JOURNAL OF BACTERIOLOGY, Feb. 1982, p. 523–528
0021-9193/82/020523-06$02.00/0

Vol. 149, No. 2

Dansyl Chloride Labeling of *Pseudomonas aeruginosa* Treated with Pyocin R1: Change in Permeability of the Cell Envelope

YOSHIHIKO URATANI

Mitsubishi-Kasei Institute of Life Sciences, Tokyo 194, Japan

Received 2 June 1981/Accepted 16 September 1981

Pyocin R1, a bacteriocin of *Pseudomonas aeruginosa,* caused an increase in binding of fluorescent label, 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride), to sensitive cells. In pyocin R1-treated cells, cytoplasmic soluble proteins and crude ribosomes as well as cell envelopes were labeled by dansyl chloride. The amount of bound dye was proportional to the multiplicity of pyocin R1 and reached a maximal level at high multiplicity. In addition, pyocin R1 rapidly caused an increase in fluorescence intensity of the hydrophobic probes *N*-phenyl-1-naphthylamine, pyrene, and perylene, which were mixed with cells. These results show that pyocin R1 damages locally a cell envelope barrier to hydrophobic solutes and allows dyes to penetrate into the intracellular space across the barrier.

Pyocin R1 is a bacteriocin produced by *Pseudomonas aeruginosa* which is morphologically like a bacteriophage tail with a contractile sheath (8, 10). Pyocin R1 rapidly inhibits active transport of substrates before cessation of macromolecular synthesis and O_2 consumption by glucose oxidation (7, 11, 13).

Pyocin R1 induces an increase in fluorescence intensity of probes immediately after the addition of pyocin R1 to the cell suspension (26). Fluorescent probe response has been found to be induced by colicin E1, a bacteriocin of *Escherichia coli*, and attributed to alteration in permeability of the outer membrane which results from membrane deenergization caused by colicin E1 (2, 3, 21). This study, using a covalently bound fluorescent dye, shows that pyocin R1 also increases permeability of the cell envelope to hydrophobic probes.

MATERIALS AND METHODS

Bacterial strains and culture condition. *P. aeruginosa* PML14 was used as a pyocin R1-sensitive strain. Bacterial cells were grown at 37°C in a medium containing 20 g of sodium glutamate, 5 g of glucose, 5.63 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of yeast extract per liter. The cells were harvested at the early to middle logarithmic phase, washed twice with a medium which contained 0.1 M $NaHCO_3$ and 5 mM $MgCl_2$, pH 8.4, and suspended in the same solution to give a cell concentration of approximately 6.5×10^8 cells/ml. *P. aeruginosa* PML15, a pyocinogenic strain, was used for the preparation of pyocin R1 by the method described by Kageyama (10).

Labeling procedure. Bacterial cells were preincubated for 5 min at 37°C with stirring, and then pyocin R1 was added. After 10 min of incubation, a fluorescent reagent, 10 mM 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride), dissolved in acetone was mixed with the pyocin R1-treated cells to give a final concentration of 50 μM. The residual 0.5% acetone did not affect the cell viability. After 30 min at the same temperature, the dansylated cells were collected by centrifugation for 10 min at 5,000 rpm in a Sorvall GLC-2 centrifuge at room temperature, washed three times with the suspension medium to remove the unreacted reagent and 1-dimethylaminonaphthalene-5-sulfonic acid produced by hydrolysis during the reaction (5), and finally resuspended in the above medium.

Preparation of cell constituents. Pyocin R1-treated cells were collected by centrifugation after labeling with dansyl chloride and broken by grinding with acetone-washed alumina at room temperature. DNase (10 μg/ml) was added, and then alumina grains were removed by low-speed centrifugation for 3 min at 1,000 rpm in a Sorvall GLC-2 centrifuge. The supernatant was centrifuged for 30 min at $60,000 \times g$ at 4°C and separated into two fractions, a sedimented fraction and a soluble fraction. Cell envelopes were separated from the $60,000 \times g$ pellet by centrifugation through two discrete sucrose layers of 0.5 and 2.0 M sucrose for 60 min at $73,000 \times g$; cell envelopes collected from the interface. The $60,000 \times g$ supernatant fraction was further centrifuged for 2 h at $100,000 \times g$ and separated into a soluble protein fraction and crude ribosomes (17).

Determination of killing activity. Surviving cells after pyocin R1 treatment were counted by the usual plating method.

Measurements of fluorescence and fluorescence polarization. Fluorescence of labeled dansyl groups and other fluorescent probes was measured by using a Shimadzu RF502 corrected recording spectrophotometer with a wavelength compensator attachment. Sample solutions were stirred in a thermostatted cuvette.

The dansyl group was excited at 330 nm, and the fluorescence was recorded at 510 nm. The slit width was set at 5 nm for both excitation and emission. The

extent of dansylation was monitored as fluorescence intensity.

Measurement of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was carried out with the above spectrofluorimeter equipped with a fixed and a rotating polarizer in the path of excitation and emission, respectively. For labeling of cells with DPH, cells were incubated with 2 μM DPH for 60 min at 40°C, centrifuged to remove free probe, and then suspended in the suspension medium. Excitation was at 360 nm, and polarized fluorescence was measured at 430 nm (14). The fluorescence polarization of the probe was calculated as fluorescence anisotropy, $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the plane of polarization of the exciting beam, respectively.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was performed as described by Mizuno and Kageyama (16). Protein concentration was determined by the method of Lowry et al. (15), with bovine serum albumin as a standard.

Chemicals. Dansyl chloride was purchased from Sigma Chemical Co. and used without further purification. 8-Anilino-1-naphthalenesulfonate (ANS) (Tokyo Kasei Kogyo Co.) purified as the magnesium salt was used (26). *N*-Phenyl-1-naphthylamine (NPN) was obtained from Tokyo Kasei Kogyo Co. and purified by recrystallization from ethanol. DPH (Nakarai Chemicals, Ltd.) dissolved in acetone was of specially prepared grade for scintillation and was used without further purification. Pyrene and perylene, the generous gifts of N. Wakayama, were dissolved in acetone. DNase was from Miles Laboratories Ltd.

RESULTS

Dansylation of pyocin R1-treated cells. Dansyl chloride reacts with basic groups including primary and secondary amines and phenolic hydroxyls which may be present in cell constituents, resulting in the formation of covalently linked fluorescent labels. Dansyl chloride, which is not fluorescent, is also hydrolyzed in an aqueous solution, forming fluorescent sulfonic acid (5).

The addition of pyocin R1 to sensitive cells was carried out at 37°C. At this temperature, the complete hydrolysis of 50 μM dansyl chloride and the full dansylation of pyocin R1-treated and untreated cells were accomplished within 30 min. Intact cells were slightly dansylated, whereas pyocin R1-treated cells were greatly labeled with dansyl chloride (Fig. 1). Pyocin R1 particles also were slightly modified with dansylation, but the amount of pyocin R1 added to the cells was so small that the contribution of dansylated pyocin to the total labeling was negligible.

Location of dansyl label in the cells. Distribution of dansyl label in pyocin R1-treated and untreated cells was investigated by separating cells into the cell envelope and the cytoplasmic components including soluble proteins and crude ribosomes. Since the separation of outer and cytoplasmic membranes from pyocin-treated cells by the method of Mizuno and Kageyama (16) was unsuccessful, the cell envelopes which contained both membranes were isolated from the above cells.

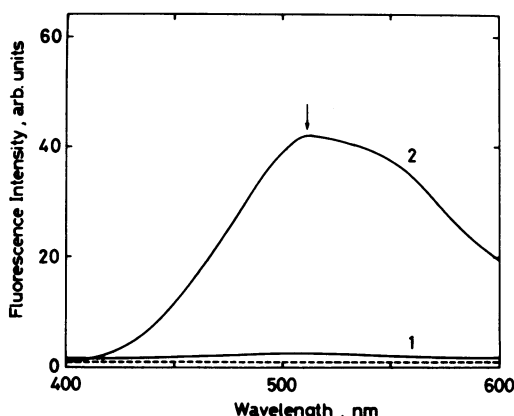

FIG. 1. Dansyl chloride labeling of pyocin R1-treated and untreated whole cells. Details of dansylation are given in Materials and Methods. Cell concentration before addition of pyocin R1 was 6.3 × 10^8 cells/ml. Cell survival after pyocin R1 treatment was 0.6%. Fluorescence emission spectra were recorded at room temperature, excited at 330 nm. Dashed curve, intact cells alone; curve 1, dansylated intact cells; curve 2, dansylated pyocin R1-treated cells. An arrow shows the maximal emission spectrum of dansyl group bound to the cell.

Dansyl label in the cellular components from intact cells was too low for analysis, but in pyocin R1-treated cells label was distributed in the cell envelope, soluble proteins, and ribosomes (Table 1).

Dansylation dependent on the amount of adsorbed pyocin R1. ANS is a hydrophobic fluorescent probe that does not covalently bind to cell components. Addition of pyocin R1 to the cell suspension containing ANS induces the enhancement in fluorescence intensity of the probe which results from an increasing interaction of ANS with pyocin R1-treated cells. Effects of pyocin R1 on fluorescent response of covalent and noncovalent labels were compared.

As reported previously (26), the fluorescence intensity of ANS after addition of pyocin R1 to the cell suspension increased linearly with cells killed. On the other hand, the increase in fluorescence intensity of dansyl groups bound to pyocin R1-treated cells was not proportional to cell death (Fig. 2A).

Fluorescence intensity from dansylated cells was replotted against multiplicity of pyocin R1 per cell (Fig. 2B). The increase in fluorescence was proportional to the number of pyocin parti-

TABLE 1. Distribution of dansyl label in cellular constituents

Fraction	F_{510}[a]	
	−R1	+R1[b]
Whole cell	1	130
Cell envelope	6	249
Soluble protein	1	24
Crude ribosomes	0	177

[a] Fluorescence intensity (arbitrary units) at 510 nm with 0.1 mg of protein/ml for each fraction.

[b] Initial cell concentration, 7.0×10^8 cells/ml; cell survival, 1%.

cles in the lower multiplicity range and reached a maximal level at higher multiplicity. These results suggest that the amount of dansylation is dependent on the number of pyocin particles adsorbed to the cell surface and that the cell is fully dansylated by the aggregated dansylation of regions caused by more than 10 particles attached to the cell.

The increase in dansylation induced by pyocin R1 occurred at a similar ratio in not only whole cells but also cell envelopes isolated from pyocin R1-treated cells after dansylation (Table 2).

Dansylation at various concentrations of dansyl chloride. The range of dansylation may depend on the dansyl chloride concentration. Dansylation at various concentrations of dansyl chloride per cell was examined after pyocin R1 treatment. The minimal multiplicity of pyocin R1 for maximal labeling decreased as the concentration of dansyl label increased (Fig. 3). In addition, the maximal amount of dansylation per cell was also enhanced. These results suggest the existence of reactive sites with different reactivities or different accessibilities of the dansyl label.

Use of other hydrophobic probes and fluorescence polarization. Instead of dansyl chloride, NPN, perylene, pyrene, and DPH were used as unreactive hydrophobic probes. These probes drastically increase the fluorescence intensity when they are transferred from aqueous solutions into a hydrophobic environment like cell membranes or pyrene excimers formed in lipid bilayers (1, 14, 21, 24).

In the presence of the intact cells, the fluorescence intensity of NPN, perylene, pyrene excimer, and DPH was low, showing that few dye molecules were bound to the intact cells. On the other hand, an increase in fluorescence intensity took place within 1 min after the addition of pyocin R1 in spite of different time courses of probes used and different rates of survival (Fig. 4).

By using DPH, one can gain information on structural changes in the membrane by measuring the fluorescence polarization (9, 14). The fluorescence polarization of DPH which was noncovalently incorporated into pyocin R1-treated and untreated cells was measured between 10 and 40°C. The fluorescence anisotropy of the probe was 0.191 for intact cells without pyocin R1 and 0.190 for cells treated with pyocin R1 at 37°C. In both cases, the fluorescence anisotropy gradually increased as the temperature was lowered, showing a small transition between 20 and 25°C. It was 0.222 for untreated cells and 0.228 for pyocin R1-treated cells at 10°C. There was no difference in the fluorescence polarization of DPH before and after pyocin R1 treatment between 10 and 40°C. On the other hand, the fluorescence intensity of DPH entrapped by pyocin R1-treated cells was 2.6 times larger than that by untreated cells at the survival of 2% after free probes were re-

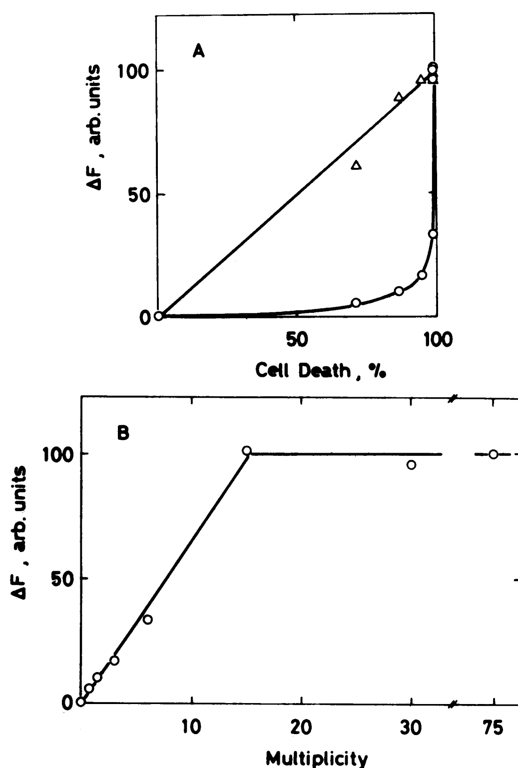

FIG. 2. Dansylation after the addition of various pyocin R1 concentrations. (A) Comparison of dansyl chloride labeling with ANS response. ANS (50 μM) was mixed with the cells 30 min after the addition of pyocin R1 at 37°C, and then the fluorescence was excited at 350 nm and measured at 510 nm at room temperature. Amplitude of fluorescence increment, ΔF, of dansyl label (○) and ANS (△) compared with the fluorescence intensity in the untreated cell suspension is plotted. Initial cell concentration was 6.0×10^8 cells/ml. (B) Dansylation as a function of multiplicity of pyocin R1. Multiplicity was calculated from the zero term of the Poisson distribution. Data were taken from (A).

TABLE 2. Effect of pyocin R1 concentration on dansylation of cell envelope

Fraction	R1 MOI[a]	F_{510}[b]
Whole cell[c]	0	20
	4	294
	28	884
Cell envelope	0	4
	4	121
	28	436

[a] MOI, Multiplicity of killing particles/cell.
[b] Fluorescence intensity (arbitrary units) at 510 nm per 10^9 cells/ml or per 0.1 mg of protein/ml.
[c] Initial cell concentration, 10^9 cells/ml.

moved by centrifugation. Similarly, the 2.5-fold increased fluorescence of perylene retained in cells treated with pyocin R1 was compared with that in untreated cells at the same survival. Otherwise, the addition of pyocin R1 to DPH-labeled cells did not cause any changes in fluorescence intensity and fluorescence polarization at 37°C.

In addition, comparison of SDS-gel electrophoresis patterns of the cell envelopes from pyocin R1-treated and untreated cells showed that no change in major protein bands of the cell envelope was detected after pyocin R1 treatment (data not shown).

DISCUSSION

Intact *P. aeruginosa* cells reacted with a small quantity of dansyl chloride and other hydrophobic probes in spite of the presence of a large number of potentially reactive sites. This shows that an outer surface of the cell envelope acts as a permeability barrier to lipophilic reagents and hydrophobic probes and prevents those agents from approaching the reactive sites. The impermeability of the outer membrane to hydrophobic agents has also been observed in intact *Salmonella typhimurium* (19, 22). The cell envelope of *P. aeruginosa* consists of outer and cytoplasmic membranes and a peptidoglycan layer between the two membranes (16, 18). From comparison of membrane composition between the two membranes, it appears that lipopolysaccharides located in the outer membrane may form a hydrophobic barrier of the cell envelope. On the other hand, the outer membrane of *P. aeruginosa* has water-filled pores with an exclusion limit of over 600 daltons for hydrophilic molecules (4, 6). However, it is unlikely that these pores permit passage of hydrophobic substances of molecular weights less than 600.

Pyocin R1 permitted movement of dansyl chloride and other hydrophobic probes across the outer surface barrier, which indicates that pyocin particles deprive the outer membrane of a barrier to hydrophobic substances. Multiplicity-dependent labeling suggests that loss of the outer membrane barrier should take place in regions where pyocin particles have adsorbed to the cell surface. Pyocin R1 particles undergo sheath contraction and core penetration across the outer membrane after attachment to the cell surface (K. Amako, personal communication). It has not been determined whether lipophilic substances penetrate the outer membrane through an interior pore of the core or leak around the area of the core penetration.

Pyocin R1-induced dansylation of ribosomes and cytoplasmic soluble proteins showed that the cytoplasmic membrane of *P. aeruginosa* as well as erythrocyte membranes and *S. typhimurium* cytoplasmic membranes were also permeable to dansyl chloride (22, 23). That the labeling of both whole cells and cell envelopes was dependent on the multiplicity of pyocin R1 suggests that dansylation of the cytoplasmic components may also depend on the multiplicity and

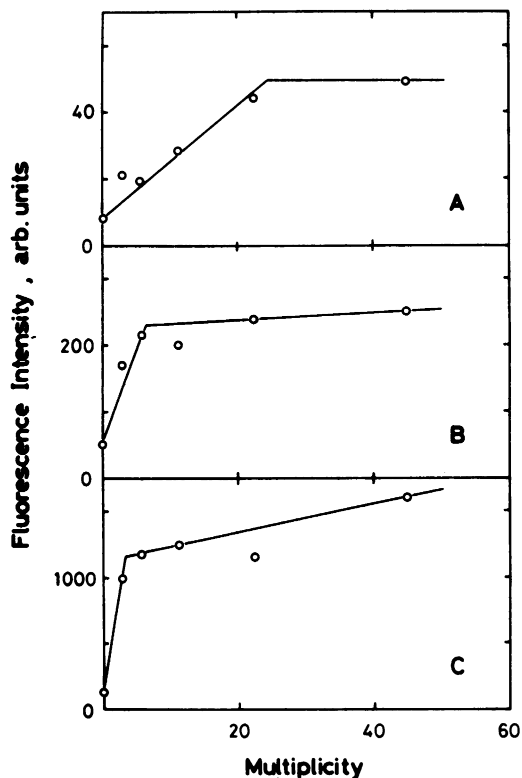

FIG. 3. Dansylation at various concentrations of dansyl chloride. Cells were dansylated at various concentration ratios after pyocin R1 treatment at different multiplicities. (A) 10 μM dansyl chloride and 6.0 × 10^8 cells/ml (1.7 nmol/10^8 cells); (B) 50 μM and 6.0 × 10^8 cells/ml (8.3 nmol/10^8 cells); (C) 50 μM and 1.2 × 10^8 cells/ml (41.6 nmol/10^8 cells). The fluorescence intensity was recalculated against 6.0 × 10^8 cells/ml.

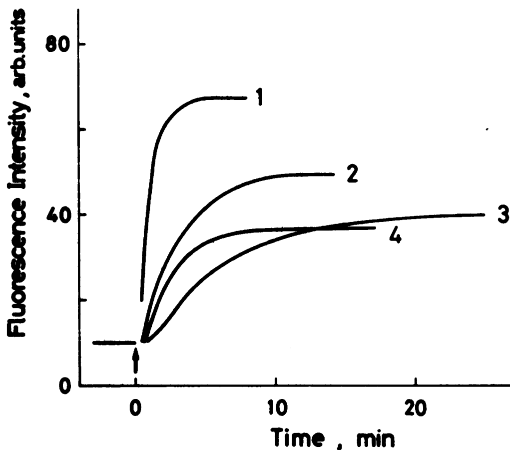

FIG. 4. Fluorescence response of hydrophobic probes after the addition of pyocin R1. Fluorescent dyes of 5 μM NPN (curve 1), 2 μM pyrene (curve 2), 1 μM perylene (curve 3), or 2 μM DPH (curve 4) were mixed with intact cells for 3 min, and then pyocin R1 was added. Excitation and measured emission were at 330 and 410 nm for NPN, at 334 and 480 nm for pyrene excimer, at 410 and 470 nm for perylene, and at 360 and 430 nm for DPH, respectively. Initial cell concentration, 5.8 × 10^8 cells/ml; cell survival, 0.2% for NPN, 2.2% for pyrene, 5.4% for perylene, and 1.5% for DPH; temperature, 37°C.

that labeling reagents may enter into the intracellular space across the cytoplasmic membrane in the vicinity of attachment spots of pyocin R1 particle.

The difference observed in labeling by ANS and dansyl chloride may be interpreted as follows. Both labels pass through the outer membrane into a periplasmic space after pyocin R1 damages the outer surface barrier. Thereafter, ANS, a noncovalent hydrophobic probe, diffuses into and nonspecifically associates with hydrophobic regions on the surface of the outer and cytoplasmic membranes. Therefore, the fluorescence response of ANS correlates with cell death. On the other hand, dansyl chloride in aqueous solution initiates two reactions: (i) dansylation with basic groups and (ii) hydrolysis producing an unreactive compound. The hydrolysis reaction may cause the localized labeling at low multiplicity. High multiplicity corresponding to the increased number of entrances allows dansylating labels to spread over the whole cell faster than hydrolysis, resulting in full dansylation in the cell. In a similar manner, the higher concentration of dansyl chloride may shift the deflection point toward lower multiplicity by reacting with all sites before hydrolysis.

The fluorescence response of hydrophobic probes used instead of dansyl chloride was rapidly caused by pyocin R1. A similar rapid response of ANS, which is presumably less hydrophobic than probes like NPN, pyrene, and DPH, has been observed after addition of pyocin R1 to sensitive cells (26). The rapid probe response reveals that the change in permeability of the outer membrane should occur soon after pyocin R1 addition, although the time course can not be confirmed by the method of dansylation. The fluorescence intensity of DPH and perylene incorporated into pyocin R1-treated cells was much larger than that into untreated cells. It shows that pyocin R1 causes an increased binding of these probe molecules to the cells. It has been recently reported that membrane deenergization by energy poison and colicin K results in the increased binding of parinaric acid and azidopyrene to the cell envelope of *E coli* (25, 27). In addition to these recent findings, results of a transition in fluorescence polarization of DPH and a deflection in fluorescence intensity of ANS around 20°C (26) indicate that hydrophobic probes are distributed in the cell envelope where the phase transition of lipid is observed (20) in spite of pyocin R1 treatment. There is no difference in fluorescence polarization of DPH and in SDS-gel patterns of major protein bands in the cell envelope before and after pyocin R1 treatment, indicating that pyocin R1 does not cause a detectable gross structural alteration in the cell envelope.

In summary, the action of pyocin R1 on the permeability barrier of the cell envelope to hydrophobic substances is as follows. Pyocin R1 particles added to sensitive cells are adsorbed to the cell surface and rapidly damage the permeability barrier of the outer membrane in the vicinity of the attachment spot to change the permeability to hydrophobic substances locally. Subsequently, these molecules cross the outer membrane into the periplasmic space, resulting in an increased binding of the molecules to the hydrophobic regions in the outer and cytoplasmic membranes. The pyocin R1-induced fluorescence increase of hydrophobic probes results from the above events on the cell envelope caused by pyocin R1.

ACKNOWLEDGMENTS

I thank T. Mizuno for his guidance on the preparation of cellular constituents and N. Wakayama for his helpful advice on the measurement of fluorescence polarization.

LITERATURE CITED

1. **Cogan, U., M. Shinitzky, G. Weber, and T. Nishida.** 1973. Microviscosity and order in the hydrocarbon region of phospholipid and phospholipid-cholesterol dispersions determined with fluorescent probes. Biochemistry **12**:521–528.
2. **Cramer, W. A., and S. K. Phillips.** 1970. Response of an *Escherichia coli*-bound fluorescent probe to colicin E1. J. Bacteriol. **104**:819–825.
3. **Cramer, W. A., P. W. Postma, and S. L. Helgerson.** 1976.

An evaluation of N-phenyl-1-naphthylamine as a probe of membrane energy state in *Escherichia coli*. Biochim. Biophys. Acta **449**:401-411.

4. **Decad, G. M., and H. Nikaido.** 1976. Outer membrane of Gram-negative bacteria. XII. Molecular-sieving function of cell wall. J. Bacteriol. **128**:325–336.
5. **Gray, W. R.** 1967. Dansyl chloride procedure. Methods Enzymol. **11**:139–151.
6. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and difinition of the permeability barrier. J. Bacteriol. **136**:381–390.
7. **Iijima, M.** 1978. Mode of action of pyocin R1. J. Biochem. **83**:395–402.
8. **Ishii, S., Y. Nishi, and F. Egami.** 1965. The fine structure of a pyocin. J. Mol. Biol. **13**:428–431.
9. **Jocobson, K., and D. Papahadjopoulos.** 1975. Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH, and concentrations of bivalent cations. Biochemistry **14**:152–161.
10. **Kageyama, M.** 1964. Studies of a pyocin. I. Physical and chemical properties. J. Biochem. **55**:49–53.
11. **Kageyama, M.** 1978. Effect of pyocin R1 on the glucose metabolism of sensitive cells of *Pseudomonas aeruginosa*. J. Biochem. **84**:1373–1379.
12. **Kageyama, M., K. Ikeda, and F. Egami.** 1964. Studies of a pyocin. III. Biological properties of the pyocin. J. Biochem. **55**:59–64.
13. **Kaziro, Y., and M. Tanaka.** 1965. Studies on the mode of action of pyocin. I. Inhibition of macromolecular synthesis in sensitive cells. J. Biochem. **57**:689–695.
14. **Lentz, B. R., Y. Barenholz, and T. E. Thompson.** 1976. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 1. Single component phosphatidylcholine liposomes. Biochemistry **15**:4521–4528.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Mizuno, T., and M. Kageyama.** 1978. Separation and characterization of the outer membrane of *Pseudomonas aeruginosa*. J. Biochem. **84**:179–191.
17. **Mizuno, T., and S. Mizushima.** 1974. Characterization of stuck ribosomes induced by neomycin *in vivo* and *in vitro*. Biochim. Biophys. Acta **353**:69–76.
18. **Nikaido, H.** 1973. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of Gram-negative cell walls, p. 131–208. *In* L. Leive (ed.), Bacterial membranes and wall. M. Dekker Inc., New York.
19. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta **433**:118–132.
20. **Overath, P., M. Brenner, T. Gulick-Krzywicki, E. Shechter, and L. Letellier.** 1975. Lipid phase transitions in cytoplasmic and other membranes of *Escherichia coli*. Biochim. Biophys. Acta **389**:358–369.
21. **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.
22. **Schindler, P. R. G., and M. Teuber.** 1978. Ultrastructural study of *Salmonella typhimurium* treated with membrane-active agents: specific reaction of dansylchloride with cell envelope components. J. Bacteriol. **135**:198–206.
23. **Schmidt-Ullrich, R., H. Knüfermann, and D. F. H. Wallach.** 1973. The reaction of 1-dimethylaminonaphthalene-5-sulfonyl chloride (DANSCl) with erythrocyte membranes. A new look at "vectorial" membrane probes. Biochim. Biophys. Acta **307**:353–365.
24. **Soutar, A. K., H. J. Pownall, A. S. Hu, and L. C. Smith.** 1974. Phase transitions in bilamellar vesicles. Measurements by pyrene excimer fluorescence and effect on transacylation by lecithin: cholesterol acyltransferase. Biochemistry **13**:2828–2836.
25. **Tecoma, E. S., and D. Wu.** 1980. Membrane deenergization by colicin K affects fluorescence of exogenously added but not biosynthetically esterified parinaric acid probe in *Escherichia coli*. J. Bacteriol. **142**:931–938.
26. **Uratani, Y., and M. Kageyama.** 1977 A fluorescent probe response to the interaction of pyocin R1 with sensitive cells. J. Biochem. **81**:333–341.
27. **Wolf, M. K., and J. Konisky.** 1981. Increased binding of a hydrophobic, photolabile probe to *Escherichia coli* inversely correlates to membrane potential but not adenosine 5′-triphosphate levels. J. Bacteriol. **145**:341–347.