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# Evidence for a Microviscosity Increase in the *Escherichia coli* Cell Envelope Caused by Colicin E1†

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ABSTRACT: Colicin E1 added at a low multiplicity to sensitive cells causes an increase in the polarization of fluorescence of the cell-bound probe *N*-phenyl-1-naphthylamine. The dye seems localized in the hydrocarbon regions of the cell envelope as inferred from its known properties and from the measurement of an abrupt change in fluorescence polarization at approximately 16°. An order-disorder transition in the lipid of cells of similar fatty acid composition has been documented by others to occur in this temperature range. Because the fluorescence lifetime of the cell-bound dye also increases in the presence of colicin E1, it is concluded that colicin causes an increase in the rotational relaxation time of the dye. Colicin E1 added at high multiplicities (10 μg/ml) causes a similar increase in fluorescence polarization from 8 to 26° and does not alter the magnitude of the order-disorder transition or change its midpoint by more than 1°. It is calculated from the Perrin equation that colicin causes the rotational relaxation time to increase from 3.6 to 6.5 nsec and from 6.6 to 10.5 nsec when added at 21 and 12.5°, respectively. The increase in rotational relaxation time could be explained by (1) a colicin-induced increase in microviscosity in the cell envelope or (2) a redistribution of the dye to regions of the envelope with higher microviscosity. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which causes a decrease in intracellular ATP under the conditions of the fluorescence experiment, also causes an increase in fluorescence polarization and rotational relaxation time when added in the absence of colicin. The question of whether a colicin-induced increase in microviscosity in the cell envelope could be a physical mechanism responsible for the inhibitory effects of colicin E1 is discussed.

There is very little known about the physical and chemical details of colicin-induced structural changes in the cell envelope which are associated with or are the direct cause of specific biochemical inhibitory events. One approach to gaining information on this problem has been through the use of fluorescence probes. Colicin E1, which has been purified as a protein of mol wt 56,000 (Schwartz and Helinski, 1971), causes an increase in fluorescence intensity and a blue shift in emission maxima of the probes 8-anilino-1-naphthalenesulfonate and *N*-phenyl-1-naphthylamine (Cramer and Phillips, 1970; Phillips and Cramer, 1973; Cramer *et al.*, 1973). The basic properties of the colicin E1 induced fluorescence changes discussed in the above references are as follows. (1) The existence of a probe fluorescence increase caused by colicin E1 correlates very well with the circumstances under which the adsorbed colicin is lethal. In particular, colicin E1 does not cause a probe fluorescence change when added to *colicinogenic* or *tolerant* strains. (2) The fluorescence intensity increase arises from cell-bound dye which in the case of the uncharged *N*-phenyl-1-naphthylamine is very likely localized in the cell envelope. (3) Dye binding and emission spectra studies show that the increase in probe fluorescence caused by colicin E1 is mostly due to a change in the environment of the bound dye and not to an increase in dye uptake. (4) The rate of the fluorescence increase is similar to the rate of the colicin E1 induced decrease in intracellular ATP and potassium levels. (5) The fluorescence

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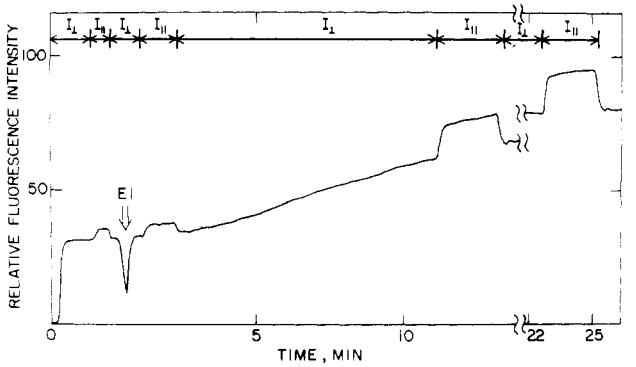


FIGURE 1: Colicin E1 induced increase in fluorescence and fluorescence polarization. The cells were grown and concentrated as described in the Methods. The experiment was done at 22° using 2 $\mu$M *N*-phenyl-1-napthylamine and 0.1 $\mu$g/ml of E1. The survival level was $1.8 \times 10^{-2}$ ($m \approx 4$). Fluorescence levels were measured with the analyzer Polaroid filter perpendicular ($I_\perp$) and parallel ($I_\parallel$) to the vertically polarized light defined by the excitation polarizer prism.

change is inferred to be an indicator of primary structural changes in the cell envelope caused by colicin E1, since the fluorescence increase occurs with undiminished amplitude under conditions where there is no decrease in ATP level and relatively little decrease in intracellular potassium levels. (6) This fluorescence change was not affected by a decrease of at least 30-fold in ambient $O_2$ level. (7) The fluorescence change paralleled or followed the decay of trypsin reversal of colicin E1 lethality. One simple explanation of this experiment is that the fluorescence change occurs as the colicin penetrates into the cell envelope and becomes inaccessible to trypsin. (8) An involvement of envelope lipid in the transmission mechanism of colicin E1 is suggested in biphasic Arrhenius plots of the initial rate of the fluorescence change. The transition temperatures of the Arrhenius plots vary with culture growth temperature in a manner consistent with the changes in fatty acid composition. A decrease in order or rigidity of the environment around the dye molecules near the transition temperature is implied by discontinuities observed in the fluorescence polarization plotted as a function of temperature.

The experiments reported in the present work are directed toward elucidating the nature of the primary structural changes in the cell envelope caused by colicin E1. It is shown in this work that colicin E1 causes an increase in the rotational relaxation time of bound probe molecules, which might be explained in terms of an increase in the microviscosity of part of the cell envelope.

## Methods

(1) Strains and Media, and Growth Conditions. *Escherichia coli* B/1,5 was grown and maintained in M9 medium containing (grams/liter): $NH_4Cl$, 1.0; $MgSO_4$, 0.13; $KH_2PO_4$, 3.0; $Na_2HPO_4$, 6.0; glucose, 1.0; and trace metals (Anraku, 1967). The cells were routinely grown at 20° for 6–7 generations to a titer of about $5 \times 10^8$ cells/ml and concentrated 5- to 10-fold to $3–5 \times 10^9$/ml in the growth medium without glucose for fluorescence experiments. Colicin E1 was purified by the method of Schwartz and Helinski (1971).

(2) Fluorescence polarization measurements were made with the instrument previously described (Cramer *et al.*, 1973). The excitation light was defined by a Bausch and Lomb high-intensity monochrometer with ultraviolet (uv) grating and deuterium source, and Corning 7-39 and 363 nm interference filters. Emission was defined by a 2-mm thick $NaNO_2$ liquid filter and a Corning 7-39 filter. With this filter combination the background of scattered and reflected light is 0.4% of full scale in the recording shown in Figure 1. The polarization values were corrected for this background. The polarization parameter, $p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, is defined relative to vertically polarized exciting light. The output was offset and amplified by fivefold at a time constant of 10 sec for accurate measurement of polarization (see Figure 3 of Cramer *et al.*, 1973). The cuvet was constantly stirred during the measurements using a magnetic stirring bar. *N*-phenyl-1-naphthylamine (Eastman) was used without further purification at a concentration of 2 $\mu$M. Higher dye concentrations caused an upward drift in the base line, particularly at temperatures above 25°. The limiting polarization, $p_0$, of *N*-phenyl-1-naphthylamine measured in glycerol at 20° was found to be 0.38.

(3) Fluorescence lifetimes were measured using a modified TRW nanosecond spectral source as previously described (Lytle, 1973), with a nitrogen light source and RCA 1P28A photomultiplier tube. The excitation and emission were defined by the same filter combinations employed in the polarization measurements. The excitation pulse intensity–time profile was digitized and then convolved in a laboratory computer with a family of synthetic exponential decays. The resultant curves were drawn on a Calcomp Plotter and transferred to a Xerox transparency. The experimental emission pulse was superimposed on the transparency to determine the best fit and the lifetime. For the $N_2$ lamp, the decays are generated every nanosecond and the lifetime can be reliably determined to $\pm 0.5$ nsec. Components in the emission with lifetimes less than 2 nsec were probably not resolved. An example of a lifetime measurement using the convolutes can be seen in Figure 1 of an earlier paper (Lytle, 1973).

## Results and Discussion

The change in polarization of fluorescence of cell-bound *N*-phenyl-1-naphthylamine is measured as a function of time after addition of colicin E1 in Figure 1. The downward spike seen in the traces of Figures 1 and 3 as colicin or FCCP[1] is added is simply a consequence of the photomultiplier tube high voltage being turned off during the addition. At 22° the fluorescence intensity increases over a period of about 20 min after addition of colicin at a multiplicity of 4 as calculated from the survival level, and during this time the polarization of the fluorescence also increases. The polarization value is 0.063 before colicin addition and 0.086 at the end of the experiment. There is no change in polarization in a control sample incubated 20 min without colicin. Polarization values obtained (a) before colicin addition and (b) after the maximal increase in fluorescence intensity is reached in the presence of 10 $\mu$g/ml of colicin are plotted as a function of incubation temperature in Figure 2. It is clear that the polarization increases after colicin addition at all temperatures. A transition in the fluorescence polarization parameter is seen in the absence of colicin. The transition is centered at 16° and extends from approximately 13–19°. An earlier measurement (Cramer *et al.*, 1973) showed a midpoint of about 14°. The measurements of Figure 2 utilized additions of an excess of colicin (10 $\mu$g/ml) so that the amplitude of the fluorescence intensity polarization change would definitely be saturated at all temperatures. The amplitude of the polarization increase obtained is the same as at low multiplicities with an addition of 0.1 $\mu$g/ml of colicin, as shown in Figure 1. Thus, the polarization change is not a secondary consequence of overloading the cells with colicin. The polarization increase occurs at the lowest multiplicities of active colicin where the biochemical consequences of colicin addition can be measured.

[1] Abbreviation used is: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

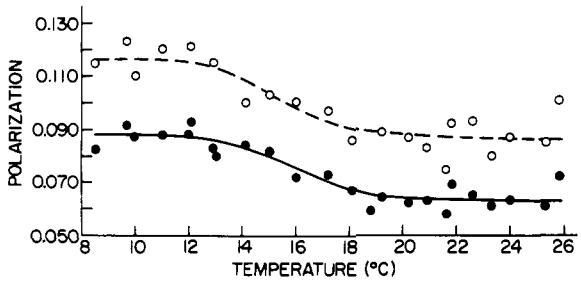


FIGURE 2: Polarization of cell-bound *N*-phenyl-1-naphthylamine as a function of temperature. The polarization was measured before (●) and after (○) colicin E1 addition. Each pair of values at a given temperature was obtained using a separate sample of cells. Experimental conditions and measurements were made as shown in Figure 1, except that 10 μg/ml of colicin E1 was used. Temperatures were measured with a United Systems Corp. digital thermometer to an accuracy of 0.02°F. The range of survival levels was $6 \times 10^{-4}$ to $3.9 \times 10^{-7}$.

TABLE I: Fluorescence Polarization and Average Lifetimes Measured above and below the Order–Disorder Transition; Rotational Relaxation Times, ρ, Calculated According to the Perrin Equation.

| Temp (°C) | E1 | FCCP | $p$ | $\tau$ (nsec) | $\rho$ (nsec) |
|---|---|---|---|---|---|
| 21.1 | − | − | 0.063 | 7.0 | 3.6 |
| 20.9 | + | − | 0.086 | 8.5 | 6.5 |
| 21.3 | − | + | 0.127 | 7.0 | 9.2 |
| 12.5 | − | − | 0.089 | 8.3 | 6.6 |
| 12.9 | + | − | 0.117 | 9.0[a] | 10.5 |
| 12.3 | − | + | 0.154 | 8.3 | 14.8 |

[a] Lifetime in the absence of colicin was 8.0 nsec instead of 8.3 nsec in this experiment.

One obvious interpretation of the transition showing a decrease in fluorescence polarization is that this represents an order–disorder transition in the fatty acid chains of the membranes, since (a) the probe is located in the hydrocarbon region of membranes, away from the membrane–water interface (Radda, 1971; Colley and Metcalfe, 1972); (b) fluorescence polarization is expected to be an indicator for the rotational motion of the probe if the fluorescence lifetime and rotational relaxation time are initially similar; (c) the fatty acid composition of the cells used in our present experiments has been measured (Cramer *et al.*, 1973) and resembles that of fatty acid auxotrophs grown on a *cis*-18:1 fatty acid supplement, where it has been documented (Overath *et al.*, 1970; Wilson *et al.*, 1970; Overath and Träuble, 1973; Träuble and Overath, 1973) that an order–disorder transition in the membrane lipid occurs near 15°, using a number of different physical techniques including fluorescence probes. The one physical technique which shows disparate transition temperatures is that of X-ray diffraction (Esfahani *et al.*, 1971). The width of the transition observed in Figure 2 is 5–6° which also is very similar to that observed by Overath and Träuble (1973) in cells, membranes and phospholipids of *E. coli*. The transition observed in Figure 2 is, moreover, better defined than that seen in whole cells by intensity changes in *N*-phenyl-1-naphthylamine fluorescence (Overath and Träuble, 1973), so that fluorescence polarization may be a more sensitive technique for measurement of lipid phase transitions in whole cells. Further comparative studies on the phase transition observed in whole cells using different techniques are needed to establish the relative sensitivity of the polarization technique.

Although colicin causes an increase in the polarization, Figure 2 shows that the transition region in the fluorescence polarization is changed very little in the presence of 10 μg/ml of colicin. It is concluded that colicin does not change the midpoint or the width of the order–disorder transition in the cell envelope by more than one degree.

The polarization, $p$, defined in Methods, is determined for spherical fluorescent molecules from the relation (Perrin, 1926, 1929; Weber, 1953):

$$(1/p) - (1/3) = [(1/p_0) - (1/3)][1 + (3\tau/\rho)] \quad (1)$$

where $p_0$ is the limiting polarization of a rigid randomly oriented population of emitters and $\tau$ is the lifetime of fluorescence; $\rho$ is the rotational relaxation time of the sphere, defined as the mean time required for movement of one axis of the sphere in space through an angle of arccos $1/e$. The Perrin equation may be only a crude approximation when applied to the *N*-phenyl-1-naphthylamine molecule, but it will be used here to discuss the variables on which the polarization of the cell-bound dye will depend, and to show qualitatively the changes in rotational relaxation time caused by colicin. Furthermore, the rotational relaxation times calculated from static polarization measurements are likely to be in error if the fluorescence is heterogeneous. In particular, a small background contribution of short lifetime and resulting high polarization will shift the average static polarization values and rotational relaxation times calculated therefrom to higher values. (We thank Professor G. Weber for a discussion leading to clarification of this point.) We thus wish to emphasize that we will use eq 1 to calculate relative rotational relaxation times in the presence and absence of colicin.

Using eq 1 to discuss the variables which will determine the polarization, the rotational relaxation time of an equivalent sphere is $\rho = 3\eta V/kT$ (Perrin, 1926, 1929; Weber, 1953), where $\eta$ and $V$ are the equivalent viscosities and volume, respectively. Thus

$$(1/p) - (1/3) = (1/p_0) - (1/3)[1 + (kT\tau/\eta V)] \quad (2)$$

The independent experimental variables in eq 2 which affect the value of the polarization, $p$, are the temperature, $T$, the lifetime, $\tau$, and the effective microviscosity, $\eta$. At a given temperature eq 2 shows that the increase in polarization observed in Figures 1 and 2 could be the consequence of a decrease in emission lifetime or an increase in rotational relaxation time and microviscosity.

The fluorescence lifetime was measured by a pulse technique before and after colicin addition at temperatures above and below the order–disorder transition observed in Figure 2. The lifetime before colicin addition at 21° is approximately 7.0 nsec and it increases to 8.5 nsec 20 min after addition of colicin at a concentration of 10 μg/ml (Table I). At 12–13°, the lifetime increases from about 8.3 to 9.0 nsec in the presence of colicin (Table I). It should be emphasized that the lifetime increase at 20° is unchanged if a colicin concentration of 0.1 μg/ml (a multiplicity of 3–4) is used. Preliminary measurements of the lifetimes by a phase method give slightly lower absolute values, evidently because of a short-lived emission component from the cells alone, but show approximately the same increase in lifetime upon colicin addition (experiment done in collaboration with G. Mitchell and G. Weber). Since the lifetime increases in the presence of colicin, the increase in polarization observed after addition of colicin must be caused by an increase in rotational relaxation time. The increase in rotational relaxation time caused by colicin multiplicities as low as 3–4 is calculated from eq 1, using a value of 0.38 for $p_0$. The rotational relaxation time increases from 3.6 to 6.5 nsec after colicin addition at

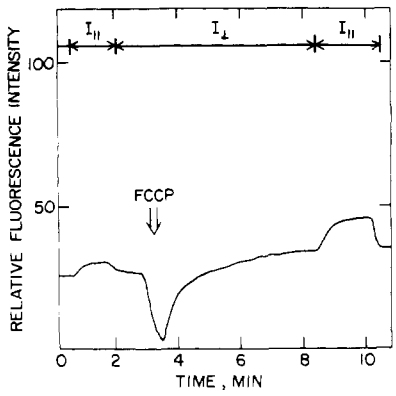


FIGURE 3: FCCP-induced increase in fluorescence and fluorescence polarization. The cells were grown and concentrated as in Figure 1 and the experiment was done at 21.1° using 2 $\mu$M *N*-phenyl-1-napthylamine. The fluorescence polarization increased from an initial value of 0.067 to a final value of 0.127 after the addition of $5 \times 10^{-6}$ M FCCP.

21°, and from 6.6 to 10.5 nsec at 12–13° (Table I). The increase in rotational relaxation time must be the consequence of an increase in microviscosity in the neighborhood of a significant fraction of the bound dye molecules. The increase in effective microviscosity could result either from (1) a real increase in microviscosity in the cell envelope caused by colicin, or (2) from a redistribution of dye molecules to regions of the envelope, perhaps intrinsic membrane protein, with unchanged and higher values of microviscosity. The first possibility seems more likely at the present time since the order–disorder transition sensed by the dye shows approximately the same amplitude in the presence as in the absence of colicin (Figure 2), indicating that the dye concentration in the hydrophobic lipid region remains approximately constant. There is a question, however, as to why an increase in microviscosity in the lipid region is not paralleled by an increase in the transition temperature, although there is a precedent for this in the case of cholesterol (see discussion below). Another reason for inferring an increase in microviscosity is that colicin also causes an increase in rotational relaxation time of the fluorescence probe 8-anilino-1-naphthalenesulfonate (data not shown) which is probably bound at the envelope–water interface (Radda, 1971; Colley and Metcalfe, 1972). Rather than postulate that colicin causes the two dyes bound in different parts of the cell envelope to seek regions of higher microviscosity, it seems more reasonable at the present time to assume that the colicin causes a general and widespread increase in microviscosity of the cell envelope.

Regarding possible mechanisms of colicin action, an increase in envelope microviscosity would support the conclusion that phospholipase A activity is not associated with early events in the action of colicin E1 (Cramer and Keenan, 1974). An increase in lysophosphatidylethanolamine would be expected to cause a decrease in membrane microviscosity below the transition temperature, since the microviscosity at 20° of an egg lysolecithin dispersion has been found to be about two-thirds that of a similar egg lecithin dispersion (Cogan *et al.*, 1973). In the case of colicin E1, the primary effects seem to be exerted on the cell envelope and affect potassium permeability (Luria, 1964), active transport (Fields and Luria, 1969; Lusk and Nelson, 1972), and apparently membrane-bound ATPase (Feingold, 1970). There is at present no evidence to support an enzymatic mechanism for colicin E1, and it would seem that any other degradative enzymatic activity affecting the membrane besides phospholipase A would also tend to decrease the order and the microviscosity, rather than increase it. Although an enzymatic mechanism cannot be excluded, it would seem worthwhile to consider a physical mechanism as well. Such a mechanism was originally proposed for colicin acting from the surface of the cell (Luria, 1964; Nomura, 1964; Changeux *et al.*, 1967). A modification of this model might include colicin E1 penetration into the cell envelope as an integral part of colicin action (Cramer *et al.*, 1973).

Regarding the nature of the physical mechanism, it might be hypothesized that colicin E1 causes the increase in microviscosity through a steric effect, by inserting itself into the membrane or envelope and acting somewhat like cholesterol in increasing microviscosity. It has been shown with fluorescence polarization techniques that cholesterol added to dipalmitoyllecithin at a molar ratio of 1:3 or 1:6 markedly increases the microviscosity of the dispersion without significantly changing the temperature range of 30–45° in which the microviscosity transition takes place (Cogan *et al.*, 1973). Calorimetric measurements have also shown that there is little effect of cholesterol on the transition temperature or the heat of transition below a cholesterol concentration of 20 mol % (Ladbrooke, *et al.*, 1968; Oldfield and Chapman, 1972). At higher concentrations of cholesterol, however, the phase transitions are eliminated, as shown by fluorescence measurements (Papahadjopoulos *et al.*, 1973), as well as by other techniques (Chapman, 1973). Colicin E1 acts on the *E. coli* cell envelope at a much lower molar concentration to increase the microviscosity, but the colicin molecule is much larger than cholesterol. An alternate or simultaneous physical effect of colicin E1 on the cell envelope might also involve a change in aggregation or conformational state of the membrane protein, as originally suggested by Singer and Nicolson (1972).

Could an increase in membrane or envelope rigidity be responsible for the inhibitory effects on active transport and the lowering of potassium and ATP levels? It is certainly well documented that the decrease in membrane fluidity which occurs below the transition temperature in *E. coli* is associated with an increase in the activation energy for transport (Overath *et al.*, 1970; Wilson *et al.*, 1970). Nothing can be said at present as to whether the colicin-induced increase in microviscosity will affect transport systems in the same way as a liquid crystal–gel transition in the fatty acid chains. Regarding the decrease in intracellular ATP level caused by colicin E1, we note that the uncoupler of oxidative phosphorylation FCCP causes an increase in the intensity of fluorescence of 8-anilino-1-naphthalenesulfonate bound to *E. coli* (Cramer and Phillips, 1970), as well as in intensity and polarization of fluorescence of *N*-phenyl-1-naphthylamine (Figure 3) and the calculated rotational relaxation time (Table I). In the presence of 5 $\mu$M FCCP the rotational relaxation increases from 3.6 to 9.2 nsec and from 6.6 to 14.8 nsec at 21 and 12.5°, respectively. FCCP at a concentration of 5 $\mu$M will cause the intracellular ATP level to decrease by about 50% under the conditions of the fluorescence experiment. The action of the substituted carbonyl cyanide phenylhydrazone compounds is certainly not identical with that of colicin E1, as FCCP does not generally cause an efflux of potassium (S. K. Phillips and W. A. Cramer, unpublished data) or an increase in probe lifetime (Table I), and carbonyl cyanide *m*-chlorophenylhydrazone and colicin E1 are known to have synergistic effects on the permeability of *E. coli* cells to protons (Feingold, 1970). However, the similar effects of FCCP and colicin E1 in decreasing ATP levels and increasing probe rotational relaxation time are of interest.

A question arises regarding the generality of the physical mechanism suggested here for colicin E1. Colicins E2 and E3 do not show an obvious fluorescence probe response with 8-ani-

lino-1-naphthalenesulfonate (Cramer and Phillips, 1970) or *N*-phenyl-1-naththylamine (J. Lacktis, unpublished data) and have been proposed to act through enzymatic mechanisms (E2: Almendinger and Hager, 1972; E3: Boon, 1971, 1972; Bowman *et al.*, 1971; Samson *et al.*, 1972). It should be noted, however, that the increase in endonuclease I accessibility to cytoplasmic DNA proposed by Almendinger and Hager to be caused by receptor-bound colicin E2 does not exclude a physical mechanism as part of the induction process. Furthermore, a mutant has been obtained in which lethal effects of colicin E2 associated with inhibition of cell division can be dissociated from DNA degradation (Beppu *et al.*, 1972). In the case of colicin E3, it seems unlikely that a physical mechanism such as that discussed here is a significant factor in its mode of action, since the *in vitro* studies do indicate that E3 can interact directly and catalytically with ribosomes in the absence of a membrane.

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