JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 341-347
0021-9193/81/010341-07$02.00/0

Vol. 145, No. 1

Increased Binding of a Hydrophobic, Photolabile Probe to *Escherichia coli* Inversely Correlates to Membrane Potential but Not Adenosine 5'-Triphosphate Levels

MARCIA K. WOLF AND JORDAN KONISKY*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

We describe conditions for a quantitative determination of azidopyrene binding to *Escherichia coli* cells. In addition, we define conditions whereby irradiation of azidopyrene in the presence of cells leads to irreversible association of probe with cells. This is presumably due to the light-dependent generation of reactive nitrenes and subsequent incorporation of nitrenopyrene moieties into cellular components. These methods allowed us to determine that the amount of azidopyrene bound to cells was inversely correlated with the magnitude of the cellular membrane potential, but was not correlated with high or low adenosine 5'-triphosphate levels per se. Cells bound more azidopyrene if the $\Delta\psi$ was low. Cell-bound azidopyrene was found to be entirely associated with the inner and outer membrane. We suggest that the decreased association of hydrophobic probes upon energization of whole cells reflects a rapid transition in structural properties of the cell envelope.

In recent years, fluorescent molecules have been extensively used as probes of biological membranes (for reviews, see references 1, 2, and 30). These hydrophobic and amphiphilic probes associate with membranes when added to cells or artificial systems, and their resultant fluorescence properties can be used to monitor a variety of membrane characteristics.

When added to *Escherichia coli* cells, the fluorescence characteristics of a number of these probes change in response to the energy state of the cells. The specific properties of cellular energy responsible for these changes have not been well characterized. In general, the addition of effectors which result in deenergization of cells leads to increased fluorescence from probes present in the cell suspension. This is true of negatively charged 8-anilino-1-naphthalenesulfonate (ANS) (13, 14, 25) and *cis*-parinaric acid (36), positively charged 3,3'-dihexyloxacarbocyanine (7), and neutral probes, such as *N*-phenyl-1-naphthylamine (NPN), 1,6-diphenyl-1,3,5-hexatriene, pyrene, and azidopyrene (13-15, 25, 26, 38). In the one case studied, results indicate that the colicin E1-induced reduction in cellular ATP levels is not necessary for the colicin-induced fluorescence response (28).

Changes in the amount of probe bound to cells have been implicated to form at least part of the fluorescence changes seen in the case of NPN (26), azidopyrene (25), and *cis*-parinaric acid (36). It seems likely that the enhanced binding of acriflavine to deenergized cells (24, 33) is a related phenomenon. In the above cases, the degree of binding was estimated by centrifugation of the probe/cell mixture and a determination of fluorescence intensity either in resuspended cell pellets or in the supernatant. However, it is important to point out that such determinations must be regarded as semiquantitative since dye binding may not strictly correlate to fluorescence from cell-associated probe existing in a heterogenous environment. Furthermore, this general approach is not suitable for an attempt at probe localization since manipulations involved in cell fractionation might lead to a substantial redistribution of probe.

Nieva-Gomez and Gennis have described the use of azidopyrene as a fluorescent probe that can be covalently affixed to cells in situ (25). Upon irradiation, a highly reactive nitrene is generated, which becomes covalently attached to neighboring molecules. They found that whereas changes in fluorescence intensity were reversible in response to alterations in the energy state of cells before photolysis, the fluorescence intensity of cell-associated probe did not respond to changes in energy status after photolysis. It was suggested that photolysis caused irreversible probe binding, and that the amount of probe bound increased dramatically if the cells were deenergized.

In a continuation of this approach, we have used [^{3}H]azidopyrene to directly quantitate the binding of this hydrophobic molecule to *E. coli* cells. We show that the level of probe binding negatively correlates with the magnitude of the cellular membrane potential. Thus, a condition

of high membrane potential corresponds to low levels of bound azidopyrene, whereas cells having a low membrane potential bind high levels of probe. Binding is not affected by the level of cellular ATP. Probe associates with outer and inner membranes in cells with a high or low membrane potential. This system should be useful for elucidating the cellular changes that result in altered probe binding.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* K-12 strain JK1 (W3110 *rpsL* λ^-), strain AN180, and its *uncA401* derivative (AN120) were used. Medium 63 (23) was supplemented to 0.15% glucose and 0.15% casein hydrolysate for JK1; 0.4% glucose, 10 μg of arginine per ml, 1 μg of thiamine per ml, and 19 μM proline for AN120; and 0.4% glycerol, 0.15% casein hydrolysate, and 1 μg of thiamine per ml for AN180. If glutamine uptake was to be measured, 10 μM glutamine was added.

Photoactivation and binding assay of azidopyrene. Cells were harvested at mid-log phase by centrifugation at 12,000 × *g* for 5 min at 4°C and suspended in M63 salts at room temperature to 100 Klett units (no. 42 filter). This is equivalent to 7.7×10^8 cells per ml. Portions of 5 ml were added to 150-ml beakers (2-inch [ca. 5-cm] diameter) or 10 ml to 400-ml beakers (3-inch [ca. 7.5-cm] diameter) and incubated for 5 min. This and all subsequent manipulations were carried out with continuous gentle shaking at 37°C. Appropriate effectors were added, and incubation was continued for another 5 min before addition of [^{3}H]azidopyrene (in methanol) in the dark. The final concentration of azidopyrene was 2 μM and approximately 5,000 cpm/ml. The final methanol concentration never exceeded 0.2%. After 5 min of incubation in the dark, the samples were placed 26 cm under a prewarmed General Electric sunlamp (275 W, 110 to 125 V) and irradiated for 10 min. This treatment led to an increase in solution temperature of less than 2°C over the 10-min period. Subsequent steps were performed under ambient light. Quadruplicate portions of 0.5 ml were removed and centrifuged for 2 min in an Eppendorf model 3200 centrifuge at room temperature, and the resulting pellets were then washed twice in M63 salts containing 6 mg of bovine serum albumin (BSA) per ml. The presence of BSA in the wash solution greatly increased the reproducibility of the data. It is possible that BSA served to scavenge free probe. The samples were resuspended in 0.5 ml of this same M63/BSA buffer, filtered onto membrane filters (Celotate, Millipore Corp.; 0.5-μm pore size, prewashed with the M63/BSA buffer), and washed twice with 3 ml of this same buffer. The filters were dried, and radioactivity was determined by counting the samples in 10 ml of toluene with 4 g of 2,5-diphenyloxazole per liter. Azidopyrene binding is expressed as the percentage of counts per minute added that remained on the filter. The total counts added was determined by spotting 0.05 or 0.1 ml of the photolyzed suspension onto a dry filter. Nonspecific binding of photolyzed azidopyrene to filters was 2.5% and was determined after photolysis in M63 salts in the absence of cells. Data are presented without any correction for such nonspecific adsorption.

Amino acid uptake. Proline uptake was measured during illumination in cultures under conditions identical to those used to measure azidopyrene binding, except that [^{3}H]proline replaced [^{3}H]azidopyrene. [^{3}H]proline was added immediately after the beginning of irradiation to a final concentration of 19 μM and specific activity of 30 Ci/mol. Samples (0.05 ml) were filtered, using membrane filters (no. BA85, Schleicher & Schuell Co.; 0.45-μm pore size) that had been prewashed with 0.1 M lithium chloride. Filters were washed once with 3 ml of 0.1 M lithium chloride and dried; the radioactivity was then determined as described above with [^{3}H]azidopyrene. The nonspecific uptake at zero time was determined by pipetting cells and then [^{3}H]proline onto a prewashed filter and immediately beginning the vacuum and wash. The zero-time value was subtracted from all time points.

Glutamine uptake was measured in a similar assay, but [^{14}C]glutamine replaced proline and was added to 10 μM and 5 Ci/mol. The filters were washed once with 10 ml of 0.01 M Tris (pH 7.3)–0.15 M NaCl–0.5 mM $MgCl_2$, and the incubation was not carried out under the sunlamp.

ATP assay. ATP levels were determined by the luciferin-luciferase assay as described by Berger and Heppel (6), with the following modifications. The perchloric acid extract was frozen overnight. Such storage did not result in any loss of ATP activity. Potassium perchlorate was removed by centrifugation for 10 min at 2,600 × *g* instead of by filtration. Cell extracts or ATP standards were added after endogenous light emission had subsided. Light emission was detected by a Beckman LS-100 scintillation counter.

Starvation. Starvation of JK1 was achieved by using α-methylglucoside and sodium azide according to the procedure of Purdy and Koch (29). ATP levels were depleted in AN120 simply by washing the cells twice in M63 salts, resuspending them to 100 Klett units in the same buffer, and incubating them for 1 h with shaking at 37°C. After incubation, they were washed twice in M63 salts and resuspended to 100 Klett units in that same buffer.

Probe localization. Strain JK1 was grown and photolyzed as detailed above for the binding assay except for the following changes. Portions of 100 ml were photoactivated in 1,000-ml beakers. The extent of binding was comparable to that obtained with smaller portions. After the sunlamp was turned off, glucose was added to the starved samples, and incubation continued for 5 min. The cells were harvested and washed twice with M63/BSA buffer containing 0.4% glucose. Membrane separations were performed as described by Smit et al. (34) with the modifications of Crowlesmith et al. (9), except for the following changes. After the cells were washed once with ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, they were suspended in 1 ml of the HEPES buffer containing 40 μg of RNase and DNase. This cell suspension was passed once through a French pressure cell at 12,000 lb/in^2. The lysate was centrifuged for 20 min at 4°C and 1,000 × *g*, and the entire supernatant was layered onto a sucrose gradient of three steps (10 ml of 2.02 M, 13 ml

of 1.44 M, and 14 ml of 0.77 M). All sucrose solutions contained HEPES buffer. Centrifugation was for 12 h in a Beckman SW27 rotor running at 27,000 rpm. The gradients were fractionated by pumping distilled water onto the top and collecting fractions from the bottom. The radioactivity in each sample was quantitated by trichloroacetic acid precipitation. An equal volume of ice-cold 10% trichloroacetic acid was added to the samples before filtration onto membrane filters (no. BA85; Schleicher & Schuell Co.; 0.45-μm pore size) which had been prewashed with 10% trichloroacetic acid. The filters were washed twice with ice-cold 10% trichloroacetic acid and placed directly into 10 ml of Biofluor. Within a few hours, the filters dissolved, and the sample was counted. This method allowed quantitative (greater than 90%) recovery of tritium counts present in the lysate.

Azidopyrene. 1-[^{3}H]azidopyrene and 1-azidopyrene were supplied by Michael Kilbourn, University of Illinois. The concentration of azidopyrene in stock solutions was determined from its molar extinction coefficient of 49,000 at 357 mm (λ_{max}). Using thin-layer chromatography on silica gel, we found that 1-[^{3}H]-azidopyrene (stored at 4 Ci/mmol over 1 year) underwent decomposition to form a preparation containing 48% 1-[^{3}H]azidopyrene, 33% [^{3}H]aminopyrene, and 19% unidentified products. In this regard it is important to point out that except where specifically noted in the text, experiments were carried out with [^{3}H]-azidopyrene containing little or no decomposed azidopyrene. Since other workers have reported that 1-[^{3}H]azidonaphthalene (stored at 3 Ci/mol) sustained extensive radiation damage (5), it is possible that decomposition of radiolabled azides may be a general problem in the storage of such chemicals.

Reagents. [^{3}H]proline was purchased from Amersham/Searle; [^{14}C]glutamine and Biofluor were purchased from New England Nuclear Corp. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), L-ascorbic acid, phenazine methosulfate (PMS), and BSA (fraction V, 96 to 99% albumin) were obtained from Sigma Chemical Co. D-(−)-Lactate (lithium salt) was purchased from Boehringer Mannheim Corp., and casein hydrolysate was from ICN.

RESULTS

Azidopyrene binding assay. An assay was developed to quantitate the amount of [^{3}H]azidopyrene irreversibly bound to cells after photolysis. The probe is allowed to equilibrate with cells in the dark. After 5 min the probe is associated with the cells to the same level as after optimal photolysis (data not shown), but this binding is reversed if the energy state of the cells is changed before photolysis. This assay is illustrated (Fig. 1) by an experiment in which the time required for optimal irreversible probe binding was established. For this experiment washed cells were either starved (see Materials and Methods) or stored at 4°C for approximately 2 h before addition of azidopyrene. These suspensions were then illuminated, and at varying times portions were removed to the dark. Each portion was washed twice with the M63/BSA buffer and then energized by the addition of glucose before filtration. Upon energization, only irreversibly bound probe (i.e., probe which is photolyzed to produce a nitrene and presumably becomes covalently bound) remains with the cell and is retained on the filter. As can be seen, starved cells retained more probe than unstarved cells, but in both cases photoactivation was complete after 10 min of irradiation. Although the amount of probe associated with cells was the same whether or not the cells were subsequently irradiated (data not shown), irreversible binding was dependent upon illumination (Fig. 2). It is evident that in contrast to

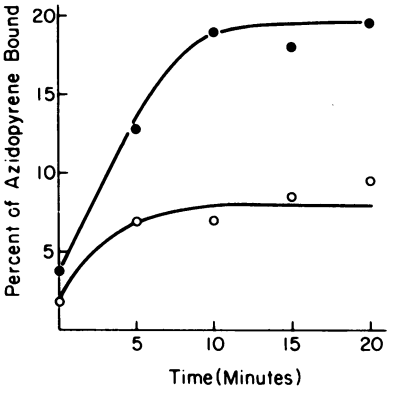

FIG. 1. *Time course of photoactivation of [^{3}H]azidopyrene. Starved (●) or unstarved (○) cells of strain JK1 were irradiated in the presence of [^{3}H]azidopyrene for the indicated times and then energized and assayed in the dark for the amount of bound azidopyrene as described in Materials and Methods.*

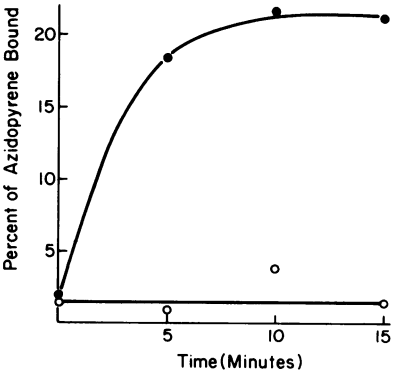

FIG. 2. *Light-dependent binding of azidopyrene to starved cells of strain JK1. Starved cells were incubated for the indicated times with irradiation (●) or in the dark (○) and then reenergized and assayed in the dark as described in Materials and Methods.*

irradiated cell suspensions, cells stored in the dark before energization by glucose and subsequent filtration retained little probe.

Azidopyrene binding correlates to the membrane potential ($\Delta\psi$). To determine whether azidopyrene binding correlates to the cellular membrane potential or ATP levels, an *E. coli uncA* mutant was used (Table 1). In this mutant, the membrane-associated ATPase is defective, and therefore there is no conversion between membrane potential and ATP. This allows ATP and $\Delta\psi$ to be varied independently. Although ATP levels can be determined directly, we found it impossible to measure $\Delta\psi$ by using triphenylmethylphosphonium ion. The accumulation of this hydrophobic cation in cells treated with EDTA is a direct measure of the membrane potential (32, 37), but such EDTA treatment causes a large increase in azidopyrene binding (data not shown). We therefore chose to use proline uptake as an indirect measure of $\Delta\psi$. This approach is justified since it is known at pH 7.0 that active transport of proline is dependent on the generation of $\Delta\psi$ (31).

Probe binding was inversely correlated to the membrane potential and not to cellular ATP levels. Conditions of high $\Delta\psi$ (high proline transport) resulted in low probe binding, whereas conditions of low $\Delta\psi$ (CCCP-treated cells) led to a relatively high amount of probe being bound. In contrast, 15- to 18-fold differences in ATP levels led to no significant differences in binding of azidopyrene, whether $\Delta\psi$ was high or low. The ATP formed in such cells was available as a source of energy (Table 2). Glutamine transport is known to be dependent on phosphate bond energy derived from ATP (6), and it is clear that high ATP levels correlate with high glutamine uptake.

In the above experiments, it is not unambiguously established that probe binding correlates to $\Delta\psi$ and not to some metabolic product derived from catabolism of glucose or lactate. However, the addition of the artificial electron-donating system ascorbate/PMS resulted in low azidopyrene binding (Table 3). As expected, this effect was abolished by inhibition of electron transport

TABLE 1. *Azidopyrene binding, proline uptake, and ATP levels in an uncA mutant*

Addition	ATP[a] (nmol/mg of cell protein)	Proline uptake (nmol/mg of cell protein per min)	% Azidopyrene bound[b]
None	0.5	3.0	6.9 (6.8, 6.9, 6.8, 7.0)
Glucose (0.4%)	10.0	10	4.1 (4.4, 5.1, 2.9)
D-Lactate (20 mM)	0.9	16	2.5 (3.2, 2.1, 1.5, 3.2)
Glucose (0.4%) + D-lactate (20 mM)	13.5	14	2.8 (2.8, 3.1, 2.8, 2.5)
CCCP (60 μM)	0.4	<1	12.8 (10.9, 14.3, 13.4, 12.5)
Glucose (0.4%) + CCCP (60 μM)	7.2	<1	13.6 (12.8, 13.3, 13.9, 14.5)

[a] ATP was determined in parallel cell suspensions containing [^{3}H]azidopyrene or [^{3}H]proline (see Materials and Methods). Sampling was 30 min after the start of illumination. The data are expressed as the average of triplicate determinations from each suspension (i.e., the average of six values).

[b] Since the preparation used in this experiment contains substantial amounts (see Materials and Methods) of decomposed azidopyrene (in the form of aminopyrene), these values correspond to the sum binding of azidopyrene plus aminopyrene. Of these, only azidopyrene would be covalently bound to the cells. Similar binding values are obtained when azidopyrene preparations containing no decomposition products are used. Individual values used in computing average are given in parentheses.

TABLE 2. *Glutamine uptake and ATP levels in an uncA mutant*

Addition	ATP (nmol/mg of cell protein)	Glutamine uptake (nmol/mg of cell protein per min)
None	0.3	<1
Glucose (0.4%)	14.0	34
D-Lactate (20 mM)	0.8	2.5
D-Lactate (20 mM) + CCCP (60 μM)	2.0	1.0
Glucose (0.4%) + CCCP (60 μM)	7.5	15

TABLE 3. *Effect of ascorbate/PMS on proline uptake and azidopyrene binding*[a]

Addition	Proline uptake (nmol/mg of cell protein)	% Azidopyrene bound[b]
None	2.0	9.4 (10.9, 9.2, 8.0)
Ascorbate (20 mM) + PMS (0.1 mM)	10.0	5.3 (5.6, 5.8, 4.8, 4.9)
Ascorbate (20 mM) + PMS (0.1 mM) + KCN (3 mM)	1.5	8.2 (9.0, 8.2, 8.9, 6.7)
Ascorbate (20 mM) + PMS (0.1 mM) + CCCP (60 μM)	<0.2	10.5 (13.7, 9.1, 8.9, 10.4)

[a] Strain used was AN180 grown as described in Materials and Methods with 0.4% glycerol as carbon source.

[b] Individual values used in computing average are given in parentheses.

by cyanide or collapse of the membrane potential by CCCP. These results indicate that it is the membrane potential and not some aspect of metabolism that affects probe binding.

Since a full understanding of the phenomenon under investigation requires localization of cell-associated probe (see below), the determinations described in this section were carried out under conditions in which the probe was irreversibly bound to the cells via photolysis. It is important to point out that such photolysis had no adverse effects on the ability of cells to be energized. For example, active transport of proline was linear throughout the 10-min period of photolysis, and ATP levels were similar whether assayed before or after photolysis.

Probe localization. To determine the location of cell-associated probe, both starved and unstarved cells containing bound azidopyrene photoproduct were disrupted in a French pressure cell, and the total cell lysate was subjected to sucrose gradient centrifugation under conditions which separate inner and outer membranes from other cell constitutents.

Probe associated with either starved or energized cells was found almost entirely in two peaks which migrated well ahead of unbound photolyzed azidopyrene (Fig. 3, arrow). It was established in several ways that these peaks coincided exactly with the position of inner and outer membranes. When the optical density at 280 nm (OD_{280}) profiles of the gradients shown in Fig. 3 were determined, three peaks were observed (data not shown). Two of these coincided with the peaks of radioactivity, whereas the third was centered at fraction 41 and probably represents absorbance from cellular nonmembrane material. When cells grown with [^{3}H]acetate to label membrane phospholipids were subjected to a similar treatment and analysis, the obtained profile of radioactivity was identical to that seen in Fig. 3 except for an additional peak of radioactivity near the top of the gradient. Whereas only the peak nearest the bottom in Fig. 3 (fractions 7 to 14) contained detectable colicin Ib receptor activity (an outer membrane marker, 21), 89% of the total NADH oxidase activity (an inner membrane marker) was confined to the peak contained in fractions 21 to 28, and 11% was in the peak at fractions 7 to 14.

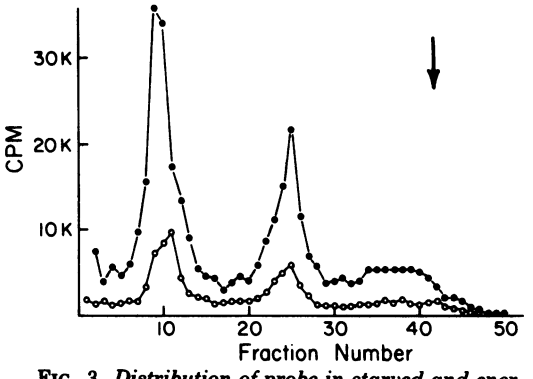

FIG. 3. *Distribution of probe in starved and energized cells of strain JK1. [^{3}H]azidopyrene (final concentration, 2 μM; 35,000 cpm/ml) was added to suspensions of cells which had been starved (●) or not starved and energized by addition of 0.4% glucose (○). After photolysis and cell breakage as described in Materials and Methods, equal amounts of (OD_{280} units) lysate were loaded onto separate sucrose gradients and subjected to centrifugation. Sedimentation was from right to left. Gradients were fractionated, and radioactivity was determined as described in Materials and Methods. OD_{280} recovery is typically 100%. Greater than 90% of the counts added to the gradient were precipitable by trichloroacetic acid. The arrow indicates the position to which photolyzed free azidopyrene migrates under these conditions. The cells used in this experiment bound 12% (energized) and 24% (starved) of the azidopyrene present in the cell suspension.*

These data together with a comparison of our results with published profiles of similar preparations (9, 34) unambiguously establish that azidopyrene is associated almost entirely with inner and outer membranes. Furthermore, it can be seen that although the distribution of azidopyrene between inner and outer membranes is identical in starved and energized cells, membranes isolated from starved cells contain more probe.

DISCUSSION

Although azidopyrene binding is inversely correlated with the magnitude of the membrane potential in whole cells, this relationship must be indirect due to the neutral character of the probe. Furthermore, less quantitative studies utilizing negative, positive, and neutral probes are consistent with the results and conclusions presented here. It seems likely that azidopyrene binding responds to structural changes in the cell envelope occurring as a direct consequence of the membrane potential. These structural changes must be reversible in order to account for the reversibility of probe binding upon energization in the absence of photolysis. ATP cannot elicit this change in the absence of a change in membrane potential even though ATP serves as an energy donor for glutamine transport, a function occurring across the envelope.

Although the results of several studies indicate that small hydrophobic molecules penetrate the outer membrane of intact *E. coli* and *Salmonella typhimurium* poorly (22, 27), we find substantial association of azidopyrene with

whole cells of an *E. coli* K-12 strain. We estimate that energized and deenergized cells bind on the average approximately 6×10^4 and 1.8×10^5 probe molecules per cell, respectively. Our results demonstrate that in both energized and deenergized cells, such cell-bound probe is associated with inner and outer membranes. The critical question i what structural transitions account for decreased probe binding upon generation of a membrane potential across the inner membrane.

Helgerson and Cramer (13) found that EDTA-treated cells showed high binding of the neutral hydrophobic probe NPN compared with that observed in energized cells. They proposed that increased probe uptake results from a permeability change in the outer membrane which allows NPN access to the inner membrane. This explanation is difficult to reconcile with the reversible nature of the probe response. Thus, although a simple permeability change could explain how probe gets into the inner membrane, it does not explain how probe is expelled upon energization. We think it likely that increased association with deenergized cells results at least in part from increased binding to the cell envelope via the generation of new available binding sites in response to a decrease in $\Delta\psi$.

One can only speculate as to how a change in inner membrane $\Delta\psi$ can bring about structural changes which could result in increased binding of azidopyrene. Possible binding to protein, phospholipid, lipopolysaccharide, or other envelope components may be shielded in energized cells. Since dissolution of azidopyrene into the fatty acid domains of phospholipids and lipopolysaccharide would require displacement of fatty acid chains, one can consider the possibility that the energy state of the inner membrane influences the lateral pressure of fatty acid chains (8, 11). This might influence their interaction with azidopyrene.

Just how the magnitude of the membrane potential across the inner membrane is able to influence outer membrane structure remains to be explained. It is possible that ionic species in proximity to the outer membrane might respond directly to the inner membrane $\Delta\psi$. Membrane potential-dependent changes in the inner membrane might be communicated to the outer membrane through sites of adhesion (3) or apposition (20). It is also possible that the energy-dependent translocation of membrane components from the inner to the outer membrane confers a specific structural state and that this condition results in suboptimal interaction with azidopyrene. In this regard it is interesting to note that the translocation of phospholipids from the inner to the outer membrane of *E. coli* is inhibited by reagents that disrupt the proton motive force, but not by arsenate, which reduces cellular ATP (10). It is further possible that $\Delta\psi$ might influence the pressure of the inner membrane against the outer membrane (35) and in so doing affect the volume of the periplasmic space. Such changes might well be translated into altered probe binding. Although the effect of membrane energization of the magnitude of the Donnan potential across the outer membrane (35) is not known, one could imagine that this potential might indeed affect binding of azidopyrene to the cell envelope.

Nitrenes, the purported reactive species of azido probes, are very reactive and can undergo a variety of reactions which lead to covalent attachment to neighboring molecules (19). Although there have been reports that azido probes do not insert into phospholipids with saturated fatty acids (4, 12), recent work has demonstrated covalent attachment of azido probe derivatives to both intrinsic proteins (5, 16–18, 39) and lipids (5). Studies are under way to determine the specific location of azidopyrene in energized and deenergized *E. coli* K-12 cells. These studies should lead to an understanding of the relationship between envelope structure and the energy state of cells.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 10106 from the National Institute of Allergy and Infectious Diseases. J.K. is the recipient of Public Health Service research career development award K04-AI 100049 from the same institute. M.W. is a recipient of Public Health Service predoctoral traineeship GM 7288 from the National Institutes of Health.

LITERATURE CITED

1. **Andersen, H. C.** 1978. Probes of membrane structure. Annu. Rev. Biochem. **47**:359–383.
2. **Azzi, A.** 1975. The application of fluorescent probes in membrane studies. Q. Rev. Biophys. **8**:237–316.
3. **Bayer, M. E.** 1968. Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. **53**: 395–404.
4. **Bayley, H., and J. R. Knowles.** 1978. Photogenerated reagents for membrane labeling. I. Phenylnitrene formed within the lipids bilayer. Biochemistry **17**:2414–2419.
5. **Bercovici, T., and C. Gitler.** 1978 5-[^{125}I]iodonaphthyl azide, a reagent to determine the penetration of proteins into the lipid bilayer of biological membranes. Biochemistry **17**:1484–1489.
6. **Berger, E. A., and L. A. Heppel.** 1974. Different mechanisms of energy coupling for the shock-sensitive and shock-resistant amino acid permeases of *Escherichia coli*. J. Biol. Chem. **249**:7747–7755.
7. **Brewer, G. J.** 1976. The state of energization of the membrane of *Escherichia coli* as affected by physiological conditions and colicin K. Biochemistry **15**:1387–1392.
8. **Conrad, M. J., and S. J. Singer.** 1979. Evidence for a

large internal pressure in biological membranes. Proc. Natl. Acad. Sci. U.S.A. **76**:5202–5206.

9. **Crowlesmith, I., M. Schindler, and M. J. Osborn.** 1978. Bacteriophage P22 is not a likely probe for zones of adhesion between the inner and outer membranes of *Salmonella typhimurium*. J. Bacteriol. **135**:259–269.
10. **Donohue-Rolfe, A. M., and M. Schaechter.** 1980. Translocation of phospholipids from the inner to the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:1867–1871.
11. **Galey, W. R., J. D. Owen, and A. K. Solomon.** 1973. Temperature dependence of nonelectrolyte permeation across red cell membranes. J. Gen. Physiol. **61**:727–746.
12. **Gupta, C. M., R. Radhakrishnan, G. E. Gerber, W. L. Olsen, S. C. Quay, and H. G. Khorana.** 1979. Intermolecular crosslinking of fatty acyl chains in phospholipids: use of photactivable carbene precursors. Proc. Natl. Acad. Sci. U.S.A. **76**:2595–2599.
13. **Helgerson, S. L., and W. A. Cramer.** 1976. Changes in *E. coli* cell envelope structure caused by uncouplers of active transport and colicin E1. J. Supramol. Struct. **5**: 291–308.
14. **Helgerson, S. L., and W. A. Cramer.** 1977. Changes in *Escherichia coli* cell envelope structure and the sites of fluorescence probe binding caused by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone. Biochemistry **16**: 4109–4117.
15. **Helgerson, S. L., W. A. Cramer, J. M. Harris, and F. E. Lytle.** 1974. Evidence for a microviscosity increase in the *Escherichia coli* cell envelope caused by colicin E1. Biochemistry **13**:3057–3061.
16. **Heller, R. A., R. Klotzbücher, and W. Stoffel.** 1979. Interactions of a photosensitive analog of cholesterol with hydroxymethylglutaryl-CoA reductase (NADPH) and acyl-CoA:cholesterol acyltransferase. Proc. Natl. Acad. Sci. U.S.A. **76**:1721–1724.
17. **Hu, V. W., and B. Wisnieski.** 1979. Photoreactive labeling of M13 coat protein in model membranes by use of a glycolipid probe. Proc. Natl. Acad. Sci. U.S.A. **76**: 5460–5464.
18. **Jacobs, S., E. Hazum, Y. Schechter, and P. Cuatrecasas.** 1979. Insulin receptor: covalent labeling and identification of subunits. Proc. Natl. Acad. Sci. U.S.A. **76**:4918–4921.
19. **Knowles, J. R.** 1972. Photogenerated reagents for biological receptor-site labeling. Acc. Chem. Res. **5**:155–160.
20. **Konisky, J.** 1979. Specific transport systems and receptors for colicins and phages, p. 319–359. *In* M. Inouye (ed.), Bacterial outer membranes: biogenesis and functions. John Wiley & Sons, Inc., New York.
21. **Konisky, J., B. S. Cowell, and M. J. Gilchrist.** 1973. Colicin Ia and Ib binding to *Escherichia coli* envelopes and partially purified cell walls. J. Supramol. Struct. **1**: 208–219.
22. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**:109–129.
23. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Nakamura, H.** 1966. Acriflavine-binding capacity of *Escherichia coli* in relation to acriflavine sensitivity and metabolic activity. J. Bacteriol. **92**:1447–1452.
25. **Nieva-Gomez, D., and R. B. Gennis.** 1977. Affinity of intact *Escherichia coli* for hydrophobic membrane probes is a function of the physiological state of the cells. Proc. Natl. Acad. Sci. U.S.A. **74**:1811–1815.
26. **Nieva-Gomez, D., J. Konisky, and R. B. Gennis.** 1976. Membrane changes in *Escherichia coli* induced by colicin Ia and agents known to disrupt energy transduction. Biochemistry **15**:2747–2753.
27. **Nikaido, H.** 1979. Nonspecific transport through the outer membrane, p. 361–407. *In* M. Inouye (ed.), Bacterial outer membranes: biogenesis and functions. John Wiley & Sons, Inc., New York.
28. **Phillips, S. K., and W. A. Cramer.** 1973. Properties of the fluorescence probe response associated with the transmission mechanism of colicin E1. Biochemistry **12**:1170–1176.
29. **Purdy, D. R., and A. L. Koch.** 1976. Energy cost of galactoside transport to *Escherichia coli*. J. Bacteriol. **127**:1188–1196.
30. **Radda, G. K.** 1975. Fluorescent probes in membrane studies. Methods Membr. Biol. **4**:97–188.
31. **Ramos, S., and H. R. Kaback.** 1977. The relationship between the electrochemical proton gradient and active transport in *Escherichia coli* membrane vesicles. Biochemistry **16**:854–859.
32. **Rottenberg, H.** 1979. The measurement of membrane potential and pH in cells, organelles, and vesicles. Methods Enzymol. **55**:547–569.
33. **Silver, S.** 1965. Acriflavine resistance: a bacteriophage mutation affecting the uptake of dye by the infected bacterial cells. Proc. Natl. Acad. Sci. U.S.A. **53**:24–30.
34. **Smit, J., Y. Kamio, and H. Nikaido.** 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. **124**:942–958.
35. **Stock, J. B., B. Rauch, and S. Roseman.** 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **252**:7850–7861.
36. **Tecoma, E. S., and D. Wu.** 1980. Membrane deenergization by colicin K affects fluorescence of exogenously added but not biosynthetically esterified parinaric acid probes in *Escherichia coli*. J. Bacteriol. **142**:931–938.
37. **Tokuda, H., and J. Konisky.** 1978. Mode of action of colicin Ia: effect of colicin on the *Escherichia coli* proton electrochemical gradient. Proc. Natl. Acad. Sci. U.S.A. **75**:2579–2583.
38. **Weber, G., S. L. Helgerson, W. A. Cramer, and G. W. Mitchell.** 1976. Changes in rotational motion of a cell-bound fluorophore caused by colicin E1: a study by fluorescence polarization and differential polarized phase fluorometry. Biochemistry **15**:4429–4432.
39. **Yip, C. C., C. W. T. Yeung, and M. L. Moule.** 1980. Photoaffinity labeling of insulin receptor proteins of liver plasma membrane preparations. Biochemistry **19**: 70–76.