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Properties of the Fluorescence Probe Response Associated with the Transmission Mechanism of Colicin E1†

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ABSTRACT: Addition of colicin E1 to sensitive *Escherichia coli* in the presence of the fluorescence probe 8-anilino-1-naphthalenesulfonate (ANS) causes a blue shift of approximately 8 nm in the fluorescence emission spectrum of the bound dye. The colicin-induced fluorescence increase of the more lipophilic probe *N*-phenyl-1-naphthylamine (NPN) is very similar to that of ANS, is independent of potassium and sodium concentration from 0 to approximately 100 mM, and is not affected by preincubation in *N,N'*-dicyclohexylcarbodiimide which prevents the colicin-induced decrease in the intracellular ATP level. The decrease in ATP caused by colicin E1 is found to be unaffected by an increase in the potassium concentration of the medium. The decay of trypsin reversal of colicin lethal effects is slightly faster than the colicin-induced ATP decrease at 35°, and much faster than the rate of the colicin-induced increase of NPN fluorescence at 13°. The rate of the increase in probe fluorescence caused by colicin E1 is unaffected by a decrease of at least 30-fold in ambient oxygen concentration. It is concluded that the fluorescence probe response caused by colicin is an indicator of oxygen-independent structural or conformational changes in the cell envelope which are responsible for permeability changes and possible activation of ATPase. These structural changes occur at the same time or after the colicin becomes inaccessible or insensitive to trypsin.

Recent studies on the mode of action of colicin E3 have shown that the colicin itself has enzymatic properties as a specific ribosomal nuclease (Boon, 1971, 1972; Bowman *et al.*, 1971). These experiments imply that colicin E3 may exert an effect directly on its biochemical target, and to do so may penetrate the cell envelope at least to the level of the cytoplasmic membrane. These experiments imply that a long-range transmission mechanism previously hypothesized to operate between the colicin at a receptor site on the surface of the cell and target sites on the internal membrane (Nomura, 1964; Luria, 1964) may not be necessary to explain the mode of action of colicin E3.

Attempts to find an intrinsic DNase activity associated with colicin E2 have thus far been negative (Nomura, 1964; Almendinger and Hager, 1972), although this colicin at high concentrations can modify the DNA melting curve (Ringrose, 1972). It has been proposed that DNA degradation associated with the action of colicin E2 *in vivo* is caused by colicin binding to surface receptors and subsequently initiating an increase in the accessibility of periplasmic endonuclease I to the bacterial chromosome (Almendinger and Hager, 1972). This mechanism for colicin E2 could involve colicin-induced structural changes in the cell envelope or membrane.

In the case of colicins like E1 and K it seems likely that the mode of action involves perturbing the cell envelope in some way. This is simply because cells treated with colicin E1 rapidly lose potassium (Nomura, 1963; Wendt, 1970) and the potassium loss can occur in the absence of any decrease in intracellular ATP (Feingold, 1970). It is possible that envelope structural changes associated with colicin E1 could be the consequence of phospholipase activity associated with or induced by colicin E1 (Cavard *et al.*, 1968).

We have previously used the dye 8-anilino-1-naphthalenesulfonate (ANS)[1] as a probe for colicin-induced structural changes in the envelope of sensitive cells (Cramer and Phillips, 1970). Dyes such as ANS are useful as indicators of local changes in conformation or structure because their fluorescence is very sensitive to solvent parameters, in particular to solvent polarity, viscosity, and rigidity (references to the original work on this subject and the most recent general reviews are Weber and Laurence, 1954; McClure and Edelman, 1966; Turner and Brand, 1968; Stryer, 1968; Radda, 1971a,b; and Brand and Gohlke, 1972). Colicin E1, but not colicins E2 or E3, caused a large increase in the fluorescence of ANS bound to sensitive cells. That the ANS might be an indicator of structural changes caused by colicin E1 was indicated by the absence of any fluorescence change in cells colicinogenic for E1 and the qualitative similarity of the colicin-induced changes in fluorescence and intracellular ATP level (Cramer and Phillips, 1970). It was not possible to decide from this work whether the probe fluorescence response was (a) a primary

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; NPN, *N*-phenyl-1-naphthylamine; DCC, *N,N'*-dicyclohexylcarbodiimide.

response to colicin E1, or (b) perhaps a secondary consequence of the decrease in intracellular ATP and/or potassium levels. It is shown below that a fluorescence probe response caused by binding of colicin E1 to sensitive cells can occur in the absence of any decrease in intracellular ATP or any large decrease in potassium level. The changes in intracellular ATP and fluorescence probe response occur more slowly than the decay of trypsin reversibility, and with a rate that is unchanged by a decrease of at least 30-fold in ambient oxygen concentration.

Methods

Bacteria. The *Escherichia coli* K-12 strains used were W3110 thy$^-$ obtained from Dr. Irwin Tessman, NP3 from Dr. Luther Williams, and the deletion mutant KB5 from Dr. Barry Rolfe. *E. coli* B/1,5 was obtained from Dr. Simon Silver.

Media and Growth Conditions. Cultures were grown at 37° to a titer of approximately 5×10^8 cells/ml in (a) nutrient broth medium, (b) M9 medium, and (c) medium A containing (grams/liter): (a) Difco nutrient broth, 8; NaCl, 5; pH 7.0; (b) NH_4Cl, 1.0; $MgSO_4$, 0.13; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; glucose, 4.0 or 1.0 as noted; pH 7.0; (c) $(NH_4)_2SO_4$, 1.0; sodium citrate, 0.5 $MgSO_4 \cdot 7H_2O$, 0.1; K_2HPO_4, 7.0; KH_2PO_4, 3.0; glucose or glycerol, 4.0; pH 7.0. For fluorescence measurements the cultures were resuspended in M9 medium without a carbon source at a titer of approximately 5×10^9 cells/ml except where otherwise noted.

Preparation of Colicin. The procedure used was that of Schwartz and Helinski (1971).

Fluorimetry. Fluorescence was measured in a direction orthogonal to the excitation beam and from the front face of a 1 × 2 cm cuvet in order to minimize scattering losses. The cuvet was stirred and thermostated. The light detector was an EMI 9558A S-20 2-in. photomultiplier tube connected to a lock-in amplifier (PAR Model 122) tuned to the frequency of the chopped exciting light. The output from a potentiometer on the wavelength drive provided the wavelength input to an X–Y recorder for emission spectra. The wavelength axis was calibrated with the lines (435.8 and 546.1 nm) from a low-pressure mercury lamp. For measurements of spectra (Figure 1) the emission was defined by a high intensity Bausch and Lomb monochromator with a half-bandwidth of 4.8 nm in series with a 2-mm thick 2 M $NaNO_2$ filter, and the excitation by a Bausch and Lomb 150-W Xenon lamp used with the B&L high intensity monochromator, a 363-nm interference filter (limiting half-bandwidth of 16 nm), Corning 7-39 filter, and a 25% neutral density filter. The emission spectra were not corrected for the system spectral response. The principal effect of lack of correction of system response on the emission spectra is distortion of the overall shape of the spectrum; however, the emission peaks are shifted only slightly, if at all. For example, we find that the emission peak and effective half-bandwidth of ANS in ethanol are 478–480 nm and 3.8×10^2 cm^{-1}, respectively, as compared with 480 nm and 4.74×10^2 cm^{-1} in the work of Turner and Brand (1968), where correction was made for the system response.

For measurements of fluorescence yield changes in Figures 2–5, 7, and 8 a Bausch and Lomb 45-W deuterium lamp was used for excitation instead of the Xenon lamp, and a Corning 3-75 filter replaced the emission monochromator.

Measurements of ATP and Fluorescence Levels in a Common Sample. Aliquots were taken from the cuvet while the time course of the colicin-induced fluorescence change was being measured. These aliquots were immediately diluted in boiling buffer and assayed as described previously (Cramer and Phillips, 1970) using the firefly enzyme method with the luminescence being detected within seconds after adding the boiled cell extract to the cuvet so that the contribution of ADP to the signal is minimal.

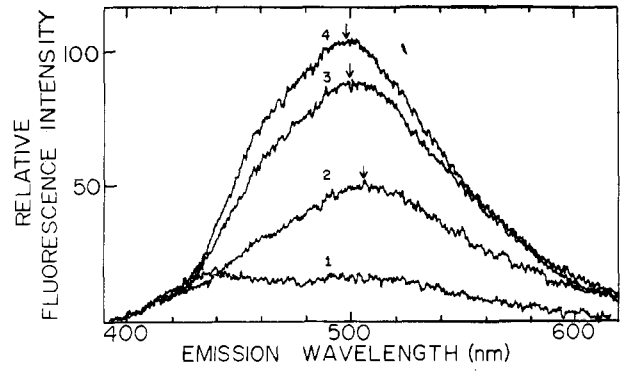

FIGURE 1: ANS fluorescence emission spectra from cell suspensions with and without colicin E1. *E. coli* B/1,5 was grown, concentrated, and resuspended in medium A. Trace 1, cells alone; 2, cells plus 60 μM ANS; 3, 3 min after adding 4 μg/ml of colicin E1; 4, 7 min after colicin E1 addition. The emission maxima of spectra 2, 3, and 4 are at approximately 506, 500, and 498 nm. The time required to scan a spectrum was about 1.5 min; temperature, 36°; cell survival after colicin addition, 1×10^{-4}.

Simultaneous Potassium and Fluorescence Measurements. A 1 × 2 cm cuvet with an enlarged area at the top to accommodate specific ion electrodes was used. The exciting light entered the 1 cm face and the fluorescence was detected from the 2-cm cuvet face in these experiments. The fluorescence probe *N*-phenyl-1-naphthylamine used in these experiments did not have any significant absorbance at the concentration used. The top of the cuvet was designed to receive an Orion 92-19 potassium specific electrode, an Orion 90-02 reference electrode, and a mechanical stirrer. The electrode output was calibrated at the time of the experiment in known concentrations of potassium. The electrode had no effect on cell viability in these experiments. The output of the electrode was amplified by a Corning Model 111 pH meter (millivolt mode) connected to a strip chart recorder.

Simultaneous Fluorescence and Oxygen Measurements. Oxygen concentration was measured with a Yellow Springs Instrument Co. No. 5331 oxygen electrode fit into a Lucite vessel which is part of the bath assembly of the YSI Model 53 oxygen monitor. The probe fluorescence could be detected through the transparent cylindrical Lucite vessel, with blocking filters and photomultiplier tube placed as close as possible to the vessel.

Chemicals. 8-Anilino-1-naphthalenesulfonate, the acid (P-10296), and magnesium salt (10990), together with *N*-phenyl-1-naphthylamine (351) which is ANS without the sulfonate group, were purchased from Eastman Organic Chemicals, Rochester, N. Y. The emission spectra of acid and magnesium ANS were found to be identical in 95% ethanol, with an emission peak at 480 nm. *N,N'*-Dicyclohexylcarbodiimide was purchased from Calbiochem (2971).

Results

Colicin-Induced Blue Shift in the Emission Spectrum of Cell-Bound ANS. The increase in fluorescence of ANS bound to sensitive cells caused by colicin E1 appeared to be mostly due to an increase in the quantum yield of fluorescence of the

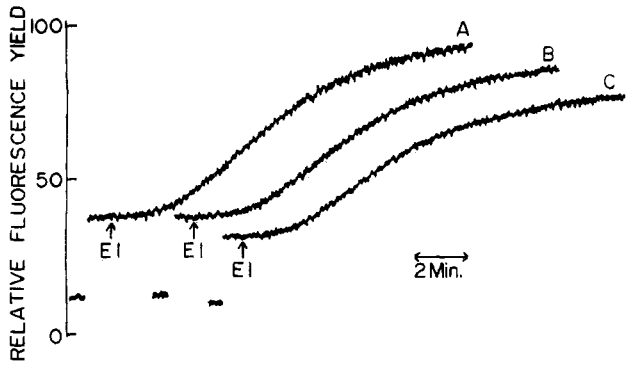

FIGURE 2: Colicin E1-induced fluorescence increase in media with different potassium ion concentrations. *E. coli* NP3 was grown in M9 medium with 0.4% glucose. Cells were concentrated and resuspended in the following media: curve A, M9 except entirely buffered with sodium phosphate; curve B, M9 medium; curve C, M9 except entirely buffered with potassium phosphate. NPN was added to 2 μM and 1.5 μg/ml of colicin E1 was added in each case; temperature, 25°; survival: A, 3×10^{-3}; B, 6×10^{-3}; C, 7×10^{-3}.

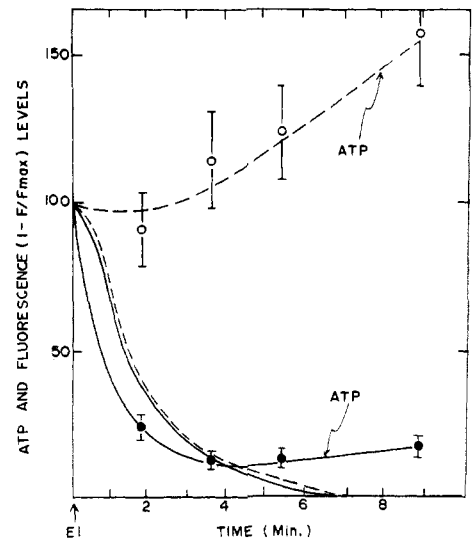

FIGURE 3: Colicin-induced fluorescence and ATP changes in control cells and cells incubated in DCC. *E. coli* B/1,5 was grown in medium A plus 0.4% glycerol to about 7×10^8 cells/ml. The fluorescence traces are replotted as $1 - F/F_{max}$, where F_{max} is the maximum fluorescence level reached. Error bars indicate the estimated error (±15%) in ATP values: (solid curves) fluorescence and ATP changes of a control suspension; (dashed curves) fluorescence and ATP changes of a suspension incubated with 100 μM DCC for 30 min at 37° before assay; NPN concentration, 2 μM; temperature, 37°; cell survival after addition of 1.5 μg/ml of colicin E1: control, 3×10^{-4}; DCC-treated sample, 3×10^{-4}.

bound dye, since the increase in dye binding was not sufficient to account for the fluorescence increase (Cramer and Phillips, 1970). An increase in the quantum yield of fluorescence of the bound ANS can be caused by a decrease in the local polarity and an increase in the microviscosity (McClure and Edelman, 1966; Radda, 1971a,b). Under these circumstances there should be a blue shift in the fluorescence emission spectrum because of the smaller interaction of the ANS excited state with the local dipole field and a smaller extent of solvent dipole rearrangement in a medium of higher viscosity. There is a small blue shift in the peak of the emission spectrum of bound ANS after addition of colicin E1. The shift is approximately 8 nm, from 506 to 498 nm (Figure 1), although there is some uncertainty in these peak values because of noise. The extent of the blue shift is 7–12 nm in different experiments. The ratio of the fluorescence yield in Figure 1 of cells alone (trace 1): cells with ANS (trace 2): cells with ANS and colicin (trace 4) is 1:2.5:4.7, as determined by the area under the respective curves. It is difficult to estimate the absolute values of the quantum yields in the presence and absence of colicin since the relative contribution of polarity and viscosity effects to fluorescence emission of the cell-bound ANS is not known. If the organic solvent data of Turner and Brand (1968), which correlate effective solvent polarity with quantum yield and emission maxima, are used to estimate the quantum yield of cell-bound ANS, maxima of 506 and 498 nm in the absence and presence of colicin would correspond, respectively, to quantum yields of 0.08 and 0.14.

N-Phenyl-1-naphthylamine (NPN) as a Probe for the Colicin E1 Response. The only difference in the chemical structures of ANS and NPN is the sulfonate group attached to the naphthalene ring of ANS which is not present on the ring of NPN. NPN is often more convenient to use as a probe, since it binds more tightly to *E. coli* and so can be used at lower concentrations. It is also much more lipophilic and therefore more surely localized in the cell envelope. The fluorescence yield of NPN in solution is dependent on solvent polarity and viscosity (Radda, 1971a). Figure 2 shows the colicin E1 induced fluorescence change of 2 μM NPN bound to sensitive cells in media with different potassium concentrations. The magnitude of the colicin-induced fluorescence change and its time course are very similar to that shown previously with ANS at concentrations in the range of 60 μM (Cramer and Phillips, 1970). The emission peak of the bound NPN in the presence of colicin is at 415–420 nm, shifted to shorter wavelength by approximately 5 nm upon addition of colicin (data not shown), a shift barely resolved by the monochromator. NPN is the fluorescence probe used in all experiments reported below.

Probe Response with Colicinogenic and Tolerant Strains. It has been demonstrated that colicin E1 did not cause any fluorescence change of ANS using colicinogenic cells (Cramer and Phillips, 1970). The tolerant mutant KB5 (Onodera *et al.*, 1970) has also been checked for colicin E1 induced fluorescence and ATP changes, and it is completely negative with respect to both parameters (data not shown). Thus, the colicin E1 induced fluorescence change is a parameter which behaves very much like those associated with colicin killing.

Relation of Colicin-Induced Fluorescence, Potassium, and ATP Changes. A principal question concerning the colicin-induced fluorescence probe response is whether it represents a primary response of the cell membrane or envelope to the colicin, or whether it is a secondary consequence of other cellular events, in particular the decrease in intracellular potassium and ATP levels. The loss of intracellular potassium could be the result of a positive colicin-induced change in the membrane potential. This change in polarity would be in the correct direction to allow an increase in the binding of ANS. The increase in ANS fluorescence in energized submitochondrial fragments or upon deenergization of mitochondria has been attributed to increased binding of ANS caused by such a positive change in the membrane potential (Skulachev, 1971). The colicin-induced fluorescence increases shown previously and above (Figures 1 and 2) are, on the contrary, mostly independent of such electrostatic effects. This is because (a) the colicin-induced increase in bound ANS can at most account for one-third of the fluorescence increase with this negatively

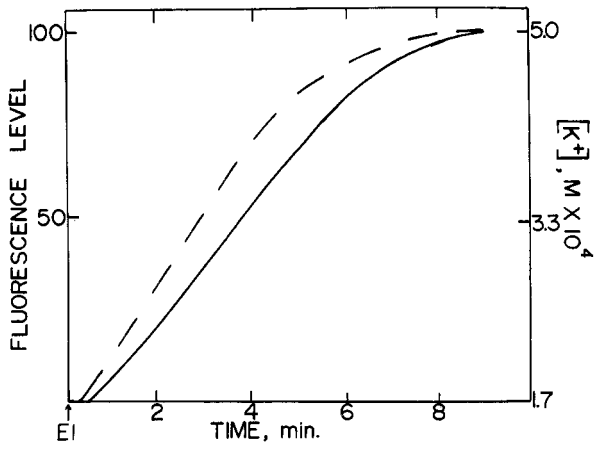

FIGURE 4: Simultaneous measurement of the kinetics of the colicin-induced fluorescence increase (solid curve) and potassium efflux (dashed curve). *E. coli* W3110 thy⁻ was grown in nutrient broth and resuspended at a concentration of 3×10^9/ml in 0.15 M NaCl–0.08 M Na_2SO_4–1 mM $MgSO_4$, pH 7.0. Potassium and reference electrodes were inserted into a cuvet as described in Methods; NPN concentration, 6 μM; temperature, 33°; fraction of cells surviving 9 min after addition of 2 μg/ml of colicin E1, 7×10^{-2}.

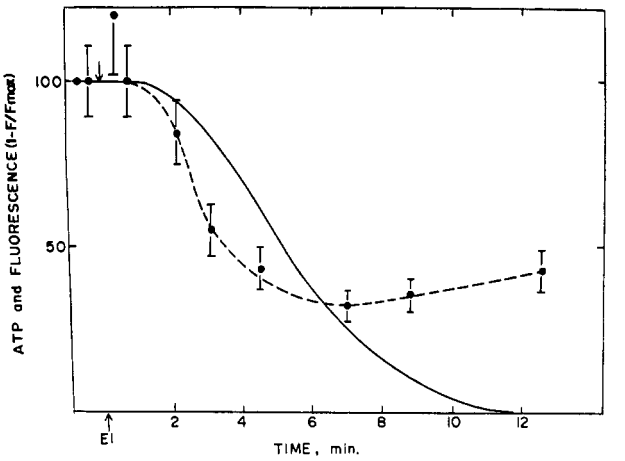

FIGURE 5: Kinetics of the colicin-induced ATP decay (dashed curve) and fluorescence increase (solid curve). *E. coli* NP3 was grown and resuspended in M9 medium with 0.4% glucose. Fluorescence is plotted as $1 - F/F_{max}$. At the indicated times 0.1-ml aliquots were taken and immediately diluted into boiling buffer; temperature, 30°; fraction of cells surviving 13 min after addition of 1 μg/ml of colicin E1, 3×10^{-2}.

charged dye (Cramer and Phillips, 1970); and (b) the fluorescence response of the much more lipophilic probe NPN, which is expected to be electrically neutral without the sulfonate group, is similar to the ANS response (compare Figure 2 with Cramer and Phillips, 1970). The possibility that dissipation of potassium or sodium gradients is directly responsible for a structural change in the envelope to which the fluorescence probe responds appears remote, as the probe response is independent of external potassium concentrations from 0 to 91 mM, and external sodium concentrations from 109 mM to 0 (Figures 2A–C). The media used in Figure 2 are identical except for the exchange of potassium for sodium in the phosphate salts. The potassium concentrations are 0, 22 mM, and 91 mM and the sodium concentrations 109 mM, 84 mM, and 0 in Figures 2A, 2B, and 2C, respectively. The intracellular potassium concentration of cells grown to early log phase in a medium containing glucose at 2.5–10 times the concentration used in these experiments is about 200 mM (Schultz and Solomon, 1961).

The colicin-induced fluorescence probe response can also occur independently of any decrease in intracellular ATP level (Figure 3). The colicin-induced ATP decrease can be prevented by preincubation of the cell suspension in 100 μM *N,N'*-dicyclohexylcarbodiimide (DCC), as shown previously by Feingold (1970). DCC has been shown to be an inhibitor of membrane-bound ATPase in *S. faecalis* (Harold *et al.*, 1969). The time course of the fluorescence change in Figure 3 (plotted as $1 - F/F_{max}$) is almost exactly the same in the presence and absence of DCC, in spite of the fact there is no ATP decrease in the sample pretreated with DCC. Although the raw data are not shown, the absolute amplitudes of the fluorescence changes are also the same. The initial ATP levels in the DCC-treated sample are the same as those of the control within experimental error (±15%), though there seems to be a higher level of ADP present in the DCC-treated sample. From his studies with DCC, Feingold (1970) concluded that the colicin-induced potassium loss and inhibition of biosynthesis were not caused by the decrease in intracellular ATP levels alone. It was suggested that the ATP decrease might be caused by activation of a membrane-bound ATPase. We can conclude from Figures 2 and 3 that the fluorescence probe response is not caused by either the decrease in intracellular potassium *per se* or necessarily by the ATP decrease. It might be proposed instead that the fluorescence probe response reflects structural or conformational changes in the cell envelope which are responsible for the loss of potassium and possible activation of ATPase.

One problem with the above hypothesis is that when the kinetics of the potassium efflux and fluorescence increase (Figure 4) or of the ATP decrease and fluorescence increase (Figure 5) are measured in separate experiments there is a slight lag in the fluorescence response. In the experiment shown in Figure 4 a potassium electrode was used to monitor the extracellular potassium in the medium which increased from 1.7×10^{-4} to 5×10^{-4} M during the course of the colicin-induced potassium efflux, the potassium and fluorescence changes being measured simultaneously.

Figure 5 shows the kinetics of the colicin-induced ATP change in more detail than previous measurements (Cramer and Phillips, 1970). The ATP decrease which attains a minimum of 30% of the control level occurs somewhat more rapidly than the fluorescence increase, with the latter normalized to the maximum fluorescence increase and plotted as $1 - F/F_{max}$ for purposes of comparison. The increase in ATP level which begins 5 min after the colicin addition is often seen in the concentrated cell suspensions used for the fluorescence experiments (Cramer and Phillips, 1970). If the loss of intracellular potassium is responsible for the decline in ATP levels (Feingold, 1970), the rate and extent of the ATP decline should be diminished in the presence of high external potassium concentrations. It was found that the rate and extent of the ATP decrease in the high (91 mM) potassium medium were very similar to those shown in Figure 5 (data not shown).

Relation between the Time Course of Trypsin Reversal of Colicin E1 Killing, ATP Decline, and the Fluorescence Probe Response. Assuming that the probe response represents primary or secondary structural or conformational changes in the cell envelope caused by colicin E1, it is important to know whether such changes occur while the lethal effects of colicin

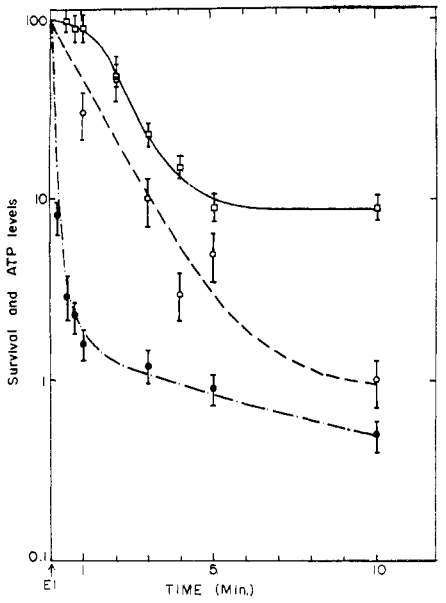

FIGURE 6: The kinetics of colicin adsorption and the decay of intracellular ATP and trypsin rescue. *E. coli* NP3 was grown in M9 medium with 0.4% glucose and respspended at 8×10^8 cells/ml. Adsorption kinetics (– · –) were measured by diluting an aliquot taken at the indicated times into M9 buffer at 37° and immediately plating after subsequent dilution. For measurement of trypsin rescue (– – –), 250 μg/ml of trypsin was added to the M9 dilution tube and the diluted aliquot was incubated for 10 min at 37°; 0.5-ml samples were taken for ATP measurements (solid curve); temperature, approximately 35°; cell survival shown in figure.

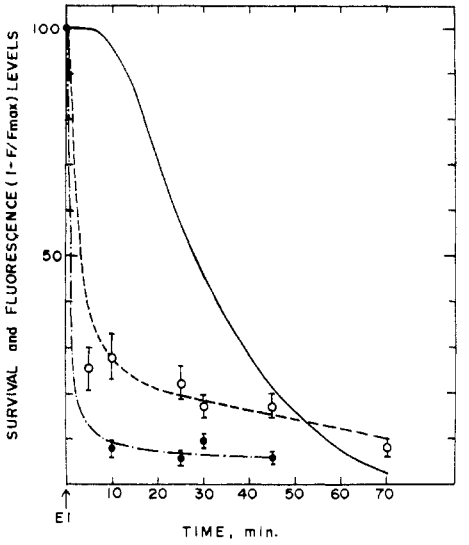

FIGURE 7: The kinetics of colicin adsorption, the decay of trypsin rescue, and the fluorescence increase at 13°. *E. coli* NP3 was grown in M9 with 0.1% glucose at 37°. Fluorescence (solid curve) plotted as $1 - F/F_{max}$; adsorption (– · –) and trypsin rescue (– – –) measured as in Figure 6; NPN concentration, 1.75 μM; cell survival shown in figure.

are trypsin reversible. The inhibitory effects of colicins K (Nomura and Nakamura, 1962) and the lethal effects on colony forming ability of colicins E2 (Maeda and Nomura, 1966; Reynolds and Reeves, 1969) and E3 (Nomura and Maeda, 1965) are partly reversible by trypsin added 0.5–1 hr after the colicin. It was inferred from these experiments that the inhibitory and lethal effects of these colicins are exerted from the outer, trypsin-accessible surface of the cell envelope (Nomura, 1964; Luria, 1964). It has been observed, however, that in the absence of metabolic inhibitors the decay of trypsin reversibility may occur much more rapidly (Reynolds and Reeves, 1969). Figure 6 shows that the decay of trypsin reversibility as measured by cell survival occurs somewhat faster than the decrease in the intracellular ATP level. In control experiments it was shown that trypsin treatment itself had no effect on cell viability and inactivated free colicin by at least 95% in 40 sec, and that doubling the trypsin concentration or increasing the incubation time in trypsin to 1 hr did not increase the efficiency of trypsin rescue. Figure 6 also reemphasizes the point made previously (Cramer and Phillips, 1970), that adsorption which is 90% complete in 15 sec and 98% complete in 1 min does not play a role in determining the kinetics of the colicin-induced probe response or biochemical changes observed here. The relation of the cell survival functions with and without trypsin in Figure 6 is very similar to that shown previously for colicin E1 by Cavard *et al.* (1971).

Figure 7 shows a comparison of trypsin rescue and fluorescence probe response at a lower temperature (13°), where the fluorescence change is significantly slower. The decay of trypsin rescue at the lower temperature occurs much more rapidly than the change of fluorescence. It must be concluded from Figures 6 and 7 that both the ATP decline and fluorescence probe response occur after the colicin has become partly or wholly inaccessible or insensitive to trypsin.

Fluorescence Probe Measurements of the Oxygen Requirement for Colicin Action. Since potassium efflux, inhibition of RNA and protein synthesis, and the fluorescence probe response can occur in the absence of a decrease in ATP (Feingold, 1970; Figure 3 above), it seems unlikely that the main inhibiting effects of colicin E1 are a consequence of its being an uncoupler of oxidative phosphorylation, as has often been stated in the literature. One reason for the belief that colicin E1 might affect oxidative phosphorylation was the unpublished report of Levinthal and Levinthal (cited in Luria, 1964) that when cells sensitive to colicin E1 were grown under anaerobic conditions they continued to synthesize protein and RNA after addition of colicin E1. Fields and Luria (1969b), however, reported inhibition by colicins E1 and K of TMG accumulation as well as incorporation of uracil and amino acids in cells growing under conditions anaerobic enough so that glucose metabolism was fermentative rather than respiratory. Fields and Luria (1969b) also found that hemin-deficient mutants were colicin sensitive, and suggested that any protection by anaerobic conditions against colicin might be independent of oxidative phosphorylation. Feingold (1970) proposed that the oxygen requirement for colicin E1 action might involve lipid peroxidation.

The published experimental work on the oxygen requirement has thus far always involved cells grown under anaerobic conditions. This raises the question as to whether the oxygen requirement for colicin sensitivity reflects an actual requirement for the colicin interaction or whether the cell envelope changes structure under anaerobic conditions so as to render the cell resistant or tolerant, either through impaired receptor function or transmission of the response. An effect of anaerobic conditions on envelope structure seems to be a possibility since it is known that the fatty acid composition of the cells changes with decreased oxygen tension (Knivett and Cullen, 1965). The fluorescence probe assay for colicin sensitivity

allows a direct measurement of rate of the colicin effect under anaerobic and aerobic conditions. Cells that had been grown aerobically were placed in the electrode well of a water-jacketed oxygen electrode assembly. The electrode well is transparent and fluorescence emission from the well could be detected at a right angle to the excitation beam, using the fluorimeter described in Methods. A simultaneous measurement of the oxygen level and the colicin-induced fluorescence change is shown in Figure 8. After insertion of the oxygen electrode into the well, the cells rapidly deplete the suspension of oxygen. The increase in fluorescence as the oxygen trace reaches zero oxygen tension is usually observed. The fluorescence increase seen upon colicin addition in Figure 8 is very similar to that observed in a parallel experiment in the electrode well under aerobic conditions, with the aerobic experiment looking like Figure 2.

A reservation about these measurements is that the minimum detectable oxygen concentration in this experiment is approximately 3% of the ambient level, determined by the properties of the electrode membrane, although the actual oxygen concentration at the time colicin is added would be much below this if oxygen consumption continued at the same rate. In any case, the data of Figure 8 show that decreasing the oxygen concentration by at least a factor of 30 does not reduce the rate of the colicin response. It is still possible that the oxygen requiring process has a low K_m for oxygen, but the data of Figure 8 taken together with those of Fields and Luria (1969b) make it seem at least as likely that the oxygen requirement for colicin E1 sensitivity resides in the culture growth conditions. The decrease in intracellular ATP caused by colicin E1 is then probably not caused by uncoupling of oxidative phosphorylation but, in view of the inhibitory effects of DCC (Feingold, 1970; Figure 3 above), may be caused by activation of ATPase.

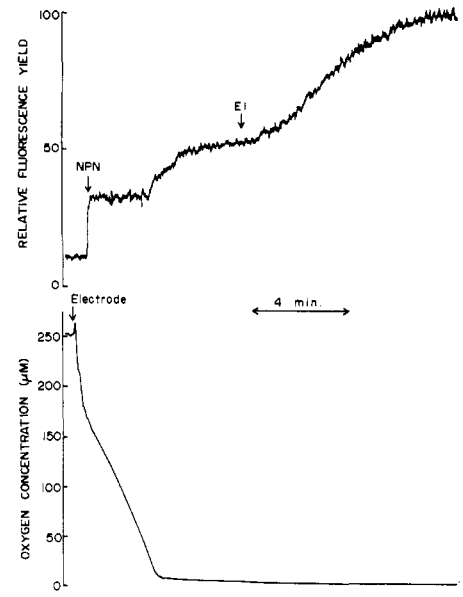

FIGURE 8: Colicin E1-induced fluorescence increase in an anaerboic suspension. *E. coli* NP3 was grown in M9 medium with 0.4% glucose and resuspended in M9 without glucose at 6×10^9 cells/ml. Fluorescence (upper trace) and oxygen level (lower trace) were measured simultaneously as described in Methods. The oxygen concentration in the open stirred suspension before insertion of the electrode which covered the suspension was assumed to be approximately 250 μM. The calibration of the subsequent decline in oxygen level of the enclosed suspension is normalized to 250 μM; NPN concentration, 4 μM; temperature, 25°; fraction of cells surviving 10 min after addition of 1 μg/ml of colicin E1, 0.10.

Discussion

From the work of Feingold (1970) and the fact that the ATP decrease shown in Figure 5 is independent of external potassium concentration it would seem that the decreases in intracellular ATP and potassium levels are not dependent upon each other, but rather the consequence of other events, most likely changes of structure or conformation in the cell membrane or envelope. The intracellular ATP decrease can conceivably be caused by a membrane perturbation activating ATPase, since bacterial ATPase is known to be an allotopic enzyme (Harold *et al.*, 1969). Since the rate and extent of the colicin-induced increase in fluorescence of the bound probe *N*-phenyl-1-naphthylamine is independent of external potassium concentration (Figure 2) and the presence of DCC which prevents the ATP decrease (Figure 3), the probe response may be an indicator of structural changes in the cell surface which alter potassium permeability and/or activate ATPase. The time required for the fluorescence yield to increase may represent the time required for the colicin effect to spread into or over the cell surface. It seems very likely that the colicin-induced structural changes involve a large part of the cell surface, since the amplitude of the colicin-induced fluorescence probe response is maximal with a colicin multiplicity of only two (Cramer and Phillips, 1970). Under these conditions the rate of the probe response as well as lethal activity saturated at multiplicities between 10 and 20. The potassium leak, as well as ATPase activation, could originate when the perturbation is still well localized in the cell surface. The difference in the kinetics of the probe response and the potassium efflux (Figure 4) could be the result of the potassium being lost through the leak in a localized region of the cell surface before the probe response has time to spread. Activated ATPase might similarly hydrolyze the maximum amount of ATP before the probe response reaches its maximum value and account for the lag in the fluorescence trace in Figure 5.

The ability of trypsin to reverse inhibitory and lethal effects of colicin over long times has often been interpreted in terms of colicin acting on the outside surface of the cell in contact with the external medium. As pointed out by Reynolds and Reeves (1969), many of the trypsin rescue experiments have been performed in the presence of metabolic inhibitors (Nomura and Nakamura, 1962; Nomura and Maeda, 1965; Maeda and Nomura, 1966). In the case of colicin E1, trypsin reversal of lethality is rapidly lost in the absence of such inhibitors (Figures 6 and 7 and Cavard *et al.*, 1971). The decay of trypsin rescue could be due to an irreversible lethal event caused by colicin acting on the outside surface of the cell, a conformational change in the colicin on the outside surface making it trypsin insensitive (Racker and Krimsky, 1958), or penetration of colicin into the cell envelope making it trypsin inaccessible. It has recently been shown that trypsin can restore colony forming ability to colicin K treated cells only if the cells have not been functionally damaged by the colicin (Plate and Luria, 1972). Penetration into the cell envelope could also be part of the irreversible inhibitory or lethal event. That colicin can penetrate into the cell envelope to the cytoplasmic membrane seems very likely from studies on the intrinsic ribosomal nuclease activity possessed by colicin E3 (Boon, 1971, 1972; Bowman *et al.*, 1971). That colicin E1

penetrates the cell envelope during the transmission of its inhibitory effects would be implied by an effect of the physical state of phospholipid on the activation energy of the transmission process (Cramer *et al.*, 1973). For these reasons we would tentatively hypothesize that the stage I–stage II transition of the colicin transmission process defined by Plate and Luria (1972) in which cells lose the ability to be rescued by trypsin corresponds to a transition when the colicin begins to penetrate into the cell envelope.

If loss of trypsin reversibility is a measure of colicin penetration, then the colicin-induced ATP decrease at 37° lags slightly behind colicin penetration (Figure 6). At 37° the colicin-induced fluorescence probe response would then lag even somewhat more behind penetration since the probe response lags behind the ATP decrease (Figure 5). At a lower temperature (13°) the fluorescence probe response would lag much behind penetration (Figure 8). Thus, if we hypothesize from the trypsin rescue experiments with colicin E1 that this rapidly is penetrating into the cell envelope, we would then propose that the colicin-induced fluorescence probe response and associated structural changes arising from most of the cell surface occur during or after the colicin penetration.

Colicin-induced membrane structural or conformational changes have been suggested to result from cooperative interaction between hypothetical membrane subunits subsequent to colicin binding (Changeux *et al.*, 1967). With the recent data on colicins E2 and E3, and the possibility of colicin penetration into the cell envelope, alternative mechanisms for induction of structural changes by colicin E1 can be proposed. (a) A possibility independent of enzymic effects is that merely by inserting itself the colicin exerts a localized strain in one or more layers of the cell envelope which is transmitted along the layer. This insertion model does not explain why E3, which seems likely to penetrate as mentioned above, does not induce a fluorescence change. The induction of a stress in membrane layers upon insertion of extra protein is also less likely if the membrane is partly fluid in nature (Singer and Nicolson, 1972). (b) From the studies on colicin E2 and E3, it seems plausible that colicin E1 may have an intrinsic or induced enzyme activity associated with it which could cause structural changes in the cell envelope. An apparent phospholipase activity has been detected in cells treated with colicins K and E1 (Cavard *et al.*, 1968). The kinetics of the phospholipid changes at high multiplicities were, however, much slower than reported changes in ATP (Fields and Luria, 1969a, and Figure 5 above), potassium (Wendt, 1970 and Figure 4 above), and fluorescence probe response (data presented above). If an enzyme activity of this nature could be definitely associated with colicin E1 action, the mechanism of the large scale E1-induced conformational changes could involve the colicin penetrating into the cell envelope and the colicin or a colicin-induced phospholipase moving in the plane of the membrane or periplasmic gap over the cell surface. This movement might be facilitated by fluid properties of the membrane (Singer and Nicolson, 1972). An involvement of fluid membrane properties in the transmission of the colicin response is suggested by biphasic Arrhenius plots of the rate of colicin action (Cramer *et al.*, 1973).

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