JOURNAL OF BACTERIOLOGY, Nov. 1970, p. 819-825
Vol. 104, No. 2

Printed in U.S.A.

Response of an *Escherichia coli*-Bound Fluorescent Probe to Colicin E1

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Received for publication 6 July 1970

The fluorescent probe, 8-anilino-1-napthalenesulfonate (ANS) binds to *Escherichia coli*, showing an enhanced fluorescence. The interaction of colicin E1 with sensitive cells causes an increase of about 100% in the fluorescence of the bound ANS, and this change at equilibrium has an apparent "all-or-none" nature as a function of E1 multiplicity. Approximately 6 to 8% of the ANS is bound to the cells at equilibrium. The colicin E1-induced fluorescence increase can be attributed partly to an increase in ANS binding and partly to an increase in the fluorescence yield of the bound ANS. The kinetics of the E1-induced fluorescence increase in sensitive cells are very similar to those of the adenosine triphosphate decrease. The phosphorylation uncoupler *p*-trifluoromethoxy-carbonylcyanidephenylhydrazone also causes a large change in the fluorescence of bound ANS. Colicin E2 or E3 does not cause any fluorescence change, nor does colicin E1 cause fluorescence change with a colicinogenic strain. ANS appears to be a probe of structural or conformational change in the cell envelope that is closely associated with the colicin E1-induced adenosine triphosphate decrease.

Colicin adsorption at the receptor sites on the surface of a sensitive cell causes an inhibitory change at the respective biochemical sites for oxidative phosphorylation, deoxyribonucleic acid synthesis, or protein synthesis. It has been proposed that the mechanism, by which information is transferred from the receptor site to the large number of biochemical sites on the cytoplasmic membrane, involves a colicin-induced change of structure or conformation in the cell envelope (13, 16). In support of this hypothesis, colicin-tolerant mutants have modified cellular envelopes that are often fragile and permeable to organic ions (15, 17). However, the predicted colicin-induced conformational change has not yet been observed.

8-Anilino-1-napthalenesulfonate (ANS) and related dyes have been used as qualitative probes of structural or conformational changes occurring in hydrophobic regions of proteins (2, 7, 21, 24). Recently this technique was applied to the erythrocyte membrane (18), the sarcoplasmic reticulum (23), and as a probe of structural changes electrically induced in nervous tissue (12, 22) and chemically induced in submitochondrial membrane fragments (1, 6). The relevant property of many of the dyes used as fluorescent probes is that their fluorescence yield and emission spectra depend on the local dielectric constant (21). As the polarity of the dye environment decreases, the fluorescence yield increases, and the fluorescence emission maxima are blue-shifted.

We used ANS as a probe for colicin-induced structural changes in sensitive cells, and observed a large increase in the fluorescence yield of the ANS bound to *Escherichia coli* upon addition of colicin E1. The kinetics of the fluorescence increase and the colicin E1-induced adenosine triphosphate (ATP) decay are very similar.

MATERIALS AND METHODS

Bacteria. Wild-type strain *E. coli* B obtained from F. C. Neidhardt was used for most of these experiments. Very similar results have been obtained thus far with several K-12 strains. Colicinogenic strain JC411 (Col E1) was given to us by S. Schwartz.

Media. The bacteria were generally grown and suspended in M9 medium containing (grams/liter): NH_4Cl, 1.0; $MgSO_4$, 0.13; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; and glucose, 4.0.

Colicin. Purified colicin E1, E2, and E3 used in these experiments were generously given to us by S. Schwartz and D. Helinski. The molecular weight of these proteins is approximately 60,000 (10, 19).

Fluorescence yield measurements. The bacteria were grown to 5×10^8/ml and concentrated to 5×10^9/ml (ca. 1 mg of total protein per ml) to obtain enough fluorescence signal. The concentration of ANS added

to the cell suspension was 6×10^{-5} M, which gave an approximately maximal fluorescence signal in the optical arrangement used for these experiments. Higher ANS concentrations gave less signal because of light absorption by the ANS. The relative fluorescence of the turbid suspension, in the presence and absence of colicin, was measured in a Shimadzu MPS-50L spectrophotometer operated in the single beam mode with a deuterium light source. The suspension in a 1 by 2 cm cuvette was stirred from the top by a metal paddle attached to the shaft of a Cer-mag no. 35 model car motor, and kept at 37 C by a water jacket and circulator. The excitation wavelength was 360 nm as defined by the monochromator and a Corning 7-39 filter to remove stray light. The fluorescence was defined by a blocking filter combination of 2 M $NaNO_2$ (2 mm thick) and a Corning 3-72 filter (5). The optical density of this blocking-filter combination is 6.0 at 360 nm, 2.5 at 420 nm, and 0.1 at 480 nm, and has more than 50% transmission at wavelengths greater than 460 nm. In a preliminary experiment we found that the peak of the ANS-*E. coli* B fluorescence emission was near 480 nm. From the known relationship between ANS fluorescence yield and fluorescence emission maximum (21), the blue shift in fluorescence caused by colicin E1 will not be more than 10 nm. This would give about 10% greater signal from the S-19 photomultiplier tube, which is cancelled by a 10% transmission decrease in the Corning 3-72 filter. The optical density of the turbid *E. coli* suspension at 360 nm was about 1.1 and with ANS about 1.5. Addition of colicin to the dye-cell suspension involved a dilution of 0.1 to 0.2% which had a negligible effect on the light transmission of the suspension. Because the transmission was constant in the presence and absence of colicin, the relative fluorescence yield change induced by colicin could be measured along the axis of the excitation light beam.

ATP measurements. ATP was measured with firefly-enzyme extract (Sigma FLE-250) in a photometer consisting of a two-inch (5 cm) photomultiplier tube (EMI 6255) and a Keithley 153-μv ammeter operated as a DC amplifier. This photometer could easily detect 10^{-9} M ATP. A 0.1-ml amount of the cell suspension was quickly placed in 1.9 ml of boiling 0.02 M Tricine-KOH, 3×10^{-3} M $MgSO_4$, *p*H 7.5, and was left there for 10 min. ATP measurements were made by adding 0.1 ml of firefly-lantern extract to 2.7 ml of the Tricine-Mg^{2+} buffer in a 1-cm cuvette. With the cuvette in the photometer, the luminescence was measured 3 to 5 sec after adding 0.2 ml of the boiled cell extract to the cuvette.

Chemicals. *p*-Trifluoromethoxy-carbonylcyanide-phenylhydrazone (*p*-CF_3O-CCP) was kindly supplied by P. Heytler, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. ANS was purchased from Eastman Organic Chemicals, Rochester, N.Y. (P10296).

RESULTS

The recorder trace of the fluorescence changes that occur upon addition of 6×10^{-5} M ANS to a concentrated *E. coli* suspension and upon subsequent addition of colicin E1 are shown in Fig. 1.

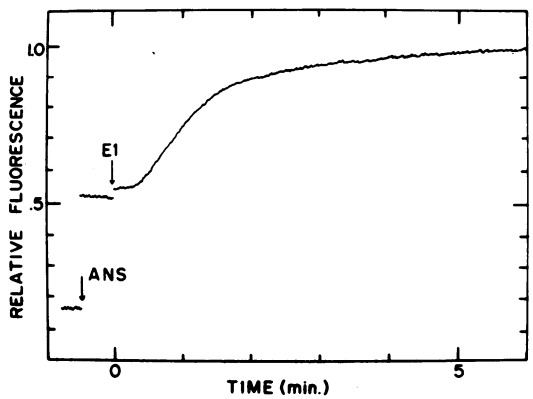

FIG. 1. *Colicin E1-induced fluorescence change in a suspension of 4.5 $\times$ 10^9/ml E. coli B and 6×10^{-5} M ANS. After ANS was added to the cell suspension in M9 medium, 10 μliters of E1 solution was added to give a concentration of 1 μg/ml E1 in the cuvette. The cell survival level assayed 10 min after E1 addition is 5×10^{-4}, which is close to the E1-resistant background with this strain, and the colicin multiplicity is inferred to be about 20 from measurements with less colicin.*

The contribution of free ANS to the fluorescence signal, in M9 medium of turbidity equivalent to that used in Fig. 1, and polarity of M9 medium is completely negligible. The true ratio of the fluorescence of cells with ANS to cells alone, in the absence of colicin, is slightly greater than the factor of 3 shown in Fig. 1, since ANS attenuates the 360-nm excitation beam. The increase of 100% in fluorescence of the ANS-cell suspension, caused by addition of colicin E1 at a concentration of ca. 1 μg/ml, requires no corrections since: (i) E1 has no effect on the fluorescence of cells without ANS (*data not shown*); (ii) the 1/500 dilution of the suspension by addition of E1 has a negligible effect on the optical density; (iii) it was experimentally determined that any colicin-induced scattering changes do not change the optical density by more than 1 part in 150. Thus, the E1-induced fluorescence change shown in Fig. 1 represents a true change in fluorescence yield of the ANS-cell suspension.

The E1-induced fluorescence change of cells with ANS is caused by ANS bound to the cells and not by an increase in the fluorescence of free ANS in solution, due, perhaps, to hydrophobic substances leaking out of the cells upon addition of E1. This is shown by a comparison of supernatants obtained before and after addition of E1 (Table 1). The origin of the E1-induced fluorescence change shown in Fig. 1 could be caused either by a colicin-induced increase in cell-bound dye or by an increase in the fluorescence yield of the dye already bound. It is difficult to discrimi-

nate between these possibilities since only a small fraction of the 6×10^{-5} M ANS is cell-bound at equilibrium in the concentrated suspension (Table 2). Table 2 shows that colicin E1 treatment causes an increase from 6 to 8% in the fraction of dye bound. The fractional increase is 0.30, or about 0.33 if it is assumed that the cell pellet contains 50% intercellular free space (4). From many such measurements the best value for the E1-induced increase in cell-bound ANS is 0.3 ± 0.2. The E1-induced fluorescence increase shown in Fig. 1 is ca. 100% when corrected for the fluorescence of cells without ANS. Thus, the increased dye uptake cannot account for all of the fluorescence increase caused by E1. There is also an increase in the average fluorescence yield of the bound dye, due, presumably, to a decrease in the local polarity of the dye-binding sites (21).

The colicin-induced fluorescence yield increase appears to be specific for E1, as there is no fluorescence change caused by addition of equivalent titers of E2 and E3 (Fig. 2). E1 also has no effect on the fluorescence yield of ANS bound to cells of the colicinogenic strain JC411 (Fig. 3), suggesting that the E1-induced fluorescence change occurs only with cells sensitive to E1.

The colicin E1-induced fluorescence increase shown in Fig. 1 shows a lag of 30 sec to 1 min, followed by an almost linear increase in fluorescence lasting for about 1 min. The rate of this initial fluorescence increase is a linear function of E1 multiplicity, saturating at a multiplicity of ca. 10 (Fig. 4). However, the total amplitude of the E1-induced fluorescence change, measured at equilibrium 10 min after E1 addition, is as great for a multiplicity of two as for one of 20 (Fig. 5). With multiplicities less than two, the amplitude of the fluorescence change is lower at 10 min but still slowly increasing. Thus, the fluorescence change at equilibrium shows an "all-or-none" response (3) to colicin E1, with the transition for zero response to maximal response coming at an E1 multiplicity less than two.

TABLE 1. *Effect of colicin E1 on fluorescence in supernatant of E. coli-ANS suspension*[a]

Culture	Relative fluorescence
Supernatant, cells + ANS	0.10
Supernatant, cells + ANS + E1	0.11
ANS	0.10

[a] Concentrated cells at 5×10^9/ml were incubated in M9 medium with 6×10^{-5} M ANS for 15 min at 37 C in the presence and absence of E1. Cell survival with E1 was 2×10^{-3}. The suspensions were then centrifuged for 10 min at 12,000 × *g*, the supernatants were decanted, and their fluorescence was assayed. The fluorescence of 6×10^{-5} M ANS in M9 medium was also assayed. The fluorescence levels are normalized to 1.0 for an E1-treated ANS-cell suspension at equilibrium.

TABLE 2. *Effect of E1 on the amount of cell-bound ANS*[a]

Suspension	Optical density at 350 nm	
	−E1	+E1
(1) Supernatant, cells + ANS	0.398	0.372
(2) Supernatant, cells	0.034	0.032
(3) (1) − (2), ANS in supernatant	0.364	0.340
(4) Pellet, cells + ANS	0.030	0.035
(5) Pellet, cells	0.005	0.004
(6) (4) − (5), ANS in pellet	0.025	0.031
(7) (3) + (6), ANS in pellet and supernatant	0.389	0.371
(8) (6)/(7), Dye bound (%)	6.4	8.4

$$\frac{\%\ \text{Dye Bound (+E1)}}{\%\ \text{Dye Bound (−E1)}} = \frac{8.4}{6.4} = 1.30$$

[a] A 5-ml sample of concentrated cells at 7×10^9/ml was incubated in M9 medium with 6×10^{-5} M ANS for 10 min at 37 C in the presence and absence of E1. Cell survival with E1 was 5×10^{-4}. The suspensions were then centrifuged for 10 min at 27,000 × *g*, and the absorbance of the supernatant at 350 nm was assayed (row 1). The cell pellet was then suspended in M9 medium for 5 min at 37 C so that most of the ANS bound to the pellet goes into solution under equilibrium conditions. This ANS was then measured by centrifuging the cells out of solution for 10 min at 27,000 × *g* and by assaying the absorbance of the resulting supernatant (4). Corrections were made for absorbance at 350 nm found in both the first (2) and second (5) supernatants which may be due to soluble reduced nicotinamide adenine dinucleotide. The corrected absorbance due to ANS alone is given in rows (3) and (6). The volume of the cell pellet was approximately 0.035 ml for E1-treated and control cells, and any correction for dye in the free space of this pellet would be small.

Assay of cell survival as a function of time after colicin addition shows that colicin adsorption is more than 99% complete within 1 min after addition (Table 3). Therefore, the initial 30 sec to 1 min delay in the E1-induced fluorescence increase in Fig. 1 may be due to a finite rate of colicin adsorption, but the subsequent 100% increase occurring after 1 min must be associated with some process which follows adsorption.

The primary biochemical consequence of colicin E1 addition to sensitive cells is believed to be a decrease in the cellular ATP pool (8). Figure 6 shows the time course of the colicin E1-in-

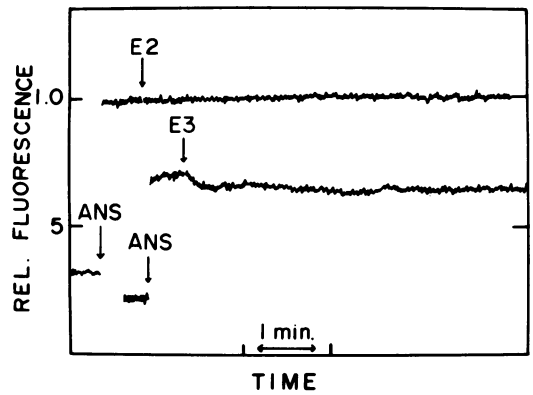

FIG. 2. *Fluorescence changes with colicin E2 and E3 added to cell-ANS suspension. Cell survival level is 3 × 10⁻⁵ for E2 and 3 × 10⁻⁵ for E3 addition. Other conditions as in Fig. 1.*

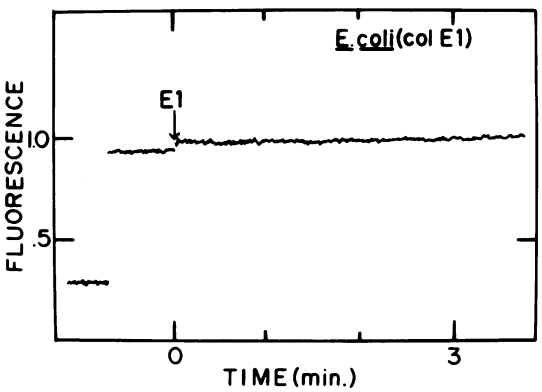

FIG. 3. *Effect of E1 on the fluorescence of ANS bound to E. coli JC411(Col E1). E1 at a multiplicity of about 40/cell was added to a concentrated suspension to which ANS had been added as in Fig. 1 and 2. Cell survival level was 100%.*

duced ATP decrease and the fluorescence increase with both measurements made in the same suspension. Centrifugation experiments determined that the ATP measured here is intracellular. The fluorescence signal, F, is plotted as $1 - F/F_{max}$ to facilitate comparison with the ATP decrease. NaF (10^{-3} M) was added to this suspension to inhibit a secondary rise in the ATP level which occurs in the concentrated. partly anaerobic cell suspension and whose source appears to be glycolysis. With or without NaF the time course of the decay of the ATP pool is very similar to that of the fluorescence increase, and this is also true at multiplicities higher than that used in this experiment. Lack of overlap of the ATP points and the fluorescence curve, at times greater than about 4

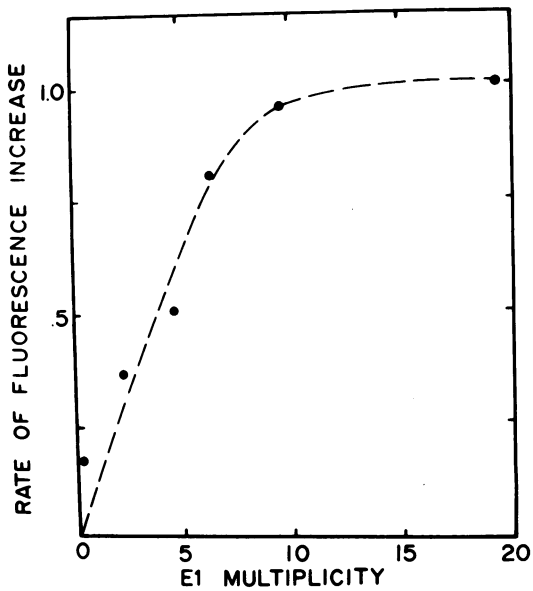

FIG. 4. *Initial rate of increase of fluorescence as a function of colicin E1 multiplicity (m). Values of $m \leq 5$ calculated from survival = e^{-m}. Values of $m > 5$ inferred from dilution factor. The initial fluorescence increase is measured as the slope of the fluorescence trace in the linear region between 30 sec and 1 min or more after E1 addition.*

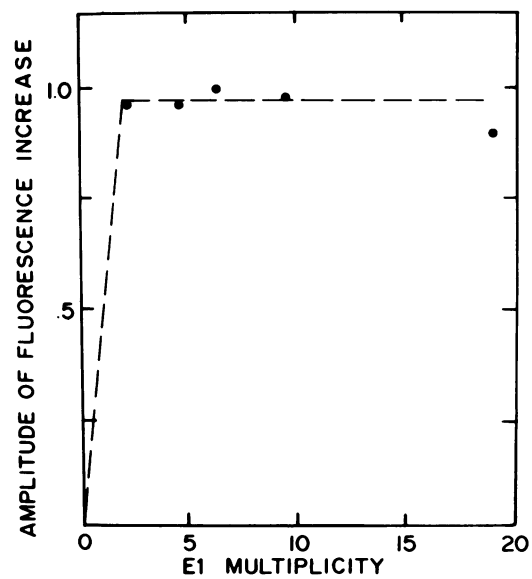

FIG. 5. *Amplitude of fluorescence change as a function of E1 multiplicity. Amplitude measured at equilibrium 10 min after E1 addition, when the fluorescence was again constant with time at a ~100% higher level. Data obtained from the same experiment as that of Fig. 4.*

TABLE 3. *Survival of colony-forming ability as a function of time after addition of colicin E1*[a]

Cell survival	Time
	min
1.0	0
1.5×10^{-4}	1
1.3×10^{-4}	3
6.3×10^{-5}	5
1.5×10^{-4}	10

[a] A 10-μliter amount of E1 was added as usual to 5 ml of concentrated cells at 4.5×10^9/ml at 37 C. Samples were withdrawn for plating at the indicated times after E1 addition.

min (Fig. 6), occurs because the ATP level never seems to decay below the 20 to 30% level after E1 treatment (8), and the function $1-F/F_{max}$ goes to zero by definition. The qualitative similarity between the initial rate of ATP decay and the fluorescence increase suggests that the two processes are closely related.

Fields and Luria (9) documented that colicin E1 is an uncoupler of oxidative phosphorylation by showing that it caused a decrease in the ATP level but no decrease in respiration rate. The known uncoupler of oxidative and photophosphorylation, *p*-CF_3O-CCP, causes a fluorescence increase in the ANS-*E. coli* complexes similar to that induced by colicin E1 (Fig. 7). In this experiment, the *p*-CF_3O-CCP was added at a concentration of 6×10^{-6} M, a concentration low enough so that its light absorption at 360 nm can be neglected. *p*-CF_3O-CCP greatly increases the proton conductance of a model phospholipid-membrane system (11), and it has been suggested (11, 14) that this is the mechanism of its uncoupling action. Fields and Luria (9) suggested that colicins E1 and K may affect oxidative phosphorylation in a related manner.

DISCUSSION

The colicin E1-induced increase in the amount and the fluorescence yield of cell-bound ANS implies that: (i) colicin E1 causes either an increase in permeability of the cell envelope to ANS or exposes more ANS-binding sites in the cell envelope; and (ii) the ensemble of ANS-binding sites within the cell or the cell envelope becomes more hydrophobic upon treatment with colicin E1. The fluorescence-yield increase induced by colicin E1 is, in this sense, a measure of structural or conformational changes occurring in the cell envelope, though we don't yet know the nature of the ANS-binding sites within the cell.

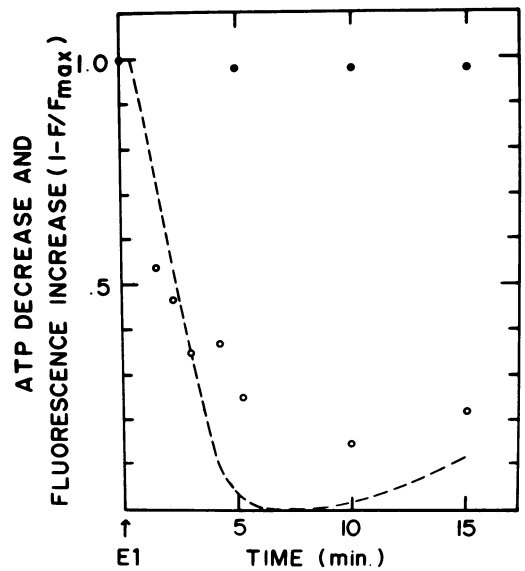

FIG. 6. *Cellular ATP and ANS-E. coli fluorescence as a function of time after E1 addition in the presence of 10^{-3} M NaF. The ATP change after E1 addition (○) is plotted as the fractional decrease relative to the control. ATP control without E1 and with NaF (●). Fluorescence (F) is plotted as $1-F/F_{max}$ (dashed line). ATP and fluorescence measurements made on the same cell suspension. Cell survival level assayed 15 min after E1 addition is 1.1×10^{-2}, with a lower E1 multiplicity purposely added because the kinetic comparison was more easily made with slower rates of change. At the lower multiplicities there was sometimes a slow decrease in the fluorescence change after 10 to 15 min.*

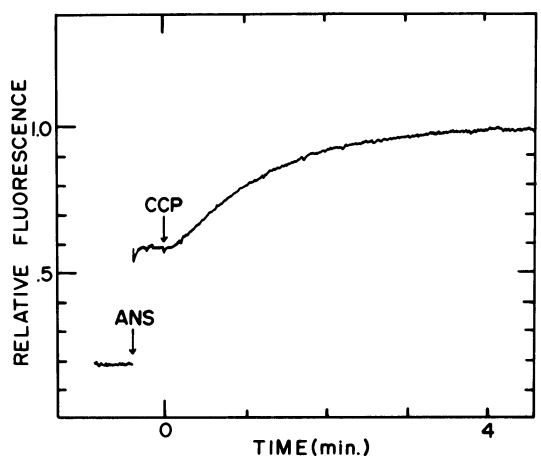

FIG. 7. *Fluorescence change of an ANS-E. coli suspension caused by addition of 6×10^{-6} M p-CF_3O-CCP. A 10-μliter amount of CCP in methanol was added to 5 ml of cells.*

The primary biochemical consequence of colicin E1 action is lowering of the cellular ATP pool. The time course of the ATP decrease and the fluorescence-yield increase or the conformational changes induced by colicin E1 are very similar (Fig. 6). It is not possible to decide from these data whether the ATP decrease or the fluorescence yield increase is a more primary event. The possibilities are: (i) ANS is a probe for a primary conformational change in the cell envelope which precedes and causes the ATP decrease; (ii) the changes seen with ANS occur only in response to the decrease in ATP level. It is also possible that the two changes are simultaneous and inseparable. The lack of a fluorescence change with colicins E2 and E3 (Fig. 2) shows that the change seen with E1 is not common to all three kinds of colicin. But since the biochemical consequences with the three colicins are distinct, primary conformational changes induced in the cell envelope might also be expected to be different. If the fluorescence change seen with ANS reflects a primary colicin-induced conformational change, ANS is a specific probe for the change caused by E1, and is not sensitive to those produced by E2 and E3. The alternative explanation of the lack of a fluorescence effect with E2 and E3, which do not inhibit energy production (16), is that the fluorescence increase is initiated by the decrease in ATP level. All of the data shown above are consistent with the latter hypothesis. Also, the rate of the ATP decrease is qualitatively proportional to E1 multiplicity, and the amplitude of the ATP decrease appears to be an "all-or-none" change (*data not shown*). Furthermore, there is evidence in other studies of acriflavine binding to *E. coli* that dye binding increases as the ATP level decreases (20). Thus, at present, it is not possible to decide whether the ANS fluorescence yield increase or the ATP decrease represent the more primary effect of colicin E1 action.

Finally, the correlation between energy level and fluorescence with the ANS probe is reversed in the colicin E1-*E. coli* system relative to the measurements made with mitochondrial membrane fragments (1, 6). In the latter system, addition of substrate under phosphorylating conditions or addition of ATP gives an increase in bound-dye fluorescence. With the colicin E1-cell interaction, however, the increase in bound-dye fluorescence is associated with a decrease in the ATP pool. Similarly, although the phosphorylation uncoupler *p*-CF_3O-CCP causes a decrease in fluorescence with the mitochondrial membrane fragments, it causes an increase in the fluorescence of ANS bound to *E. coli*.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant GB-7863, by Health Sciences Advancement Award SO4 FR06013-03 to the Department of Biological Sciences, and by a National Institutes of Health Biophysics Traineeship to S. Kent Phillips.

We are grateful to Sandra Davis and Ann P. Kong for skilled technical assistance.

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