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Distinct phases of the fluorescence response of the lipophilic probe *N*-phenyl-1-naphthylamine in intact cells and membrane vesicles of *Escherichia coli*

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The fluorescence of the lipophilic prbe *N*-phenyl-1-naphthylamine (NPN) bound to intact cells of *Escherichia coli* is quenched by the addition of glucose, succinate, D-lactate, pyruvate, formate and glycerol. Partial recovery of fluorescence occurs on anaerobiosis. Use of mutants with defects in the ATP synthase or the respiratory chain show that quenching of fluorescence may be energized either by ATP hydrolysis or by substrate oxidation through the respiratory chain. Permeabilization of the outer membrane by treatment of intact cells with EDTA, or use of a mutant with an outer membrane permeable to lipophilic substances, results in a more rapid binding of NPN and in a decrease in quenching observed on substrate addition. NPN binds rapidly to everted membrane vesicles, but does not respond to membrane energization. It is proposed that inner membrane energization in intact cells alters the binding or environment of NPN in the outer membrane. The fluorescence recovery which occurs on anaerobiosis has two components. One component represents a reversal of the changes which occur on membrane energization. The other component of the fluorescence change is insensitive to the uncoupler CCCP and resembles the behaviour of NPN with everted membrane vesicles. It is suggested that a portion of the fluorescence events seen with NPN involves a response of the probe to changes in the inner membrane.

Introduction

Uncharged lipophilic molecules such as *N*-phenyl-1-naphthylamine (NPN) have been used as probes of the fluidity or microviscosity of the lipid bilayer of biological membranes [1]. NPN fluoresces weakly in aqueous environments, but becomes strongly fluorescent in nonpolar environments. The increase in fluorescence intensity when NPN binds to intact cells of *E. coli* is accompanied by a pronounced blue shift in the emission spectrum of the probe [2], and by changes in fluorescence polarization, lifetime and rotational relaxation time [3,4]. These changes are sensitive to the state of membrane energization of the cell. Thus, addition of glucose causes a reduction in fluorescence intensity by as much as 70%, and this effect is reversed by the addition of an uncoupler, such as CCCP, by colicins Ia and E1, by inhibitors of the respiratory chain, and by depletion of

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NPN, *N*-phenyl-1-naphthylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonate.

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oxygen [2]. The mechanism responsible for the changes in fluorescence intensity on energizaton or deenergization is unclear. Nieva-Gomez et al. [7,8] have suggested that the increased fluorescence on energization is due to an increase in the amount of NPN bound to the cell envelope. By contrast, Helgerson and Cramer [3,4] have found that the changes in binding cannot account for the changes in polarization, lifetime and rotational relaxation time of the probe. They have concluded that NPN monitors structural changes in the cell envelope exterior to the inner membrane, and that deenergization of the inner membrane results in a structural change in the outer membrane which affects the environment of the probe.

In the present paper we investigate two aspects of the mechanism of the changes in fluorescence intensity of NPN which occur on membrane energization/deenergization. We show that the alteration in fluorescence intensity has two components. The first, which occurs in intact cells, is sensitive to uncouplers, and responds to membrane energization by ATP or substrate oxidation through the respiratory chain. It is lost on permeabilization of the outer membrane with EDTA and is absent in a mutant which allows free movement of nonpolar materials through the outer membrane. The first component therefore seems to involve the response of the probe to changes in the outer membrane coupled to inner membrane energization. The second component, which can also be demonstrated in everted inner membrane vesicles, is insensitive to uncouplers, but responds to the activity or state of reduction of the respiratory chain.

Materials and Methods

Bacterial strains. W3110, a wild-type *E. coli* K-12 strain and AS-1, an *acrA* mutant derived from *W3110*, were obtained from Dr. Yasuo Imae, Ngoya University, Japan. Spontaneous revertants of AS-1 to Methylene blue resistance were obtained by streaking the parent organism on nutrient agar plates containing 50 μg/ml Methylene blue. CGSC 6043 (*proC24 ompA252 his53 purE41 ilv277 metB65 lacY29 xyl14 rpsL97 cycA1 cycB2?tsx63* λ −), a porin mutant lacking outer membrane protein 3a, was supplied by Dr. B. Bachman. AN120 (*argE3 thi mtl xyl rpsL704 uncA401*) was obtained from Dr. F. Gibson, Canberra, Australia, and SASX76 (F^- *hemA met trp lac rpsL*) was obtained from Dr. A. Sasarman, University of Montreal. Quinone mutants AN384 (F^- *ubiA420 menA401 thi rpsL*) and AN386 (F^- *menA401 thi rpsL*) were supplied by Dr. I.G. Young, Canberra, Australia.

Growth of cells. Strains SASX76, AN384 and AN386 were grown in minimal salts media containing 0.4% glucose and 0.2% casamino acids. All other strains were grown in Penassay Broth (Difco). Cultures were grown from 1% (v/v) inoculum at 37 °C with aeration to stationary phase (16 h). The cells were harvested by centrifugation, washed once in 50 mM Hepes-KOH (pH 7.4) and resuspended in 3 ml of the same buffer.

EDTA treatment. Cells were harvested and washed twice in 50 mm Tris-HCl (pH 8.0). 0.25 g (wet weight) of cells were resuspended in 30 ml of 50 mM Tris-HCl (pH 7.4). EDTA was added to a final concentration of 1 mM, the cell suspension was stirred for 5 min at room temperature and then centrifuged at 12000 × *g* for 10 min. The cell pellet was resuspended in 2.0 ml of 50 mM Hepes-KOH (pH 7.4).

Preparation of everted memrbane vesicles. Cells were harvested in stationary phase and were washed once in 50 mM Tris-HCl (pH 8.0). 2.5 g (wet weight) of cells were resuspended in 20 ml of 50 mM Tris-HCl (pH 8.0)/ 5 mM $MgCl_2$. The cell suspension was disrupted by two passages through a French press (Aminco) at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 1000 × *g* for 20 min, and the membrane vesicles were then pelleted from the supernatant by centrifugation at 200000 × *g* for 1.75 h. The vesicles were washed once in 50 mM Hepes-KOH (pH 7.4)/5 mM $MgCl_2$ and resuspended in 1.5 ml of the same buffer to give a protein concentration of approx. 40 mg/ml.

Quinacrine and NPN fluorescence assays. The fluorescence of quinacrine or *N*-phenyl-1-naphthylamine (NPN) was measured at 22 °C with a Turner model 420 spectrofluorometer connected to a linear chart recorder. In experiments using intact cells the reaction mixture, in a cuvette of 1 cm light path and in a final volume of 2 ml, contained 50 mM Hepes-KOH (pH 7.4) and 2 mg

of cell protein. The assay was started by addition of 0.5–2.0 μM NPN. For quinacrine or NPN assays using everted membrane vesicles, the reaction mixture contained 10 mM Hepes-KOH (pH 7.4), 0.3 M KCl, 5 mM $MgCl_2$ and 1.0 mg of membrane protein unless indicated otherwise. The assays were started by addition of 5 μM quinacrine or 1.25 μM NPN. The energy sources and inhibitors were used at the concentrations indicated in the legends to the figures. NPN fluorescence was excited by light at 340 nm and emission was measured at 420 nm. Quinacrine fluorescence was measured using an excitation wavelength of 430 nm and an emission wavelength of 505 nm.

Determination of protein. Protein was measured by the method of Lowry et al. [9], using bovine serum albumin as a standard.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone and 1-*N*-phenylnaphthylamine were obtained from Sigma.

Results

Energization and NPN fluorescence in intact cells

The fluorescence behaviour of NPN on addition to a cell suspension is shown in Fig. 1. NPN bound to the cell envelope with an increase in fluorescence intensity. A stable fluorescence level was reached after approx. 7 min. Addition of glucose (or succinate, D-lactate, pyruvate, formate or glycerol, but not methyl α-D-glucose or 2-deoxy-D-glucose) as an energy source resulted in rapid quenching of a portion of the fluorescence. A return to the original level of fluorescence occurred when the dissolved oxygen in the reaction system had been consumed. Addition of a small amount of H_2O_2 (as a source of O_2 with the cells' endogenous catalase) to the anaerobic solution now resulted in quenching to the level given by glucose (see Fig. 3, curve 1). The intensity of the initial fluorescence change on addition of NPN to the cell suspension, and the size of the decrease produced by glucose, was increased when the uncoupler CCCP was present. An optimal response was obtained at 0.5 μM CCCP. Higher levels resulted in quenching of the fluorescence of NPN. The size of the fluorescent phase occurring after oxygen depletion was unaffected by CCCP.

Addition of azide altered the fluorescence behaviour of NPN. Similar to the effect of CCCP, preincubation of the cell suspension with 30 mM azide for 2 min prior to the addition of NPN increased the intensity of the fluorescence observed and increased the extent of quenching produced by glucose metabolism (Fig. 2). The recovery of fluorescence on anaerobiosis was much larger if azide was present (Fig. 2, curve 3). The results with azide may be explained as follows. Metabolism of endogenous substrates maintain a certain level of membrane energization, that is, an electrochemical gradient of protons across the inner membrane of the cell. Addition of glucose increases the extent of energization and this is reflected in a decrease in the fluorescence of NPN. The extent to which this occurs depends on the level of energization maintained by the endogenous metabolism of the cell. Azide, which inhibits proton translocation by the inner membrane ATP synthase [10], increases the extent of NPN fluorescence and the extent of quenching by glucose,

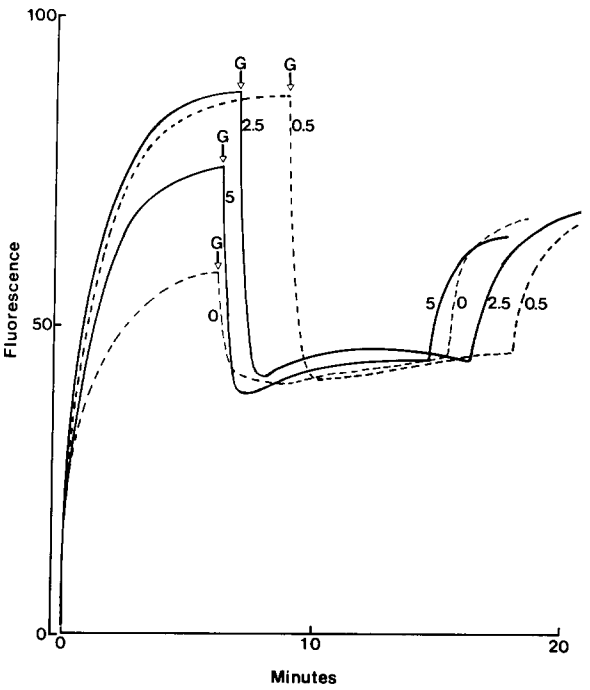

Fig. 1. Effect of uncoupler CCCP on the fluorescence of NPN with intact cells of *E. coli* AN180. The procedure is described in Materials and Methods. G, D-glucose added to 10 mM. The numbers indicate the μM concentration of CCCP preincubated with the cell suspension for 2 min prior to the addition of NPN at zero time.

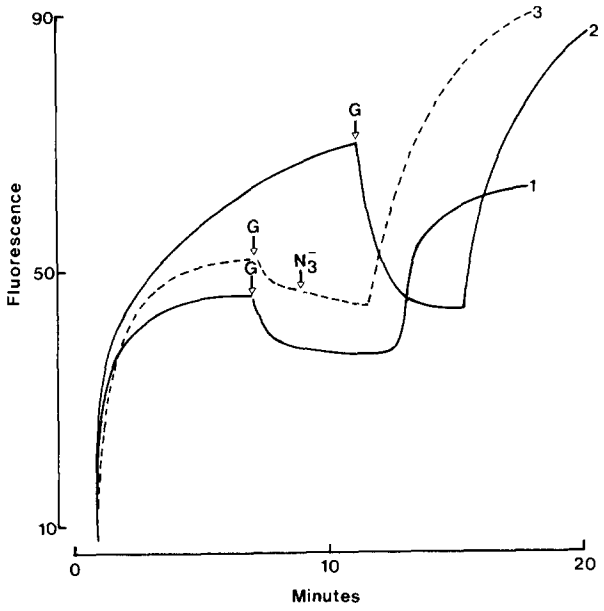

Fig. 2. Effect of azide on the fluorescence of NPN with intact cells of *E. coli* W3110. The procedure is described in Materials and Methods. G, 10 mM D-glucose; N_3^-, 30 mM azide. In curve 2, 30 mM azide was added to the cell suspension 2 min prior to the addition of NPN at zero time.

because it removes the contribution of ATP hydrolysis to the maintenance of the electrochemical gradient of protons. Azide did not affect the contribution of the respiratory chain to quenching NPN fluorescence. This contribution was removed when the system became anaerobic. Consequently there was a much larger increase in the fluorescence phase on anaerobiosis with the inhibitor present (Fig. 2, curves 2 and 3).

The data suggested that NPN fluorescence was affected by the state of inner membrane energization, and that both respiration and ATP hydrolysis contributed to this. Further evidence for the dual sources of energization was obtained as follows. *E. coli* AN120 is an *uncA* strain with a defective ATP synthase. Thus, the sole mode of membrane energization is through respiratory chain-coupled proton translocation. Addition of glucose to a cell suspension of this strain caused quenching of the fluorescence of NPN (Fig. 3, curve 1). The intensity of fluorescence was increased by low concentrations of CCCP, and the subsequent quenching by glucose was much greater. On anaerobiosis the fluorescence returned fully to its unquenched value (Fig. 3, curve 2), since the ATP could not maintain the proton gradient in this strain. The ability of low concentrations of CCCP to increase the fluorescence with NPN was due to the dissipation of the proton gradient maintained by endogenous substrates. In the presence of glucose, the rate of proton translocation by the ATP synthase and/or the respiratory chain must be sufficiently large to overcome the effect of CCCP.

Arsenate, which lowers cellular ATP levels [11] had no effect on the NPN response in the *uncA* strain (Fig. 3, curve 3). This is expected, since ATP cannot energize the membrane in this strain. (N.B. The cell suspension used in the experiment giving curve 3 was different from that yielding curves 1, 2. The control for the curve 3 without arsenate was identical to curve 3.)

E. coli SASX76 is a *hemA* mutant which cannot form cytochrome in the absence of 5-aminolevulinic acid [12]. Thus, transmembrane proton

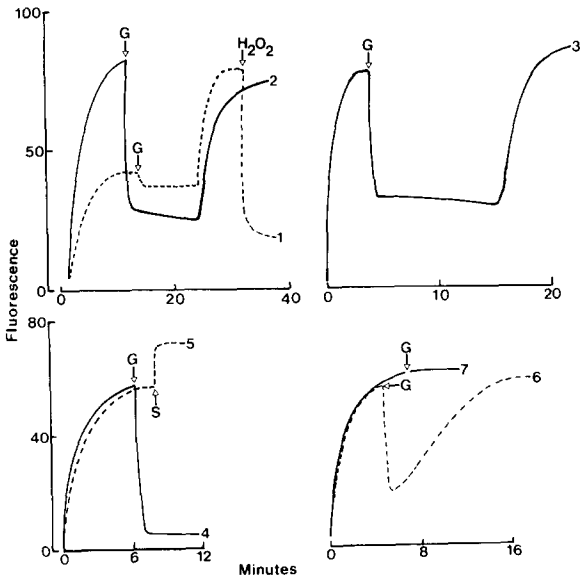

Fig. 3. Effect of inhibitors and uncoupler on the fluorescence of NPN with intact cells of *E. coli* AN120 (upper two panels) and SASX76 (lower two panels). The procedure is described in Materials and Methods. G, 10 mM D-glucose; S, 10 mM succinate; H_2O_2, 5 μl of 3% H_2O_2. The cells were preincubated for the indicated time prior to the addition of NPN at zero time with: curve 2, 2.5 μM CCCP (2 min); curve 3, 0.4 mM arsenate (6 min); curve 6, 0.4 mM arsenate (6 min); curve 7, 30 mM fluoride (2.5 min).

translocation is carried out by the ATP synthase only. The absence of respiratory chain-dependent proton translocation was reflected in the absence of a change in NPN fluorescence following addition of succinate, a respiratory chain substrate (Fig. 3, curve 5). (The rapid increase of fluorescence on adding succinate was artifactual.) Glucose produced an alteration in the fluorescence of NPN with the *hemA* strain, since it was a source of ATP (Fig. 3, curve 4). Blocking metabolism of glucose with fluoride (Fig. 3, curve 7), or depletion of the ATP pool with arsenate (Fig. 3, curve 6), affected the NPN response.

The results described in this section indicate that the decrease in NPN fluorescence caused by the addition of glucose to a cell suspension was due to the formation of an electrochemical gradient of protons driven by substrate oxidation and the hydrolysis of ATP. Partial deenergization and the partial recovery of fluorescence occurred when respiratory chain-driven proton translocation ceased on exhaustion of oxygen in the medium.

Effect of alterations in the outer membrane on NPN fluorescence

Treatment of cells of *E. coli* with EDTA results in removal of lipopolysaccharide and an increase in the permeability of the outer membrane [13]. *E. coli* W3110, a wild-type K-12 strain, was treated with EDTA and the response of the cells measured with the NPN fluorescence assay. Removal of lipopolysaccharide increased the rapidity with which NPN bound to the cell suspension and, as found by Cramer et al. [6], decreased the amount of quenching produced by the addition of glucose (FIg. 4, curves 1,2). Strain AS1 is a derivative of W3110 which has increased outer membrane permeability to nonpolar substances [14]. This is a consequence of a mutation in the *acrA* gene. Binding of NPN was rapid in untreated cells and fluorescence quenching did not occur on addition of D-lactate. However, there was an increase in fluorescence on complete depletion of oxygen in the medium (Fig. 4, curve 3). The presence of CCCP did not alter these responses. Removal of lipopolysaccharide increased the rate of the initial NPN binding only slightly (Fig. 4, curve 4). Revertant strains of AS1 which had decreased outer membrane permeability were isolated by selecting for the loss of the growth sensitivity of AS1 to Methylene blue. These strains, like the wild-type, showed quenching of the fluorescence of NPN on addition of D-lactate (Fig. 4, curve 5), and EDTA treatment increased the rapidity with which NPN was bound and diminished the extent of fluorescence quenching by substrate (Fig. 4, curve 6).

These results suggest that the integrity of the outer membrane is required to demonstrate energization-dependent quenching of the fluorescence of NPN.

Response of NPN in everted inner membrane vesicles.

NPN was bound rapidly to everted inner mem-

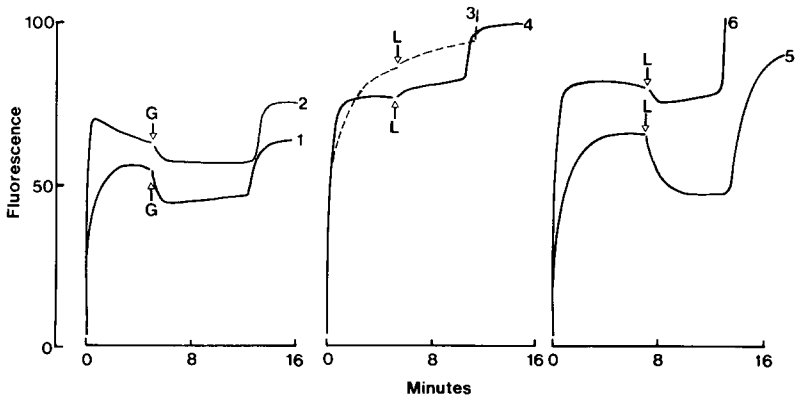

Fig. 4. Effect of EDTA treatment on the fluorescence of NPN with intact cells of *E. coli* W3110 (left panel), AS1 (middle panel) and the AS1 revertant (right panel). The procedure is described in Materials and Methods. G, 10 mM D-glucose; L, 10 mM D-lactate. The cells treated with EDTA were used for curves 2, 4 and 6.

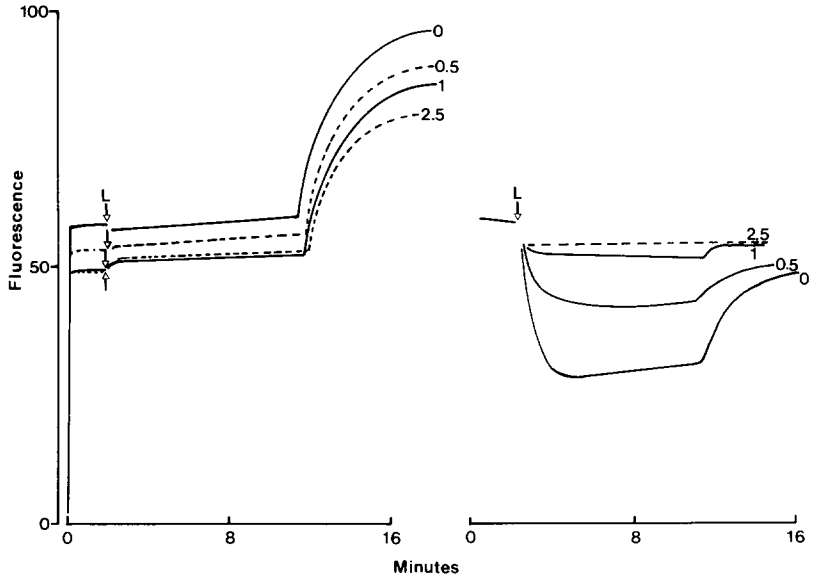

Fig. 5. Effect of the uncoupler CCCP on the fluorescence of NPN (left panel) and quinacrine (right panel) with everted inner membrane vesicles (protein, 0.5 mg/ml). The procedures are described in Materials and Methods. The numbers indicate the μM concentration of CCCP with which the vesicles were incubated for 2 min prior to the start of the experiment. L, 10 mM D-lactate.

brane vesicles (Fig. 5, left panel). Addition of respiratory chain substrates such as D-lactate did not cause quenching of fluorescence. However, when dissolved oxygen in the medium had been exhausted, there was an increase in the fluorescence of the bound probe. The NPN responses were insensitive to the uncoupler CCCP. By contrast, CCCP at the same concentrations discharged the D-lactate-dependent fluorescence quenching of quinacrine, which is a qualitative measure of the electrochemical gradient of protons [15,16]. Thus, the increase in the fluorescence of NPN which occurred when oxygen had been exhausted was not due to alterations in the proton gradient.

Menaquinone shows an increase in fluorescence intensity on reduction [17]. Since it was possible that this fluorescence change might interfere with that due to NPN, experiments were carried out using vesicles from the mutants AN384 and AN386. The absence of menaquinone in both strains, and of ubiquinone in AN386, was confirmed by thin-layer chromatography [18]. The usual increase in the fluorescence intensity of NPN occurred on anaerobiosis in suspensions of mem-

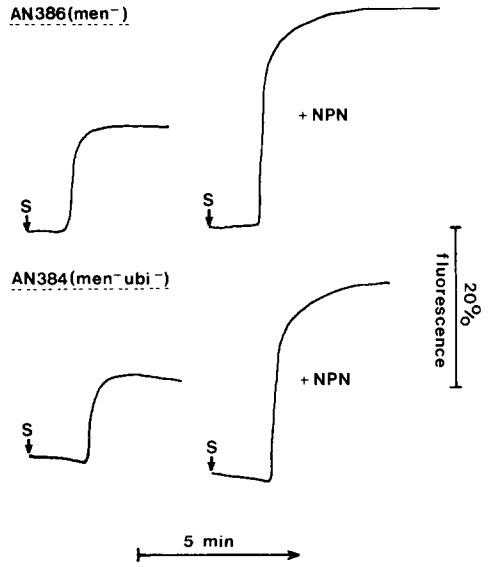

Fig. 6. NPN and endogenous fluorescence in everted membrane vesicles prepared from menaquinone-deficient strains of *E. coli*. Fluorescence was excited by light at 340 nm, and measured at 420 nm as described in Materials and Methods. The assay buffer was flushed with nitrogen prior to the addition of membrane vesicles (2 mg protein/ml). NPN was present only in the cuvettes giving the results shown on the right-hand side of the figure. S, addition of sodium succinate to 7.5 mM.

branes of these strains (Fig. 6), showing that changes in the redox state of menaquinone or ubiquinone did not affect the fluorescence response. However, an endogenous fluorescence response was observed on anaerobiosis in experiments in which NPN had been omitted. This response was about one-half of that observed in the presence of NPN (Fig. 6). The origin of the endogenous fluorescence has not been determined, but presumably it is due to a component of the respiratory chain, or to a material which is sensitive to the redox state of the respiratory chain.

Discussion

Addition of NPN to a cell suspension results in an increase in fluorescence as the probe binds to the cells. The extent of this change is dependent on the state of membrane energization of the cells by endogenous substrates. As has been observed by others [4,6,8], addition of uncoupler increases the extent of the fluorescence observed. It has been proposed that this is due to an increased level of binding of NPN [7,8] or to the removal of a permeability barrier to NPN on deenergization [4]. Addition of substrates such as glucose causes quenching of fluorescence. This has been explained as being due to a decrease in the amount of bound NPN [8] or to a change in the microviscosity of the environment about the NPN [4].

The results of the present paper show that the changes in the fluorescence of NPN described above are related to the level of membrane energization, and that energization may be brought about by the hydrolysis of ATP by the ATP synthase or by oxidation of substrate by the respiratory chain. This dual route of energization is similar to that which has been described for the active transport of certain solutes or for the energy-dependent transhydrogenation of $NADP^+$ by NADH [19].

The mechanism of the alteration in fluorescence intensity has not been completely clarified. Treatment of cells with EDTA to deplete the lipopolysaccharide of the outer membrane [13], or the use of a mutant with increased permeability to non-polar substances [14] causes a more rapid binding of NPN and a decrease or loss of the quenching effect of energization on the bound probe. NPN binds rapidly to everted membrane vesicles and is insensitive to energization by substrate oxidation or deenergization by uncoupler. These results suggest that in the absence of the outer membrane, or with the outer membrane permeabilized to lipophilic substances, the NPN reaches binding sites in the inner membrane more easily where it no longer responds to the state of energization. Thus, the response to energization seen in intact wild-type cells may reflect changes in the environment of the probe in the outer membrane, or translocation to the inner membrane. In the present study, changes in NPN fluorescence were not accompanied by changes in the concentration of free NPN, as measured by the centrifugation technique of Nieva-Gomez et al. [8].

The fluorescence of NPN on exhaustion of dissolved oxygen depends on the location of the probe. In everted membrane vesicles the recovery of fluorescence is insensitive to levels of the uncoupler CCCP which cause deenergization, as shown by the loss of the substrate-dependent quenching of the fluorescence of quinacrine, a qualitative measure of the transmembrane proton gradient [15,16]. In intact cells a component of the fluorescence recovery on anaerobiosis is also insensitive to CCCP. However, there is a greater increase of fluorescence on anaerobiosis in the presence of azide, an inhibitor of the ATP synthase [10]. This suggests that a portion of the fluorescence change on anaerobiosis in intact cells must reflect deenergization. Deenergization presumably results in a reversal of the changes in the environment of NPN which occur in the outer membrane on addition of glucose. The nature of events responsible for the uncoupler-insensitive changes in the fluorescence of NPN on anaerobiosis is unknown, but does not involve the redox state of menaquinone and ubiquinone. It is presently under investigation [20].

Acknowledgements

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