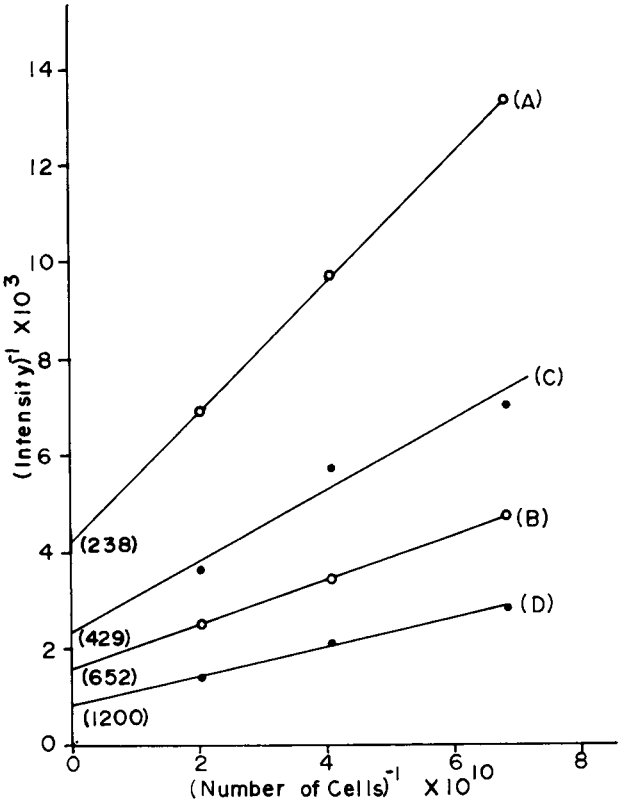

Fig. 5. Double reciprocal plot of the fluorescence intensity of PhNap bound to ML 308-225 cells versus cell titer. Additions are (A) 3 μM PhNap, (B) 3 μM PhNap + 5 μM FCCP, (C) 5 μM PhNap, and (D) 5 μM PhNap + 5 μM FCCP. Temp., 25°C. Intensities were measured using a front face cuvette geometry and were corrected for the background of scattered light from the cell suspension and the decrease in intensity due to absorption by FCCP. The values of the intensity (in arbitrary units) obtained by extrapolation to infinite cell number are shown in parentheses.

tolerant cells (31, 37), which bind colicin but are not killed, or to colicinogenic cells, which produce colicin but are immune (29). c) Dye binding and emission spectra studies with ANS show that the increase in intensity caused by colicin El is due to a change in the environment of the bound probe and not to an increase in probe uptake (29). d) The rate of fluorescence increase with PhNap is similar to the rate of the colicin-El-induced decrease in intracellular ATP and potassium levels (30). e) The fluorescence change is inferred to be an indicator of primary structural change in the cell envelope caused by colicin El, since the fluorescence increases occur with undiminished amplitude under conditions where there is no decrease in ATP levels and relatively little decrease in intracellular potassium levels (30). f) The fluorescence change occurs with the same time course or more slowly than the decay of trypsin reversibility of killing by colicin El (30); therefore, the fluorescence change may occur as the colicin penetrates into the cell envelope and becomes inaccessible to trypsin. g) An involvement of envelope lipid in the transmission mechanism of colicin El is suggested in biphasic Arrhenius plots of the fluorescence intensity increase (36). h) Colicin El causes an increase in the microviscosity

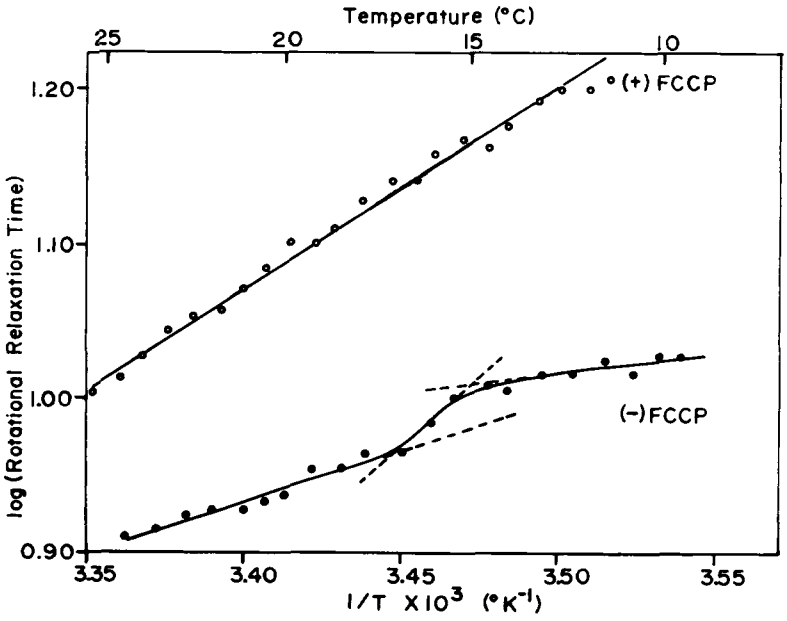

Fig. 6. Rotational relaxation time of 2 μM PhNap bound to E. coli B/1,5 cells grown at 20°C measured as a function of temperature in the presence and absence of FCCP. Polarizations and lifetimes were measured on two different samples (–) FCCP and (+) 3 μM FCCP. Data were obtained as a function of descending temperature. The cell envelope phase transition (–) FCCP has a midpoint at 16°C and a width of ~ 2.5°C. Cells grown at 20°.

of the PhNap probe environment as judged by an increase in the probe rotational relaxation time in the presence of colicin (27). The change in the fluorescence parameters of PhNap again could be due to a change in the probe environment, dye uptake from the external medium, and dye movement within the envelope.

Figure 7A shows the time course of the fluorescence intensity change of PhNap at 25° in intact cells. The fluorescence change is blocked by prior addition of vitamin B_{12}, whose transport system is known to utilize the colicin El receptor (38). This fluorescence assay is a very simple way to measure competition of different agents for the colicin receptor site (39). Fluorescence intensity changes in EDTA-treated cells are shown in Figs. 7C,D. The time course of the probe intensity change, which has always correlated well with metabolic changes caused by colicin El (30), is much faster in EDTA-treated cells (Fig. 7C). That the colicin response should be accelerated by removal of lipopolysaccharide or removal of a permeability barrier to hydrophobic molecules is not at all expected *a priori.*

It has been proposed that all of the intensity increase caused by another colicin interfering with energy metabolism, colicin I_a, is a consequence of increased uptake of PhNap (40). In the case of colicin E1, it appears that only part of the fluorescence intensity increase of PhNap is caused by an increase in PhNap binding. When B/1,5 whole cells are treated with colicin El at 25°C the intensity of cell-bound PhNap increases by a factor of 2.9, while the amount of dye bound to the cell increases by a factor of 1.9. With

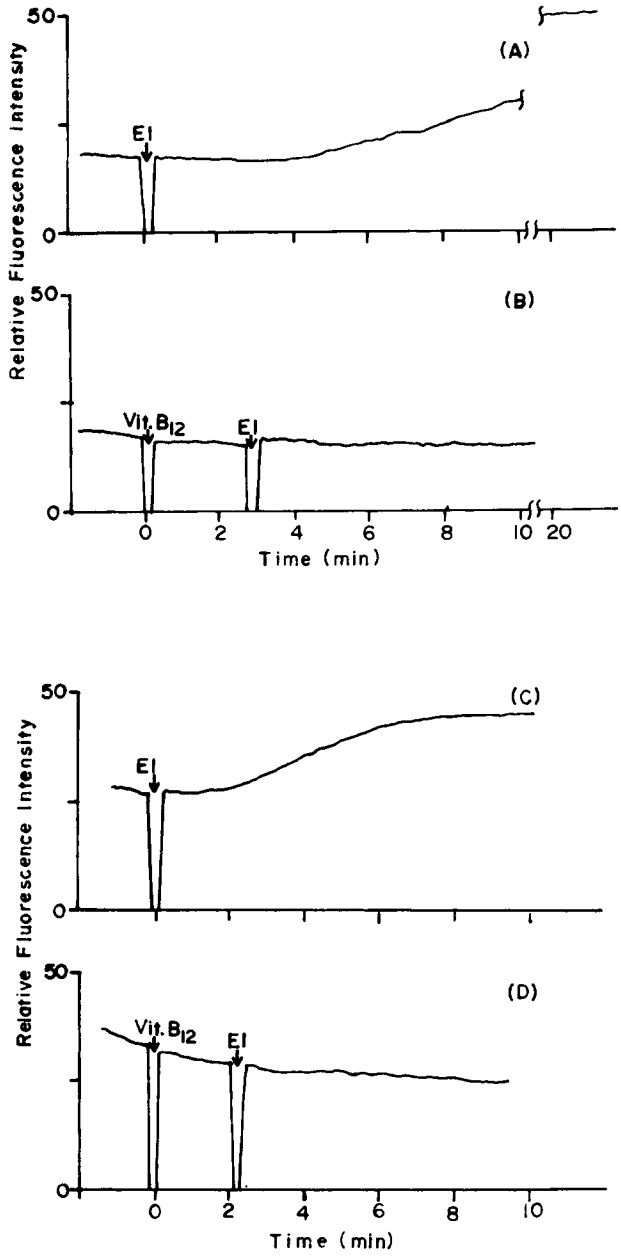

Fig. 7. Increase in PhNap fluorescence caused by colicin El in intact and EDTA-treated cells. Inhibition by vitamin B_{12} of the colicin-El-induced fluorescence increase. At the times indicated 0.1 μg/ml colicin El and 1.5 μg/ml vitamin B_{12} were added to E. coli B/1,5 whole cells (A and B) and EDTA-treated B/1,5 cells (C and D). The PhNap concentration was 3.3 μM in A and B and 2 μM in C and D. The cells were grown with glycerol as a carbon source at 37°C, harvested, and resuspended in M9 without carbon source. Fluorescence was excited at 360 nm with a band width of 3 nm and the emission measured at 410 nm with a band width of 4 nm; temperature, 25°C.

EDTA-treated B/1,5, the intensity increased by a factor of 1.6, while the amount of dye bound increased by a factor of 1.2 (Table II). In neither case can the colicin-El-induced intensity increase be accounted for solely by dye uptake into regions of the cell envelope similar to those occupied by the probe before colicin treatment.

The colicin-El-induced fluorescence increase in PhNap and ANS in the presence of whole cells and EDTA-treated cells is accompanied by increases in the fluorescence lifetime and polarization of the probes (Table III). The fluorescence polarization and lifetime of PhNap in the presence of whole cells increase from 0.084 to 0.151 and 3.75 nsec to 6.8 nsec, respectively. The fluorescence lifetimes shown in Table III were obtained with a light modulation frequency of 10 MHz and are the average of the phase and modulation lifetimes. Using the Perrin equation, the calculated rotational relaxation time (ρ value) of PhNap increased from 2.7 nsec to 11.2 nsec when whole cells were treated with colicin E1. EDTA-treated B/1,5 whole cells remain sensitive to colicin E1. EDTA treatment of B/1,5 caused the ρ value of PhNap to increase to 9.8 nsec and subsequent treatment with colicin E1 caused an increase to 13.6 nsec. The ρ value of ANS bound to B/1,5 whole

TABLE II. Colicin-El-Induced Changes in the Binding of PhNap to B/1,5 Whole Cells and EDTA-Treated Cells

Sample[a]	Probe	El[b]	Relative intensity[c]	Relative dye binding
B/1,5	5 μM PhNap	–	1.0	1.0
		+	2.9	1.9
EDTA-B/1,5	2 μM PhNap	–	1.0	1.0
		+	1.6	1.2

[a]Cells were grown with succinate as a carbon source, harvested, treated with EDTA where indicated, and resuspended at ~ 5×10^8 cells/ml in M9 without carbon source.

[b]1.0 μg/ml.

[c]Excitation, 360 nm; emission filters $NaNO_2$ + 3–75; intensity was measured with the SLM subnanosecond fluorometer; temperature, 25°C.

TABLE III. Colicin-El-Induced Changes in Fluorescence Polarization, Lifetime, and Rotational Relaxation Time of Cell-Envelope-Bound ANS and PhNap

Sample[a]	Probe	El[b]	p[c]	τ (nsec)[c]	ρ(nsec)
B/1,5	5 μM PhNap	–	0.084	3.75	2.7
		+	0.151	6.8	11.2
EDTA-B/1,5	2 μM PhNap	–	0.156	5.6	9.8
		+	0.171	6.7	13.6
B/1,5	50 μM ANS	–	0.104	2.5	2.1
		+	0.138	4.1	5.2
EDTA-B/1,5	50 μM ANS	–	0.117	2.7	2.7
		+	0.152	4.4	6.4

[a]Cells were grown and prepared as described in Fig. 7.

[b]0.1 μg/ml

[c]Polarizations and lifetimes were measured with the SLM subnanosecond flourometer; excitation, 360 nm; emission filters, $NaNO_2$ + 3–73; temperature, 25°C.

cells was calculated to be 2.1 nsec and increased to 5.2 nsec after treatment with colicin E1. EDTA treatment of whole cells of B/1,5 caused little change in the ρ value of cell-bound ANS in the absence (2.7 nsec compared to 2.1 nsec) or the presence of colicin (6.4 nsec compared to 5.2 nsec). The effect of EDTA treatment to greatly increase the ρ value of PhNap while having little effect on the ρ value of bound ANS is in agreement with the results obtained using E. coli ML 308-225 (Figs. 1 and 4 above).

Thus, treatment of sensitive whole cells and EDTA-treated cells with colicin El leads to a) an increase in the fluorescence intensity of ANS and PhNap, b) an increase in the amount of PhNap bound to the cell envelope, which is not enough to account for the intensity increase, and very little change in ANS binding; c) concomitantly, there is an increase in the measured lifetime and polarization of bound ANS and PhNap; and d) an increase in the calculated rotational relaxation time of bound ANS and PhNap, indicating an increase in the microviscosity of the probe binding sites.

Studies With Other Fluorescence Probes

Chlorotetracycline (CTC) is a chelating agent for divalent cations that is fluorescent only in the presence of added cations. The lifetime of the CTC-Mg complex increases as the solvent polarity decreases (Fig. 8), showing that the fluorescence parameters of this dye complex are dependent upon solvent environment. Caswell and Hutchinson (41) have shown that a) the fluorescence intensity increases with decreasing solvent polarity, b) the fluorescence intensity of CTC in SDS + 250 mM $MgCl_2$ is ~ four times greater than the intensity in Tris-HCl (pH 7.4) + 250 mM $MgCl_2$, and c) the intensity in the presence of Triton-X-100 and 250 mM $MgCl_2$ is the same as in Tris buffer + 250 mM $MgCl_2$. As shown in Table IV, the fluorescence intensity, lifetime, and polarization of 20 μM CTC in 10 mM Tris-HCl (pH 7.4) or 10 mM Triton X-100 are approximately the same in the presence of 10 mM $MgCl_2$. The calculated ρ value for the CTC-Mg

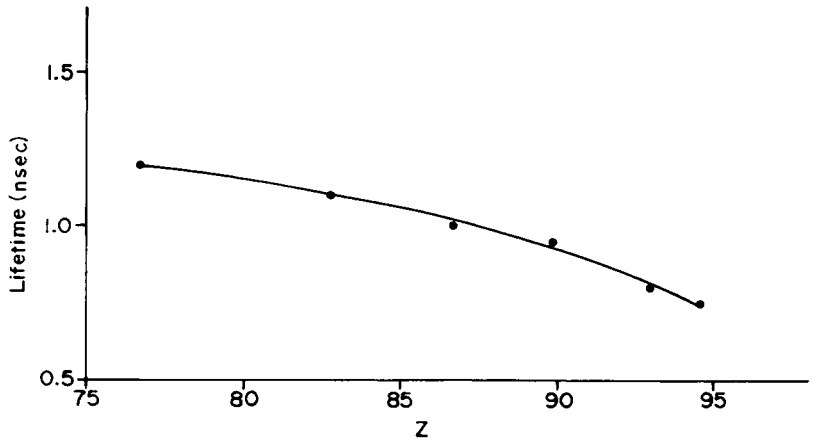

Fig. 8. Fluorescence lifetime (τ) of 20 μM CTC versus the empirical solvent polarity value Z (45) of various dioxane/H_2O mixtures. The percentage of dioxane was varied while the final concentrations of Tris-HCl (1 mM; pH 7.4) and $MgCl_2$ (10 mM) were held constant. The lifetime was measured by the phase method at a light modulation frequency of 10 MHz. Excitation wavelength, 385 nm; emission filters, $NaNo_2$ + 3–71; temperature, 25°C.

complex is 1.6 nsec in the aqueous solution and 2.3 nsec in the Triton X-100 micelle dispersion. In contrast the fluorescence of CTC in 10 mM SDS is enhanced ~ three times over the value in aqueous solution in the presence of $MgCl_2$. Using the Perrin equation and the measured lifetime (1.2 nsec) and polarization (0.331), the ρ value in 10 mM SDS + 10 mM $MgCl_2$ is calculated to be 6.6 nsec. Also shown are the relative fluorescence intensities and calculated ρ values for CTC in 10 mM SDS plus varying concentrations of $MgCl_2$. The fluorescence is a linear function of the log of the magnesium concentration up to ~ 1 mM $MgCl_2$; there is no increase in the fluorescence when higher concentrations of $MgCl_2$ are added. The ρ value increases somewhat with increasing magnesium concentration from 5.0 nsec at 0.01 mM Mg^{+2} to 6.7 nsec at 100 mM Mg^{+2}. A comparison of the fluorescence parameters of CTC bound to the E. coli cell envelope with the data obtained in detergent micelle/buffer dispersions is shown in Table IV. In micelles the fluorescence is enhanced over the level in aqueous solution only if the surface of the micelle is charged. Presumably the CTC-Mg^{+2} complex binds to the ionic head groups, resulting in a slower rotational relaxation time for the complex.

Although distinct from colicin El, colicin K (42) causes biochemical effects in the sensitive cell that are very similar to those listed above colicin El. Brewer (43) has reported the following colicin-K-induced effects on the fluorescence from cell-bound CTC: a) an approximately twofold increase in intensity when colicin K is added to cells at 37°C in the presence of 0.4% D-lactate; b) a decrease in fluorescence polarization from 0.14 to 0.06 in the presence of the colicin, and correspondingly c) a decrease in fluorescence lifetime from 1.6 nsec to 1.2 nsec; d) ρ values of 2.4 nsec and 0.4 nsec before and after treatment with colicin K, respectively, which were calculated using the Perrin equation

TABLE IV. Fluorescence Parameters for 20 μM CTC in Sodium Dodecyl Sulfate (SDS) and Triton X-100 Micelle/Buffer Dispersions

Sample[a]	Lifetime (nsec)[b]	Relative intensity[b]	Polarization[b]	ρ value (nsec)
10 mM $MgCl_2$	0.8	6.0	0.222[c]	1.6
10 mM Triton + 10 mM $MgCl_2$	0.9	5.6	0.238	2.3
10 mM SDS				
+0.01 mM $MgCl_2$	1.1	2.0	0.300	5.0
+0.1 mM $MgCl_2$	1.3	8.6	0.313	5.5
+1.0 mM $MgCl_2$	1.2	19.4	0.324	6.2
+10 mM $MgCl_2$	1.2	21.8	0.331	6.6
+100 mM $MgCl_2$	1.2	21.4	0.333	6.7

[a]All samples in 10 mM Tris-HCl (pH 7.4); temperature, 25°C.

[b]Excitation, 385 nm; emission filters, $NaNO_2$ + 3–71; lifetimes measured by phase at a light modulation frequency of 10 M Hz.

[c]This high polarization value for the probe is in contrast to probes such as PhNap and ANS, which have polarization ~ 0.01 when free in aqueous solution. CTC is not aggregating in the aqueous buffer, since the polarization was also found to be high in a dioxane (90%)/buffer solution. This high polarization of CTC may be due to alignment of the emission dipole along the long axis of the molecule; motions about this axis would cause depolarization much more slowly than about the other axis. When the structure of the long axis is broken to form iso-CTC (by treatment of CTC for 1.5 hr at pH 11), the polarization value in aqueous solution is 0.001. The limiting polarization (p_0) of chlorotetracycline is 0.48 (31).

with a p_0 value of 0.33. Using these data, Brewer suggested that in the absence of colicin, CTC is bound to the membrane; treatment with colicin K causes CTC and Mg^{+2} to move to a more fluid region, perhaps into the cytoplasm of the cell. This interpretation suggested that CTC is monitoring a structural change in the cell envelope different from that being monitored by PhNap or ANS. Both of these probes experience a less fluid environment after colicin treatment (Table III). The experiments described below were undertaken to determine a) if colicin K would cause an increase in the fluorescence of cell-envelope-bound PhNap, and b) if colicins K and El would cause the effects on cell-bound CTC that have been reported by Brewer.

When colicin K is added to E. coli B/1,5 whole cells at 37°C in the presence of 10 mM D-lactate, a rapid increase in the fluorescence from cell-envelope-bound PhNap is measured (Fig. 9). This increase is very similar to that observed when colicin El was added to the cell suspension. However, when CTC is used as the probe very little or no fluorescence increase is observed upon addition of colicin K (Fig. 9) or colicin El (data not shown). Also, when colicin El or K is added to a cell suspension under the same conditions used by Brewer, there is a slight decrease in the polarization and an increase in the lifetime of CTC (Table V). The ρ value for CTC calculated using the Perrin equation increases from 2.8 nsec to 3.1 nsec and from 3.1 nsec to 3.7 nsec upon the addition of

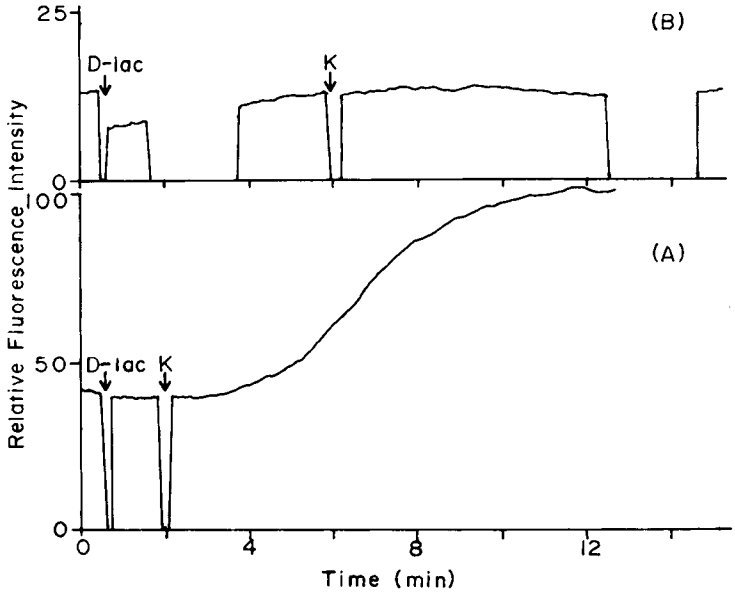

Fig. 9. The effect of D-lactate (10 mM) and colicin K (1.0 μg/ml) on the fluorescence intensity of (A) PhNap (4 μM) or (B) CTC (20 μM) in the presence of strain B/1,5 at 37°C. The cells were grown with succinate as carbon source, harvested, and resuspended in M9 without carbon source at 8.4×10^8 cells/ml. The survival after colicin K addition was ~0.01%. The gaps in the intensity recording are due either to the photomultiplier tube being turned off while the indicated additions were made or to the recorder being disconnected while the lifetime or polarization was measured (see Table V, for these data). Fluorescence was measured with SLM subnanosecond fluorometer and was excited at (A) 360 nm or (B) 385 nm with the emission defined by $NaNO_2$ + (A) 3–75 or (B) 3–71 filters. The intensity scale in (B) is expressed according to the units of (A), so the amplitude of the fluorescence in the presence of colicin K in (B) is approximately 12 units on the (A) scale. Purified colicin K was a gift from Dr. M. A. Jesaitis.

TABLE V. The Effect of D-lactate and Colicin El or K on the Fluorescence Lifetime, Polarization, and Rotational Relaxation Time of 20 μM CTC Bound to the E. coli B/1,5 Cell Envelope[a]

Sample	Lifetime (nsec)[b]	Polarization	ρ value (nsec)[c]
Cells + 20 μM CTC	1.5	0.253	4.2
+10 mM D-lactate	1.0	0.260	2.8
+0.1 μg/ml El	1.1	0.251	3.1
Cells + 20 μM CTC	1.7	0.260	5.0
+10 mM D-lactate	1.1	0.255	3.1
+1.0 μg/ml K	1.5	0.243	3.7

[a]See Fig. 9 for experimental procedure; temperature, 37°C; survival after colicin E1 was 0.2% and after colicin K ~0.01%.

[b]Lifetime measured by phase with a light modulation frequency of 10 M Hz.

[c]Calculated using the Perrin equation with $p_0 = 0.48$.

colicin E1 and colicin K, respectively. These changes are small and probably not significant due to the ± 0.5 nsec error present in the measurement of short lifetimes from CTC. At 25°C, the ρ value of CTC in the presence of strain B/1,5 is 8.7 nsec (lifetime = 1.9 nsec, polarization = 0.312), which increases to 9.7 nsec after addition of colicin E1. By comparison, the ρ value of CTC bound to SDS micelles at 25°C is 6.2 nsec, so that CTC is most likely bound at the external aqueous interface of the cell envelope. Brewer has proposed that treatment with colicin K causes CTC to move into the cytoplasm, where it occupies a much more fluid environment. This idea is not supported by the experiments presented here, since the ρ value of CTC is essentially the same after treatment with colicin. The disagreement with Brewer might be due to the use of a cell strain in that work that is resistant to chlorotetracycline. However, control experiments show that the viability of the E. coli B/1,5 that we used was not affected during the incubation times (< 20 min) of the experiments. We do not feel it is possible to draw any conclusions from chlorotetracycline probe studies about colicin-induced structural changes and magnesium movement.

DISCUSSION

The use of fluorescence probes in studies of membrane structure has its pitfalls. The great sensitivity of fluorescence parameters to changes in dye environment can itself be a problem. A slight change in dye environment can lead to a large change in fluorescence intensity. It is generally necessary to use other fluorescence parameters, such as polarization and lifetime, to confirm any inference based on measurements of intensity alone, and as well to correlate any fluorescence changes with physiological changes of the membranes or cells. The increase of PhNap fluorescence intensity appears to correlate well with de-energization measured by decay of the ATP level (30) or inhibition of active transport (figs. 2, 3 above), However, large amplitude reversal of this fluorescence change by added glucose does not correlate well with the ability of the membrane to support active transport (44). Reversal of the fluorescence change was reported by Nivea-Gomez et al. (40), who found that glucose added after colicin Ia caused a transient (in 3 min.) decrease of 60% of the fluorescence. It was proposed that glucose caused a momentary energization. The actual extent of this reenergization after addition of colicin E1 is always very small

(44). The fluorescence decrease does appear to indicate the direction of change in membrane energy level. It may be a quantitative indicator for small decreases in fluorescence, but not for fluorescence intensity changes of arbitrary amplitude.

The significance of the structural changes caused by FCCP and colicin E1 reported here firstly concerns dynamic interactions between inner and outer membrane of the E. coli envelope. It is generally assumed that the site of action of energizing or deenergizing events is the inner membrane. The assumption that the inner membrane is the sole site of action of FCCP is complicated by the discovery (Figs. 3,4) of the FCCP permeability barrier in the outer membrane. However, the similarity of the FCCP concentration dependence of the structural changes and inhibition of active transport (Fig. 1 vs Fig. 2 and Fig. 4 vs Fig. 3) implies that the relevant site of action for the structural changes is the inner membrane. In the case of colicin El, there is also a real possibility that interaction with the outer membrane causes structural changes in that membrane. However, the time course of the structural changes correlates best with the deenergization of the cell (30).

If we assume that the inner membrane is the relevant site of action of the uncouplers and colicin El, then the above work demonstrates that deenergization of the inner membrane causes structural changes in the outer membrane, where both fluorescence probes, PhNap and ANS, seem initially to be concentrated (31). A specific structural change in the outer membrane caused by deenergization is removal of a permeability barrier to hydrophobic molecules such as PhNap. These structural changes suggest that structural properties of the outer membrane are affected by the energy level of the inner membrane. Of all the different fluorescence probe effects discussed above, this is most simply shown by the effect of FCCP and colicin El on ANS fluorescence properties in EDTA-treated cells, since the complication of changes in dye binding is minimal in this case. A direct effect of FCCP on the inner membrane is implied by the changes in intensity of PhNap intensity extrapolated to infinite cell concentration, and perhaps even more dramatically by the elimination of the apparent phase transition sensed by the rotational relaxation time of PhNap.

Control of outer membrane structure by the cellular or inner membrane energization state is of obvious importance in the mechanisms of motility and transport of metabolites utilizing an outer membrane receptor. The studies presented here document the existence and some of the properties of such interaction and control.

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