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# Effects of Cations on the Outer Membrane Permeability of *Escherichia coli*

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**The outer membrane permeability to β-lactam antibiotics was determined with intact *Escherichia coli* cells treated with various concentrations of NaCl. It was found that the treatment of cells with moderate concentrations of NaCl caused dramatic increase in the diffusion rate of β-lactam across the outer membrane. This increased rate of β-lactam diffusion was not due to the high osmolarity exerted by NaCl, since the elevated osmolarity of the assay medium by sucrose had no significant effect. This increased rate of β-lactam diffusion was restored by addition of a low concentration of $MgSO_4$ or $CaCl_2$ into the assay medium. These results were interpreted as that the treatment of intact *E. coli* cells with moderate concentration of monovalent cations squeezed out divalent cations, which presumably destabilizd the outer membranes, causing electrostatic repulsion of negatively charged lipopolysaccharide molecules *in site*.**

**(Key Words: Outer membrane, Ions, Diffusion, Beta-lactam, LPS)**

## INTRODUCTION

The outer membrane of Gram-negative bacteria is located at the outer most part of the cell and functions as a barrier against the access of hazardous agents such as bile, surfactants etc (2). This barrier also protects the cells from the access of a certain kind of antibiotics including macrolide antibiotics, actinomycin D, etc (2). The barrier function of the outer membrane against small hydrophilic molecules is overcomed by the major outer membrane proteins (3,4). The exclusion limit of these diffusion pores was found to be around molecular weight (mol wt) 400 to 500 in *E. coli* and *Salmonella*. The barrier function of this membrane against hydrophobic molecules has been thought to be due to the presence of tightly packed lipopolysaccharide (LPS) and outer membrane proteins at the external leaflet of this membrane (5). In fact, the structural deletion of a part of LPS and/or quantity of the major outer membrane proteins alters the diffusion properties of this membrane against hydrophobic compounds (1).

Penicillin, an antibiotic is partially effective against Gram-negative bacteria and, hence, it has been thought that penicillin crosses the outer membrane via porin-pores in a relatively inefficient manner (7). However, the susceptibility of Gram-negative bacteria to these antibiotics was found to be somewhat influenced by the growth medium and/or the buffer used. Thus, we investigated the diffusion properties of outer membrane to

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amphipathic compound, penicillin, under various conditions.

## EXPERIMENTAL

Bacterial strain *Escherichia coli* K-12 NL1410 is used harboring R-plasmid RGN823 which produces periplasmic type I penicillinase. The Kms of penicillinase are 13$\mu$M and 10$\mu$M for benzylpenicillin (PCG) and aminobenzylpenicillin (ABPC), respectively. Cells were grown overnight in 40ml of L-broth and 24ml of this culture was diluted into 240ml of prewarmed medium. The flask was shaken (200 rpm) at 37°C for 3 hrs and cells were harvested by centrifugation. Cells were washed with desired buffered-salt solution, resuspended in the same solution, and kept at 0°C until used. A portion of cell suspension was centrifuged at 12,000xg for 10 min and the supernatant was isolated (leaked $\beta$-lactamase). Another portion of cell suspension was passed through a French pressure cell and crude extracts were obtained by centrifugation at 30,000xg for 30 min. Penicillinase activity of crude extracts was about 500 fold higher than that determined with intact cells in the medium containing 100mOs of NaCl and 10mM buffer (see below). Diffusion rate of penicillins across the outer membrane was determined essentially by the method described by Zimmermann and Rosselet (7), by the microiodometric determination of $\beta$-lactam ring hydrolysis (6). Diffusion rate of penicillin was calculated by subtracting the leaked penicillinase activity from the activity with intact cells.

## RESULTS AND DISCUSSION

Since the diffusion rate of $\beta$-lactam antibiotics was normally determined in 0.05M phosphate buffer, pH 7, we examined a possible effect of NaCl on the diffusion rate of PCG. Cells were washed two times with 5mM N-2-hydroxyethylpiperazine N′-2-ethane sulfonic acid (HEPES)-NaOH buffer, pH 7.0 containing either 100, 300 or 500mOs of NaCl and resuspended in the same buffer. Fig. 1 shows the $\beta$-lactamase activities of intact cells and that of centrifuged supernatant. The diffusion of $\beta$-lactam (intact-leakage) across the outer membrane was dramatically increased as NaCl concentration in the assay medium (as well as washing medium) was increased. The increment of penicillin diffusion at 500mOs NaCl was about 10 times as compared with that of 100mOs NaCl medium (Fig. 1A). This result suggested that high osmotic pressure exerted by added NaCl perturbed the outer membrane structure forming intramembranous cracks, though the increased diffusion rate of PCG at 300mOs (physiological osmolarity) can not be interpreted. Alternatively high ionic strength exerted by NaCl is the cause of increased permeability.

In order to test these possibilities, cells were washed with 5mM HEPES buffer pH 7.0 containing 100, 300 and 500mOs of NaCl and the total osmolarity of all solution was adjusted to 500mOs by adding appropriate amounts of sucrose. Fig. 1B shows the $\beta$-lactamase activity determined by such cells, suggesting that the osmolarity of the medium compensated with sucrose showed little effect. Therefore, it became evident that increased diffusion rate of PCG across the outer membranes treated with moderate concentration of NaCl was due to the ionic strength exerted by added NaCl.

This conclusion raises the question what happens to the outer membrane during the washing of cells with NaCl. A possibility is that NaCl squeezes out polycationic ions attached on the surface of the outer membrane forming ionic interactions with negatively charged groups, thereby decreasing the stability of the outer membrane. It is likely to occur, since the leakage of periplasmic β-lactamase was also increased as the cells were treated with moderate concentrations of salt, and the leakaged was not protected by the addition of sucrose (see Figs. 1A and 1B).

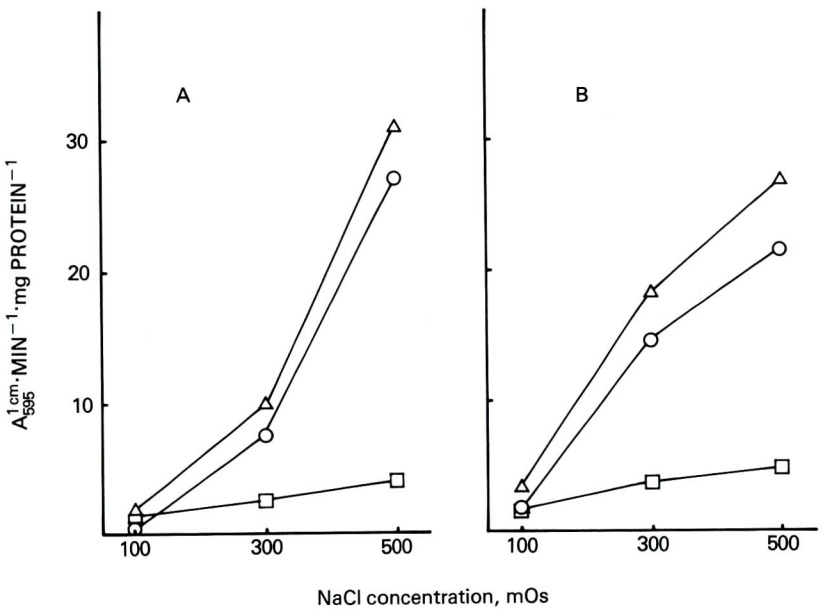


**Fig. 1** Effects of ionic strength and osmolarity on the diffusion of PCG. A, Cells were washed two times with 5 mM HEPES buffer, pH 7.0 containing 100, 300 or 500 mOs of NaCl and resuspended in the same medium. A portion of cell suspension was centrifuged at 12,000 xg for 10 min and the supernatant was isolated. Permeability rate was calculated from the β-lactamase activity in intact cells and the supernatant. B, Permeability was determined in as A, except that the osmolarity of solutions was adjusted to 500 mOs by adding appropriate amounts of sucrose.
○, diffusion rate of PCG, △, β-lactamase activity of intact cells, □, β-lactamase activity of the supernatant.

If the above assumption is correct, we should be able to lower the diffusion of PCG as well as the leakage of periplasmic penicillinase into the medium by addition of divalent cations such as $Mg^{++}$ and $Ca^{++}$. We have carried out similar experiments as Figs. 1A, and 1B, in the presence of 10 mM $MgSO_4$ and the results were shown in Fig. 2A and 2B. The diffusion of PCG and the leakage of penicillinase at relatively high concentration of NaCl was restored to levels similar to those at of low ionic concentration. Analogous results were obtained with 10 mM $CaCl_2$ instead of $MgSO_4$ (not shown). However, the effect of $MgSO_4$ for the restoration of PCG diffusion and the leakage of penicillinase was better than $CaCl_2$. Since the increased

diffusion of PCG by high ionic concentration was prevented by addition of 10mM $MgSO_4$, we wondered if the increased diffusion of penicillin once initiated could be stopped by later addition of $MgSO_4$. This possibility was tested by washing cells with 100, 300 and 500mOs of NaCl and determining the diffusion of PCG in the same buffer immediately, and in the medium containing 10mM $MgSO_4$ in addition to appropriate amounts of NaCl. Results showed that NaCl-caused increment of PCG diffusion was lowered to about one quarter of that observed with NaCl washed cells, but the diffusion could not be lowed to the level of cells washed with NaCl-$MgSO_4$ medium from the beginning.

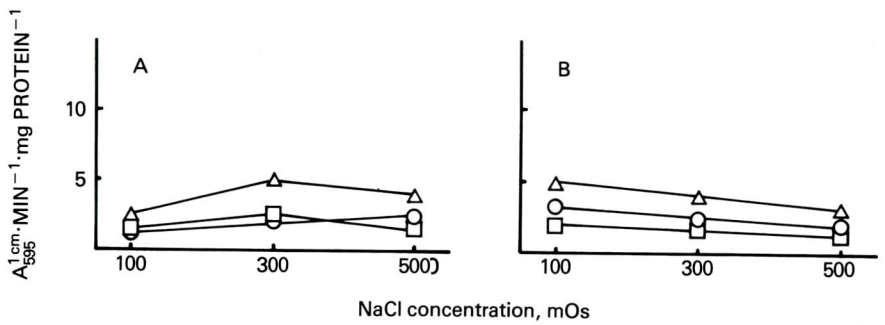


**Fig. 2** Effects of $Mg^{++}$ ions on the diffusion of PCG in the presence of NaCl. A, Experimental details were similar to legend to Fig. 1A, except that all solution contained 10mM $MgSO_4$. B, Similar to Fig. 1B except that all solution contained 10mM $MgSO_4$. Symbols are the same as in Fig. 1.

Another question is whether the increased diffusion of penicillin across the outer membranes by high ionic concentration is specific to PCG. We have tested the diffusion of ABPC under the same conditions as Fig. 1 and obtained essentially the similar results (not shown). Therefore, it is obvious that the diffusion of $\beta$-lactam antibiotics should be determined under the presence of an appropriate concentration of divalent cations even if the assay medium as well as washing medium contained physiological osmolarity of NaCl. Another question is how the diffusion of $\beta$-lactam was increased by the treatment with nearly physiological concentration of NaCl. The most likely possibility is, as mentioned above, that monovalent cations squeezed out divalent cations that crosslinked the negatively charged LPS. It is well established that a brief treatment of intact cells with a low concentration of EDTA abolishes the barrier function of the outer membrane (1). We believe that the treatment of cells with moderate concentrations of NaCl exerts a similar effect to the cells as the EDTA treatment. The final question is how PCG cross the NaCl-treated outer membrane? It is likely that the diffusion of PCG occured by dissolving in lipid domain of outer membrane, when intermolecular spaces of LPS were widen by the electrostatic repulsion of LPS molecules as a consequence of NaCl-treatment loss of divalent cations (Fig. 3). Thus, we would like point out that the present results may be applicable also as other study of outer membrane permeability in intact cells.

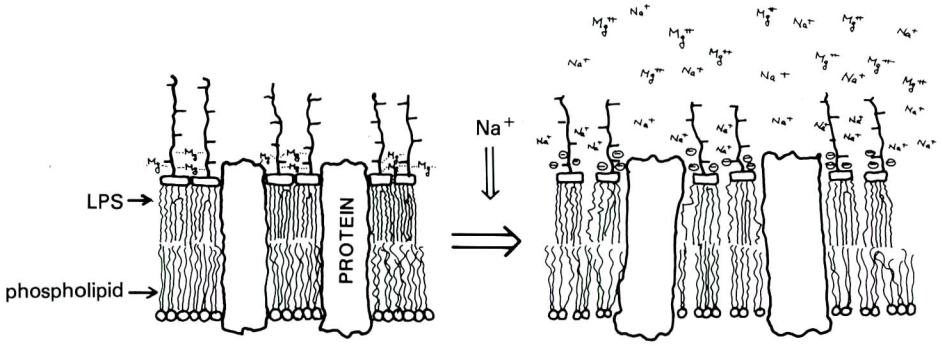


**Fig. 3** A possible structural change of outer membrane by treatment with NaCl. Detail informations were given in the text.

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