Membrane Changes in *Escherichia coli* Induced by Colicin Ia and Agents Known to Disrupt Energy Transduction†

David Nieva-Gomez,‡ Jordan Konisky,§ and Robert B. Gennis*,¶

ABSTRACT: The addition of colicin Ia to a suspension of intact *Escherichia coli* in the presence of the hydrophobic fluorescent probe *N*-phenyl-1-naphthylamine causes dramatic changes in the fluorescence of the probe. The fluorescence intensity increases several fold, the emission spectrum shifts to the blue, the fluorescence lifetime approximately doubles, and the polarization increases. These changes do not appear to result from an increase in membrane microviscosity, as has been previously postulated to be the case for the *N*-phenyl-1-naphthylamine fluorescence changes seen with colicin E1-treated cells (Helgerson, S. L., Cramer, W. A., Harris, J. M., and Lytle, F. E. (1974), *Biochemistry, 13,* 3057); rather, they result from an increased binding of the dye to the cell envelope. A variety of agents have been used to demonstrate that a very similar fluorescence response results whenever the cells are "deenergized." These agents include electron transport inhibitors (malonate, amytal, cyanide) as well as the uncouplers CCCP and azide. In addition, depleting the cells of either endogenous substrates or oxygen results in the same fluorescence response. In these cases, the fluorescence response is reversed upon addition of an oxidizable substrate or oxygen. It is clear that there are significant changes in the *Escherichia coli* envelope as energy transduction processes are disrupted and restored. The changes reported by the fluorescent probe may prove useful in deciphering structure–function relationships in the *Escherichia coli* envelope.

Fluorescent probes have been used in recent years to investigate the physical state of both biological and model membranes. The dye *N*-phenyl-1-naphthylamine (NPN)[1] has been used by Cramer and his co-workers to examine changes in the membranes of intact *Escherichia coli* induced by colicin E1 (Cramer, et al., 1973; Phillips and Cramer, 1973). Recently these studies have been extended to show that the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) causes similar changes in the fluorescence of NPN with intact *E. coli* (Helgerson, et al., 1974). The fluorescence response is characterized by an increase in the emission intensity, a blue shift in the emission peak, an increase in the fluorescence lifetime, and an increase in polarization. Based on these experiments Helgerson et al. (1974) have suggested that one of the early events in the action of colicin E1 is an increase in the microviscosity of the bacterial cell envelope, and that the disruption of membrane-associated processes such as active transport may be a consequence of this physical change. It has been suggested that the action of a few molecules of colicin E1 bound to the cell may be propagated and amplified by inducing a structural transition in the lipid bilayer. This postulated phenomenon has been cited by Singer and Nicolson (1972) as a possible example of a long-range cis-type cooperative effect in a biological membrane. Hence, these experiments may be relevant to a general understanding of the nature of protein–protein and protein–lipid interactions in the membrane, while providing information on the specific problem of the mechanism of action of the colicin.

Colicin Ia belongs to a group of colicins, including colicin E1, which induces the same general type of physiological changes in sensitive cells (Nomura, 1963; Gilchrist and Konisky, 1975). It is demonstrated in this work that colicin Ia elicits a fluorescence response from NPN in the presence of intact *E. coli* similar to that seen with colicin E1. It is further demonstrated that the fluorescence parameters of NPN in a suspension of *E. coli* cells are strongly dependent on the physiological state of the cells. Changes in fluorescence similar to those seen in colicin Ia treated cells are induced by a wide variety of agents known to disrupt energy transduction processes in bacterial cells. On the basis of spectral and binding data, the changes in fluorescence parameters are shown to be the result of an increase in the binding of NPN by the cell.

Materials and Methods

Cell Growth. E. coli K12 strains, JK1 (colicin I sensitive) and JK116 (colicin I resistant) were grown at 37 °C in M9 medium consisting of M9 salts (M9S) containing the following (in g/l.): NH_4Cl, 1.0; $MgSO_4$, 0.13; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; supplemented with glucose, 1.3. In the case of strain JK116 this medium was further supplemented with tryptophan (40 μg/ml), arginine (40 μg/ml), adenine (10 μg/ml), histidine (40 μg/ml), guanine (10 μg/ml), uracil (10 μg/ml), and thiamin (1 μg/ml). Cells were harvested at mid-log phase (Klett reading of 100 in a Klett colorimeter, 42 filter), washed with M9 salts, and resuspended in M9 salts to a density of about 5×10^8 cells/ml (Klett 80, 42 filter) for fluorescence experiments. The cells were kept at 0 °C or at room temperature prior to use. All measurements were made within 2 h after harvesting. The cells showed 100% viability after at least 3 h under these conditions. The presence of NPN or methanol at concentrations comparable to those used in the fluorescence experiments had no effect on either cell growth or viability.

† From the Department of Chemistry (D.N.-G., R.B.G.), Department of Microbiology (J.K.), and Department of Biochemistry (R.B.G.), University of Illinois, Urbana, Illinois 61801. *Received April 22, 1975.* This research was supported by grants from the National Institutes of Health (AI10106, HL16101, and GM 11223).

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[1] Abbreviations used are: ATP, adenosine triphosphate; CCCP, carbonyl cyanide *m*-chloromethoxyphenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NPN, *N*-phenyl-1-naphthylamine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; BSA, bovine serum albumin; ANS, 8-anilino-1-naphthalenesulfonate.

Starvation of Cells. The procedure used for the starvation of cells consisted of a modification of a method reported by Berger (1973). Cells grown in M9S medium supplemented with 0.13% glucose and harvested as described were washed once with M9S and twice with cold medium S consisting of: 0.05 M Tris, pH 8.0, 0.1 mM $MgSO_4$, and 7 mM $(NH_4)_2SO_4$. Cells were then resuspended in this same medium, supplemented with 5 mM dinitrophenol, and incubated with shaking at 37 °C for 6 h. The cells were then washed at least three times with excess volumes of medium S and resuspended in this same medium at the concentration specified. When starved and unstarved cells were to be compared, a fraction of the growing cell culture was separated and immediately chilled and kept in ice, while the rest of the culture was harvested and subjected to the starvation procedure. When this was completed, the unstarved fraction was washed once with M9S and twice with medium S. Both starved and unstarved cells were resuspended in medium S at the same concentration. Cell viability, as determined by plating, was essentially the same in the two suspensions.

Respiration Measurements. The rate of oxygen consumption was measured polarographically with a YSI Clark oxygen electrode apparatus. For calculations 470 ng-atom/ml was taken as the saturating concentration of oxygen in the suspension media.

Colicin. Purified colicin Ia was prepared and its concentration determined as previously described (Konisky, 1972). For the fluorescence experiments colicin Ia was added to the bacteria at an input multiplicity between 5 and 20 killing units/cell (Levisohn, Konisky and Nomura, 1968). Survival level was near 1%.

Reagents. The following reagents were obtained from the indicated sources and used without further purification: sodium azide and NPN (Eastman); amytal, malonate, chloramphenicol, and D-lactate (lithium salt) (Sigma); potassium arsenate (Matheson, Coleman, and Bell); carbonyl cyanide trichloromethoxyphenylhydrazone (CCCP) (Calbiochem); sodium succinate (Mallinckrodt); bovine serum albumin (BSA) (General Biochemicals). The purity of NPN was established by thin-layer chromatography and elemental analysis. In addition, it was demonstrated that the spectral characteristics in various solvents compared favorably with previously reported parameters (Radda, 1971).

Fluorescence Measurements. Most fluorescence measurements were made at 37 °C with cells suspended to a density of 5×10^8 cells/ml. NPN was added in absolute methanol to a final concentration of 3 μM. CCCP was also added in absolute methanol. Other reagents were added in aqueous solution. The final concentration of methanol was usually about 0.3%; in a few experiments the methanol concentration was as high as 3%. Control experiments indicated that the presence of methanol in these experiments had no significant effect. Fluorescence emission spectra and emission intensity were measured with a Perkin-Elmer MPF-III fluorimeter equipped with a circulating water bath. The temperature of the sample was measured using a YSI Model 42SC telethermometer. The excitation wavelength for all experiments was 340 nm. Fluorescence intensity was followed at 420 nm using a broad band-pass (40 nm) in conjunction with a 390-nm cutoff filter.

It should be noted that, although the background fluorescence is altered by many of the agents used in this work, this change is small relative to the alteration in the probe fluorescence. The fluorescence response is also not due to a change in the light scattering or absorbance of the sample at either the emission or excitation wavelengths.

Fluorescence polarization and lifetimes were measured using instruments in the laboratory of Dr. Gregorio Weber. The instrument used to measure fluorescence lifetimes has been previously described (Spencer and Weber, 1969). The excitation wavelength for polarization was 340 nm, as defined by a grating monochromator and a Corning 7-51 broad-band filter. The emission was defined with a liquid $NaNO_2$ filter (2M, 2 mm thick) and a Corning 3-73 cutoff filter. Parallel and perpendicular components of the emitted light were measured simultaneously. Corrections were made for the scattering and fluorescence background of the cell suspension and for the differences in phototube efficiency. Fluorescence lifetimes were measured using the phase modulation technique (Spencer, 1970). All values have been corrected for scattering and fluorescence background.

Binding Studies. Several different methods were used to determine the amount of NPN bound to the *E. coli* cells under the experimental conditions used for the fluorescence measurements. These methods included: (1) fluorescence titration—an attempt was made to find the fluorescence yield under conditions where all the dye was bound; (2) addition of BSA to a mixture of cells plus NPN—the fluorescence increase due to the formation of a BSA–NPN complex was used as a measure of the concentration of unbound NPN; (3) equilibrium dialysis; (4) filtration of the bacterial cells using a variety of filters, followed by a determination of the concentration of the NPN remaining in solution; (5) centrifugation of the cells followed by a determination of the concentration of unbound NPN. Major limitations in obtaining reliable quantitative data from most of these procedures were due to (1) the large turbidity of the cell suspension and (2) nonspecific binding of NPN to many materials such as glass or plastic tubes, filters, etc. The centrifugation technique proved to be the most reliable, and the reported results were obtained using this method. All operations were performed at 37 °C. Ten-milliliter samples were prepared containing 5×10^8 cells/ml, 3 μM NPN (final MeOH concentration was 0.3%), in M9 salts. Cyanide or colicin Ia or other agents were also added as required. These cells were centrifuged at 8000 rpm for 15 min using a Sorvall Model SS-1 bench-top centrifuge kept at 37 °C. Samples of the supernatant were used to determine the free NPN concentration. Four identical samples were used for each binding determination. Control experiments in which no cells were present were also performed and the appropriate corrections were made. NPN concentration in the supernatant was determined by measuring the fluorescence of an aliquot diluted 1:1 with a 12 mg/ml aqueous solution of BSA, and comparison with a standard curve. This method is very sensitive and the standard curves are linear over the range of NPN concentrations used (0–4 μM). The absorbance of the supernatant at the excitation wavelength (340 nm) was always within 0.02 and 0.03 absorbance unit; since the absorbance of the assay sample after dilution with the BSA solution was in the order of 0.1 absorbance unit, corrections for differences in the absorbance of the supernatants were completely negligible. The supernatants showed no absorbance at 420 nm.

Results

Fluorescence Studies. Upon the addition of colicin Ia to intact cells in the presence of NPN there is an increase in the fluorescence emission intensity (Figure 1). There is a time lag of the order of 1–2 min between the time of colicin addition and the initial fluorescence rise. This lag is longer at lower colicin concentrations (Figure 1). The rate of change in intensity increases with the amount of colicin added to the cell suspension.

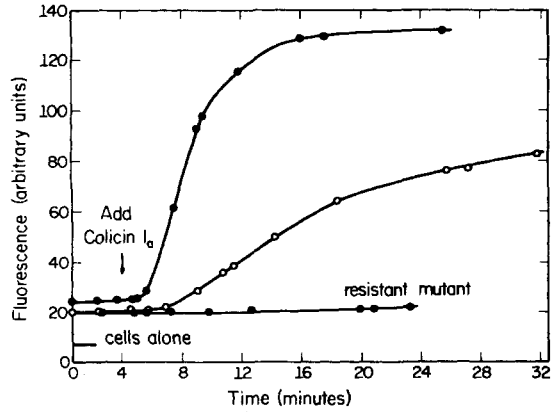

FIGURE 1: Effect of colicin Ia on the fluorescence of NPN in the presence of *E. coli* strains JK1 and JK116 (resistant). Bacteria were grown at 37 °C in M9 (0.13% glucose) medium supplemented as described under Materials and Methods, harvested at mid-log phase, and washed and resuspended in M9 medium minus glucose (ca. 5×10^8 cells/ml). NPN was added to a final concentration of 3 μM. Fluorescence intensity was measured at 37 °C using a Perkin-Elmer MPF-III fluorimeter with a circulating water bath. Excitation was at 340 nm; emission was recorded at 420 nm using a 40-nm band-pass in conjunction with a 390-nm cutoff filter. Colicin Ia was added at the indicated times. Colicin Ia was added to a final concentration of 1.5 μg/ml (—●—●—); 0.09 μg/ml (—O—O—). The final colicin Ia concentration added to the resistant strain was 1.5 μg/ml. The lowest trace shows the level of fluorescence and scattering background due to the cells prior to the addition of the dye.

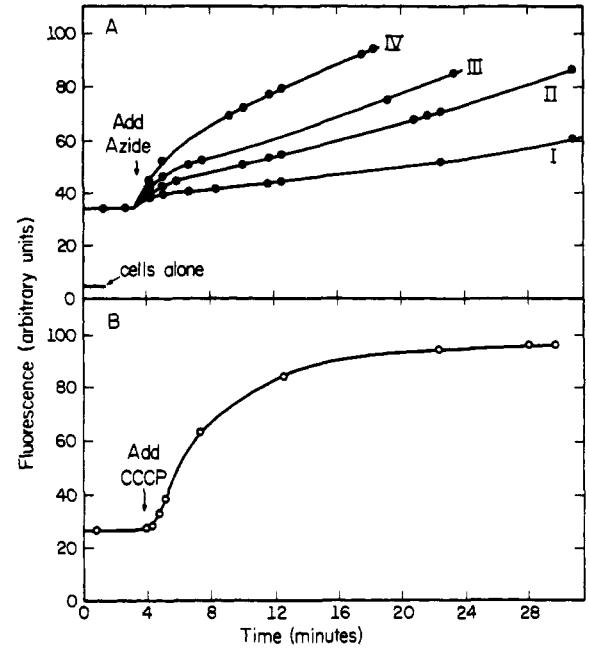

FIGURE 2: (A) The effect of sodium azide on the fluorescence of NPN in the presence of *E. coli* JK1. Samples were prepared and the fluorescence was measured at 38 °C as described in the caption to Figure 1. Sodium azide was added at concentrations of: I, 2 mM; II, 3 mM; III, 4 mM; IV, 6 mM, at the indicated time. (B) The effect of CCCP on the fluorescence of NPN in the presence of *E. coli* JK1 cells. Procedure and conditions as for part A. CCCP was added at the time indicated to a final concentration of 3.5 μM.

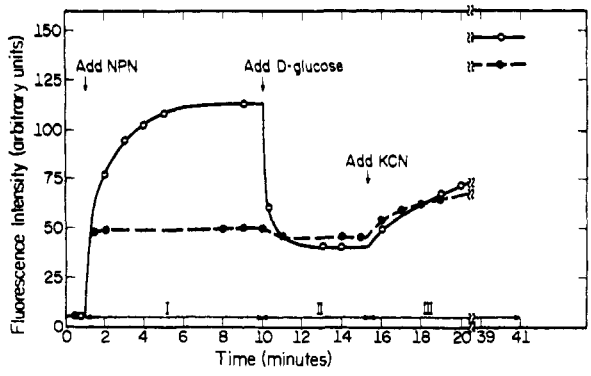

FIGURE 3: The effect of D-glucose and of KCN on the fluorescence of NPN in the presence of starved (—O—O—) and unstarved (—●—●—) *E. coli* JK1 cells. All measurements were at 37 °C. Fluorescence was measured as described in the caption to Figure 1. Respiration rates were measured as described under experimental procedures. Both starved and unstarved cells were resuspended in medium S at a concentration of 50 Klett units (42 filter). Reagents were added at the times indicated. Concentrations were: NPN, 3 μM; D-glucose, 20 mM; KCN, 3 mM. The rates of O_2 uptake in units of ng-atom min^{-1} mg^{-1} of protein, for the indicated stages of the experiment (identified with Roman numerals) were as follows: Starved Cells: I, 37; II, 373; III, 0. Unstarved Cells: I, 202; II, 500; III, 0.

The final fluorescence intensity, however, is not dependent on the amount of colicin. After a sufficient period of time the fluorescence intensity from cells treated with a low concentration of colicin reaches the same level as cells treated with higher concentrations. The extent of colicin Ia induced fluorescence increase is somewhat dependent on the particular preparation of *E. coli;* generally a three- to sixfold increase is observed with sensitive strain JK1. There is no increase in fluorescence when a colicin I resistant strain (no colicin adsorption) is employed.

A similar fluorescence response is induced by the respiration blocking agents potassium cyanide and amytal, and by the membrane uncouplers CCCP and azide (Figure 2). As in the case of colicin Ia, the rate of change in fluorescence intensity increases with the amount of reagent added, as is shown for the case of azide. Again, all traces reach approximately the same level after a sufficient period of time. Cell survival and the effects of these reagents on cellular respiration were also examined. It is noted that the rate of change of fluorescence intensity is much slower than the inhibition of respiration by cyanide, for example, which occurs virtually immediately. At 15 mM, amytal inhibited respiration by 53%.

Cells subjected to the starvation procedure described under Materials and Methods have very low levels of endogenous substrate, as evidenced by the extremely low levels of respiration. As shown in Figure 3, the fluorescence intensity of NPN in the presence of starved cells is about twice that found in the presence of unstarved cells. However, the subsequent addition of glucose to either cell suspension leads to a decrease in fluorescence intensity to approximately the same low level. The effect of a subsequent addition of KCN is also shown. As demonstrated by the respiration data (Figure 3), the observed changes in fluorescence intensity follow qualitatively the changes in respiration rate.

The addition of other respiratory substrates, such as succinate and D-lactate, to a suspension of starved cells plus NPN also induces a decrease in the fluorescence intensity. In Figure 4 the effects of sequential addition of succinate, malonate, and D-lactate are shown. As the succinate-supported respiration is blocked by malonate and subsequently restored partially by D-lactate, the fluorescence intensity is shown to follow qualitatively the rate of respiration in the prescribed manner.

A dramatic effect is observed when an oxidizable substrate such as D-lactate (20 mM) or succinate (20 mM) is added to a cell suspension, and the oxygen supply allowed to deplete. As seen in Figure 5, the fluorescence remains constant for about 15–25 min, depending on the cell preparation, at which time there is a sudden increase in fluorescence intensity. If the cu-

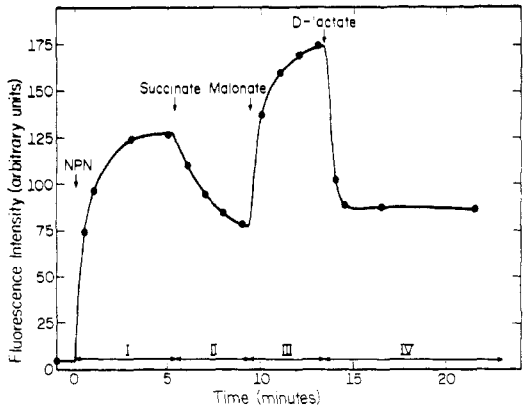

FIGURE 4: Effects of sequential addition of succinate, malonate, and D-lactate on the fluorescence of NPN in the presence of starved *E. coli* JK1 cells. Values for the rate of O_2 uptake in units of ng-atom min^{-1} mg^{-1} of protein at the difference stages of the experiment (identified with Roman numerals) were as follows: I, 52; II, 386, III, 170; IV, 659. All measurements were at 37 °C. Fluorescence was measured as described in the caption to Figure 1. Respiration rates were determined as described in experimental procedures. Cells were resuspended in medium S at a concentration of 50 Klett units (42 filter). Reagents were added at the times indicated. Concentration of NPN was 3 μM, and that of sodium succinate, sodium malonate, and lithium D-lactate was 15 mM.

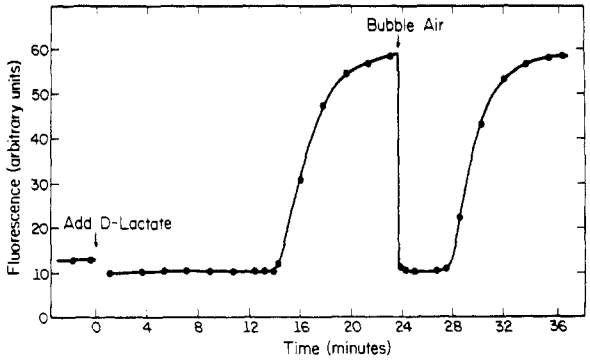

FIGURE 5: The effect of anoxia as detected by changes in the fluorescence intensity of NPN in the presence of *E. coli* JK1. Samples were prepared and fluorescence was measured at 37 °C, as described in the caption to Figure 1. D-Lactate was added to a final concentration of 20 mM. Air was bubbled into the cuvette at the indicated time by means of a Pasteur pipet.

vette is open to the atmosphere the level of this increased fluorescence oscillates with a period of the order of 1–2 min. This eventually is damped out and a new steady state is reached. If the cuvette is stoppered these oscillations are not observed or are rapidly damped out. This phenomenon has not been examined in detail. Figure 5 also demonstrates that the fluorescence increase is rapidly reversed by bubbling air through the sample. After a few minutes at this new low level of fluorescence intensity the system apparently is again depleted of oxygen, and the fluorescence accordingly increases. This cycle can be repeated several times. That this effect is due to oxygen per se and not bubbling or agitation was demonstrated by experiments in which pure oxygen or nitrogen was bubbled through the sample in the cuvette. After the addition of D-lactate, oxygen bubbling resulted in a maintenance of the fluorescence intensity at a constant, relatively low level. That is, under these conditions the fluorescence increase observed in Figure 5 did not occur. Bubbling nitrogen after the addition of D-lactate shortened the time required for the fluorescence increase, probably by flushing oxygen out of the cuvette.

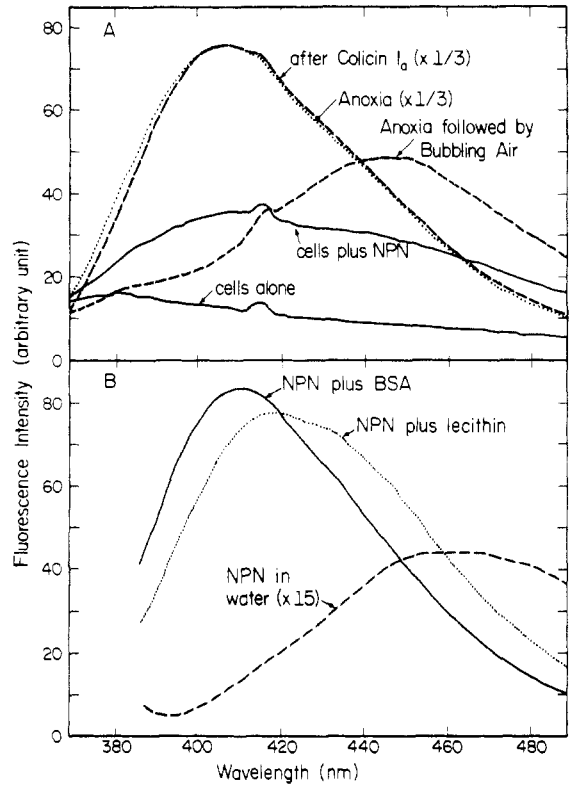

FIGURE 6: (A) The effect of colicin Ia and anoxia in the emission spectrum of NPN in the presence of *E. coli* JK1. Spectra shown correspond to (1) cells alone (no dye added); (2) cells plus 3 μM NPN; (3) cells plus NPN after the addition of colicin Ia (0.18 μg/ml); (4) cells plus NPN after anoxia induced by the addition of 20 mM D-lactate; (5) same as the previous sample only after bubbling air into the cuvette. (B) Emission spectra of NPN (3 μM); (1) in an aqueous solution of BSA (1.2 mg/ml); (2) in an aqueous dispersion of dipalmitoylphosphatidylcholine (150 μg/ml); (3) in water. Under these conditions essentially all the dye is bound to the lipid or protein. The intensity of the emission spectrum of NPN in water should be multiplied by a factor of $^1/_{15}$ to be compared with the other two spectra. The arbitrary fluorescence intensity scales in parts A and B of this figure are different. Fluorescence was measured at 37 °C using a Perkin-Elmer MPF-III fluorimeter with an excitation wavelength of 340 nm. All spectra are uncorrected.

The spectral changes of NPN emission after colicin Ia treatment and those due to anoxia are compared in Figure 6. It is evident that both of these treatments result in a nearly identical fluorescence response from the membrane probe. Bubbling of air through the sample after anoxia reverses the spectral changes, although the resulting spectrum is not identical with the original spectrum. As can be seen, a red-shifted emission spectrum is obtained after reversal of the anoxia. The blue-shifted spectrum associated with both colicin Ia treatment and anoxia is similar to those observed after cyanide, azide, or amytal addition to the cells. Only in the case of anoxia is this fluorescence response reversed or in any way affected by bubbling air through the cuvette. The fluorescence parameters of NPN are strongly dependent on the polarity and polarizability of its surroundings (Radda, 1971). In general, the fluorescence quantum yield and lifetime increase with the hydrophobicity of the medium. In part B of Figure 6 the emission spectra of NPN bound to BSA, to dipalmitoyllecithin, and in aqueous solution, are presented for comparison with the spectra in the presence of cells. Although the contribution from the cell background spectrum (also shown) has not been subtracted, the similarity between the spectra of NPN in the presence of untreated cells and after reversal of anoxia, and

TABLE I: Effect of KCN and Colicin Ia on the Binding of NPN to *E. coli*, Strain JK1 and the Fluorescence Characteristics of the Probe.[a]

	NPN in Buffer	Untreated Cells	Cells Treated with 3 mM KCN	Cells Treated with 0.26 μg/ml of Colicin Ia
Fraction of probe bound	—	0.07 ± 0.04	0.19 ± 0.04	0.29 ± 0.08
Fluorescence[b] polarization	0.017 ± 0.005	0.030 ± 0.006	0.093 ± 0.007	0.081 ± 0.002
Fluorescence[b] lifetime (ns)	2.5	2.9	—	5.8

[a] Cell concentration: 5×10^8 cells/ml. Total NPN concentration: 3 μM. Temperature was 37 °C. [b] Corrected for the scattering and fluorescence background of the cells. Scattering of the emitted light by the turbid cellular suspension results in somewhat low values for the polarization. Both polarization and lifetime measurements varied from preparation to preparation within about 10% of the reported values. Binding results also depended on the particular preparation. The binding, polarization, and lifetime measurements were all perfomed on different preparations and the reported values for each represent results for individual preparations.

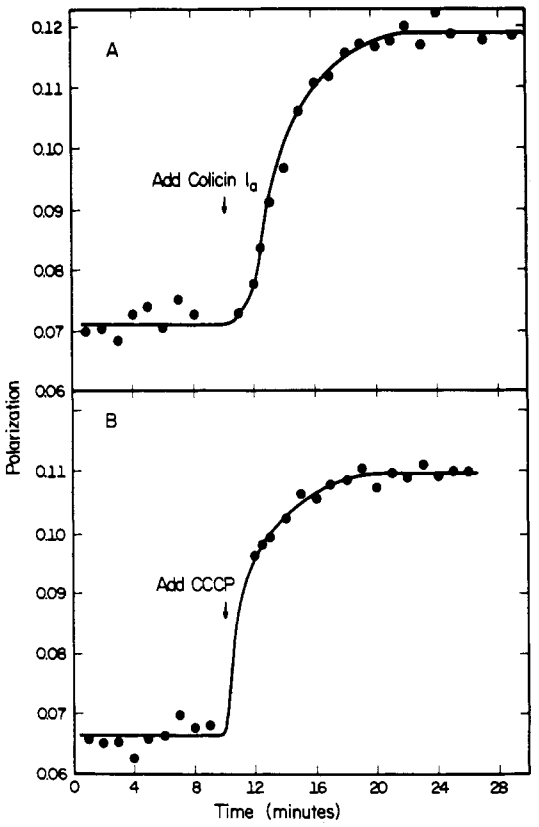

FIGURE 7: Effect of colicin Ia and CCCP on the fluorescence polarization of NPN in the presence of *E. coli* JK1. Samples were prepared as described in the caption to Figure 1. Fluorescence was measured at 37 °C. The excitation wavelength was 340 nm, as defined by a grating monochromator and a Corning 7-51 broad band filter. The emission was defined with a $NaNO_2$ filter (2M, 2 mm thick) and a Corning 3-73 cutoff filter. (A) Colicin Ia was added, as indicated, to a final concentration of 0.18 μg/ml. (B) CCCP was added as indicated to a final concentration of 1.5 μM.

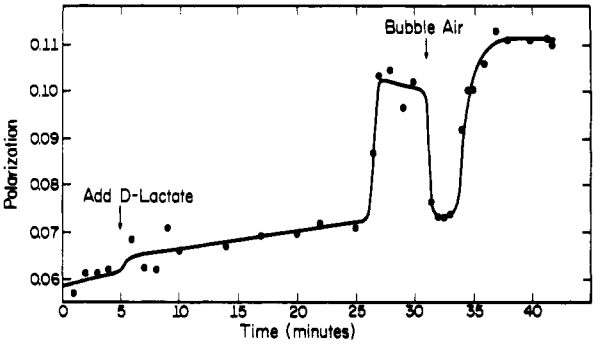

FIGURE 8: Effect of anoxia as detected by the change in NPN fluorescence polarization of NPN in the presence of *E. coli* JK1. D-Lactate (20 mM) was added to a suspension of 5×10^8 cells/ml containing 3 μM NPN prepared as described in the caption to Figure 1. Fluorescence polarization at 37 °C was measured as described in the caption to Figure 7.

the spectrum of NPN in aqueous solution is quite evident. After treatment with colicin Ia or during anoxia, the spectrum of NPN bound to cells is very similar to the spectrum of NPN bound to protein or lipid hydrophobic sites.

The increase in NPN emission intensity upon treatment with the various agents used in this study is always accompanied by an increase in the fluorescence polarization, as well as an increase in the observed fluorescence lifetime. Figure 7 illustrates the increase in polarization upon addition of colicin Ia or CCCP. These changes parallel the increase in emission intensity. Figure 8 shows the effect due to anoxia on fluorescence polarization. As can be seen, there is a precipitous rise which occurs about 25 min after the addition of the oxidizable substrate. This polarization increase is reversed by bubbling air through the sample.

Fluorescence lifetimes were measured prior and subsequent to the various treatments leading to the NPN fluorescence changes. In all cases the lifetimes increased by about a factor of two. Some of these data are included in Table I. It should be mentioned that once the state of high fluorescence intensity had been induced by one agent or treatment, other inhibitors have little effect. Also, the concentrations of agents used are not in excess and are comparable to levels routinely used in bacterial physiological experiments.

Jetten and Jetten (1975) have recently provided evidence which suggests that a transition step occurring in the colicin K, E2, and E3 mode of action sequence is energy dependent and that an energized state of the cytoplasmic membrane is required for the initiation of colicin action. Figure 9 provides support for the contention that colicin Ia action requires that treated cells be energized. As can be seen in Figure 9B, the addition of colicin Ia to starved cells does not lead to an increase in NPN fluorescence. However, the subsequent addition of glucose to colicin Ia treated starved cells results in a momentary decrease in fluorescence (energization) followed by an increase in intensity (deenergization) to a level higher than that obtained in untreated starved cells alone. As can be seen in Figure 9A, colicin addition to glucose-energized cells leads to an increase in fluorescence intensity within 2 min after colicin addition. The binding of colicin Ia to these cells is not dependent on glucose.

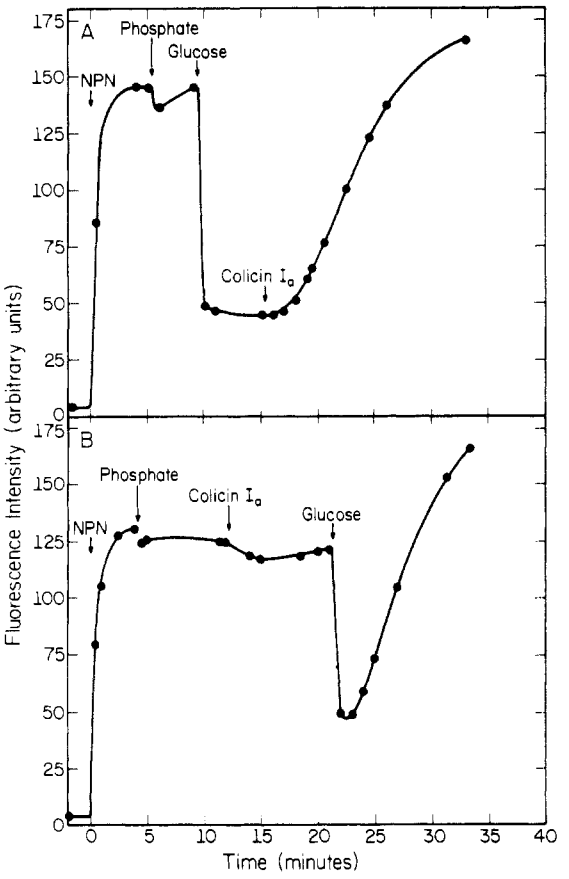

FIGURE 9: Effects of colicin Ia, D-glucose, and the order of their addition on the fluorescence of NPN in the presence of starved *E. coli* JK1 cells. Fluorescence was measured at 37 °C, as described in the caption to Figure 1. Cells were grown, harvested, and manipulated as described under experimental procedures. Cells were resuspended in medium S at a concentration of 100 Klett units (42 filter). Reagents were added at the times indicated. Concentrations were: NPN, 3 μM; potassium phosphate, pH 7, 10 mM; colicin Ia, 1 μg/ml; and D-glucose, 20 mM.

Binding Studies. Most of the procedures for measuring NPN binding to bacterial cells indicated that under the experimental conditions employed the amount of dye bound to the cells was small. Because of the technical problems mentioned (see Materials and Methods) the only method which yielded reproducible, quantitative results consisted in centrifuging the cells and bound dye and measuring the amount of NPN remaining in solution. The fraction of the dye bound to the cells as defined by this procedure was determined before and after treatment with potassium cyanide or colicin Ia. It was found that upon treatment of the cells with either cyanide or colicin Ia there is an increase in cell affinity for the probe. Prior to treatment, the bacteria exhibit very little affinity for the probe as defined by this assay. No more than 7 or 8% of the NPN was associated with the cells. After treatment with colicin Ia or KCN, approximately 30 and 20% of the probe was associated with the cells, respectively. In all cases about 2 or 3% of the NPN was found to bind to the glass centrifuge tube. These results clearly indicate that after colicin Ia or cyanide treatment there is an increase in the fraction of bound probe. This accounts for part or all of the changes observed in the NPN fluorescence parameters.

Discussion

Previous studies on the interaction of colicin E1 with *E. coli* by Cramer and co-workers have led these authors to suggest the intriguing possibility that the alteration in membrane structure reported by changes in the fluorescence parameters of NPN may arise as a direct result of colicin E1 penetration into or through the bacterial envelope. It was considered possible that this process resulted in an increase in membrane microviscosity, which might lead to a disruption of energy transduction. The results presented here provide an alternate interpretation of such data. It is suggested that the alteration of NPN fluorescence seen with colicin E1, K, or Ia may, in fact, derive from a colicin-induced deenergization of the bacterial membrane. According to this suggestion, the observed membrane changes result from colicin-induced disruption of energy transduction.

It is shown here that the fluorescence properties of NPN reflect the physiological state of cells. Whereas quenching of NPN fluorescence is observed upon energization of starved cells with a variety of substrates, agents which disrupt energy-transducing processes, such as colicin Ia, the uncouplers CCCP and azide, and the electron-transport inhibitors cyanide, amytal, and malonate, are shown to induce a similar high level of fluorescence and accompanying alterations in several fluorescence parameters. These results suggest that the changes induced by colicin can be a consequence of the disruption of the energy transduction processes in the cell rather than a manifestation of mechanistic events leading to such a disruption.

As is evident from the data in Table I, under the conditions used in this study, only a small fraction of the NPN is bound to untreated cells. Notwithstanding the wide error margins in the binding data, particularly in the case of untreated cells, it can be seen that a large increase in the fraction of NPN bound to the cells occurs upon treatment with colicin Ia and cyanide. An independent verification for this description of the phenomenon derives from the spectral data. The spectrum of NPN in the presence of untreated cells is similar to the spectrum of NPN in aqueous solution, indicating that the major contribution to the fluorescence is due to the free dye in the aqueous phase. Since the fluorescence quantum yield of NPN in water is smaller than that in phospholipid by a factor of 1/17 (Radda, 1971), it may be concluded that most of the NPN is in the aqueous phase in order for its contribution to the fluorescence to be dominant.

As mentioned above, Helgerson et al. (1974) have proposed a model in which the primary effect of colicin E1 is the induction of an increase in microviscosity in the cell membrane. The implications of such a model are of obvious importance, not only in reference to the mechanism of colicin action but to membrane physiology in general. The model is based primarily on the assumptions that the amount of bound NPN changes only by a small fraction upon colicin treatment, and that the fluorescence contribution of the unbound probe is insignificant before and after treatment. These assumptions were based on ANS binding data previously reported (Cramer and Phillips, 1970), and on the notion that the NPN molecule is more lipophilic than the ANS molecule. These assumptions are shown here to be incorrect at least under our experimental conditions. The measured values of fluorescence intensity, polarization, and lifetime all contain contributions from free NPN and bound NPN, as well as the contribution from the cell background. This latter contribution is not insignificant especially under conditions where most of the NPN is not bound. The background fluorescence may represent as much as 20% of the total under these circumstances. This is an especially serious problem when one wishes to measure the lifetime of the bound probe, particularly since the fluorescence

lifetime of the background is of the same order of magnitude as that of NPN. In addition, the substantial light scattering from the turbid sample also contributes to make a quantitative analysis of this system virtually impossible.

The spectral and binding data presented in this paper and in the previous contributions involving colicin E1 do not warrant speculation on the molecular details of the structural changes leading to the increased binding of NPN by the cells. One of the main factors preventing further insight is the uncertainty about the location of the bound probe within the cell envelope. Although it is known that at least part of the bound NPN resides in the lipid, since its fluorescence is affected by events mostly or exclusively related to the membrane lipid (Overath and Träuble, 1973), some of the dye may also be bound to hydrophobic protein sites. The changes leading to increased dye binding could be occurring in the lipid or in the membrane protein or in both. The possibility that such changes could be the result of an increase in the amount of either the reduced or oxidized form of some species in the electron-transport chain is eliminated by the fact that blockage of the chain at either the substrate side or the oxygen side leads to the same fluorescence response.

It must be emphasized that NPN is a neutral probe under any physiological pH, and therefore cannot respond electrophoretically to any changes in the trans-membrane potential or surface charges. There have appeared in the recent literature reports of the use of several charged fluorescent dyes in following the "energization" of bacterial membranes. Based on well-documented studies with mitochondrial systems (Azzi et al., 1969), the observed changes in the bacterial systems have generally been attributed to changes in the electrostatic interaction between the membrane and the charged dye molecule. During the preparation of the present report, a communication appeared describing the fluorescence response of the dye ANS in the presence of intact *E. coli* cells to several of the treatments and conditions employed in this paper, including cyanide and CCCP treatment, and the anoxia effect (Griniuviene et al., 1975). ANS behaves qualitatively in the same way as NPN under all the conditions described in this paper (Nieva-Gomez, Konisky, and Gennis, unpublished observations). Thus, in the case of *whole E. coli* cells, the response of charged fluorescent probes to the disruption or restitution of energy-transduction processes in the membrane is at least in part due to structural changes in the cell envelope in addition to any electrophoretic migration of the dye caused by redistribution of charges across the membrane.

It is clear that there are dramatic physical changes in the *E. coli* envelope occurring in response to any interference with the energy-transduction processes. Furthermore, the fluorescence response of the probe has been shown to be reversible, as in the case of addition of substrate to starved cells, addition of D-lactate to malonate-treated cells, and the addition of oxygen to anoxic cells. Hence, these effects cannot be due to the breakdown of a permeability barrier. Dramatic anoxia effects have been reported in mitochondrial preparations using the charged probes ANS (Nordenbrand and Ernster, 1971) and pyrenesulfonate (Brocklehurst et al., 1970) and with ANS in *E. coli* membrane vesicles (Reeves, et al., 1972). The changes in NPN fluorescence in response to the many agents and conditions employed in this paper seem unlikely to be understood in terms of a simple two-state model. It is possible and in some cases likely that the agents used are affecting more than one cellular component. It is realized that energy transduction actually represents many interrelated processes. It is hoped that future experiments employing both physical and chemical probes of membrane structure will serve to isolate those particular aspects which are related to these phenomena and will clarify the nature of the conformational changes which are occurring within the bacterial envelope. The questions posed concerning both the magnitude or extent of these alterations as well as their location are of clear importance to the problem of the structure–function relationships within the bacterial membrane.

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