drug resistance and the finding of the large pore in this organism, the workers hypothesized that the majority of the pore in this membrane is closed in any given time (2,4,9).

Since the pore closing and opening mechanism is not presented yet, preliminary data that conflict with the presence of the large pore was presented (6), and the results of such study are clinically and pharmaceutically important, the diffusion property of this membrane must be reexamined. We present here lines of evidence that the diffusion pore in the outer membrane of P. aeruginosa is smaller than the size of disaccharide.

MATERIALS and METHODS

Bacterial strains and growth conditions P. aeruginosa PAO1 (a laboratory strain) and BMHno.1 (a fresh clinical isolate) were grown in L-broth containing 5 mM $MgCl_2$ at 37 °C for 4 h under vigorous aeration after diluting fully grown cells with a 10-fold of fresh medium. Cells were harvested by centrifugation and washed once with a solution of 150 mM NaCl in Na-Mg-PO_4. Cells were resuspended in the same solution to about 50 percent, v/v.

Treatment of cells with the hypertonic saccharides The above cell suspension (100 µl) was centrifuged at 58,000 xg for 30 sec by an air-driven microcentrifuge (Beckman) and the pellet (about 50 mg wet wt) was mixed with 100 µl of 0.6 M saccharide in 0.5 units of G6PDH (an impermeable reference solute) and Na-Mg-PO_4. The mixture was centrifuged at 58,000 xg as above (after 3 min or 6 min for the sample for electron microscopy or for the penetration experiment, respectively). The saccharides used in this experiment were not utilized by the strains used.

Other techniques Electron microscopic technique is described in the legend to Fig 1. Details for the penetration experiment is given in the legend to Fig 2 and 3. Enzyme activity of G6PDH was determined spectrophotometrically at 340 nm. A typical reaction mixture in a 1-ml cuvette contained 1 mM glucose 6-phosphate, 5 mM $MgCl_2$, 0.4 mM NADP, 50 mM Tris, pH7.5. Saccharides were quantified by the phenol-sulfuric acid reaction (10).

RESULTS and DISCUSSION

Electron microscopic observation of the cells treated with the hypertonic saccharides P. aeruginosa PAO1 cells were treated with 600 mosM ribose, α-methylmannoside, sucrose, raffinose and stachyose and the thin sections of the saccharide-treated cells were observed under an electron microscope. The electron micrographs revealed the following results. (i) The cells treated with 600 mosM raffinose or stachyose showed the extensive shrinking of the whole cells (Fig 1a and 1b), suggesting

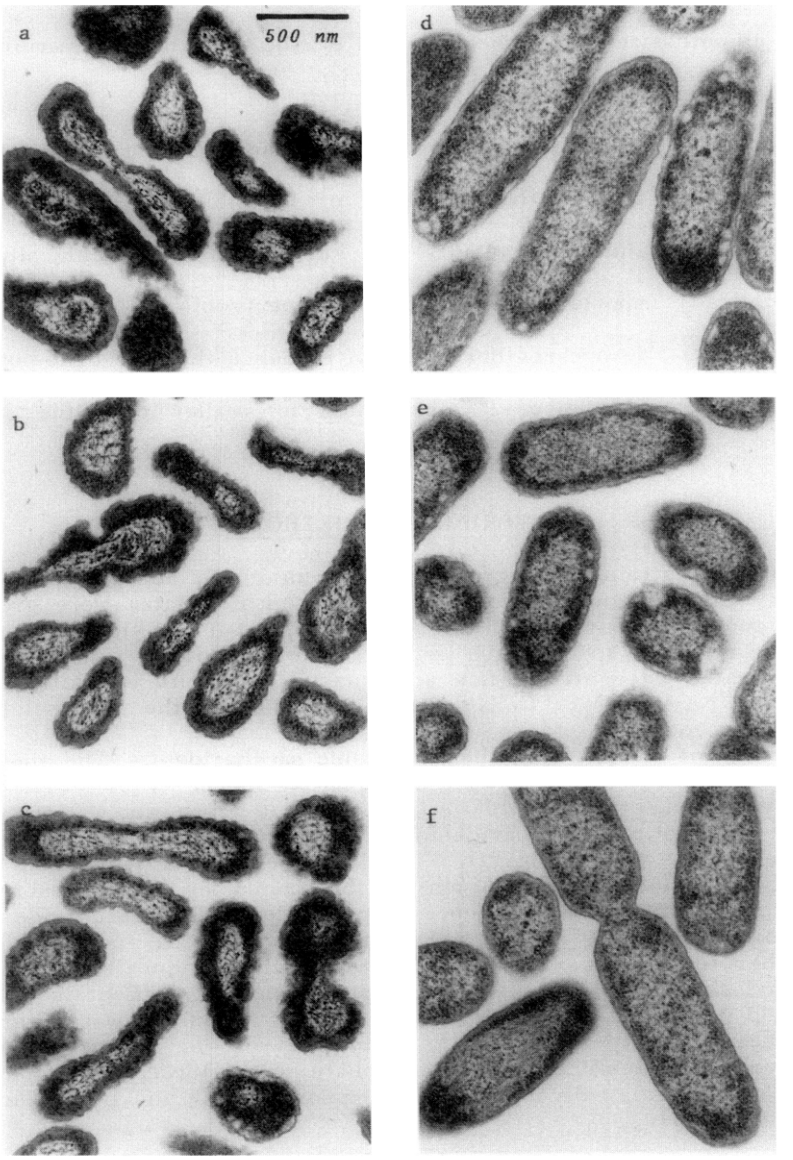


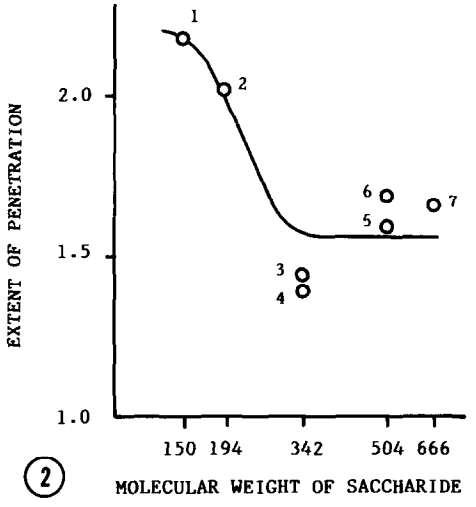
Fig. 1. Electron micrographs of the cells treated with the hypertonic saccharides. Cells of P. aeruginosa PAO1 were treated with 0.6 M saccharides as described in Materials and Methods except that G6PDH was omitted. The saccharide-treated cells (90 µl) were mixed with 10 µl of 25% of glutaraldehyde and the mixture was kept at 23°C for 15 min. The cells were centrifuged at 15,600 xg for 5 min (Eppendorf 5412) and washed 3 times with 100 mM sodium phosphate, pH7.4. The cells were treated with 1.0% of osmium tetraoxide at 23°C for 30 min and washed 4 times as above. The cells were embedded in the epoxy resin (Quetol-812) after dehydration. Thin section was made by an ultramicrotome LKB8800 (LKB) and was stained doubly with 2% of uranyl acetate and 2% of lead solution for 20 and 5 min,consecutively. Thin section was observed under a JEM-1200EX electron microscope. **a**, Cells treated with 600 mosM Stachyose. **b**, Cells treated with 600 mosM raffinose. **c**, Cells treated with 600 mosM sucrose. **d**, Cells treated with 600 mosM sucrose in the presence of 5 mM EDTA. **e**, Cells treated with 600 mosM α-methylmannoside. **f**, Cells treated with 300 mosM α-methylmannoside.

that these saccharides exerted osmotic pressure to the outer membrane. (ii) The cells treated with 600 mosM sucrose also showed the shrinking of the outer membrane but a little less than the cells treated with raffinose or stachyose (compare Fig 1c with 1a and 1b), indicating that sucrose is nearly impermeable through this membrane. A little plasmolysis was also seen. (iii) The cells treated with 600 mosM sucrose in the presence of 5 mM EDTA showed plasmolysis (Fig 1d) but no visible shrinking of the outer membrane . The results indicated that the outer membrane was permealized to sucrose by the EDTA treatment. (iv) The cells treated with ribose or α-methylmannoside showed plasmolysis (compare Fig 1e with Fig 1f), suggesting that these saccharides passed through the outer membrane and exerted the osmotic pressure to the inner membrane (the ribose treated cells appeared to be indistinguishable from the cells in Fig 1e, photograph not shown).

Penetration of saccharides into the expanded periplasmic space

Since the electron micrographs shown above clearly indicated that only ribose and α-methylmannoside penetrate through the outer membrane, an experiment was designed to confirm this result by the direct permeability assay. Cells of P. aeruginosa PAO1 were treated with 0.6 M saccharides as described above and the extent of saccharide penetration was measured (for the details of the experiment and the calculation of the solute permeability, see the legend to Fig 2). The result shown in Fig 2 revealed that only ribose and α-methylmannoside diffused significantly into the cells, so far examined. On the other hand the diffusion of disaccharides was very low compared with that of the monosaccharides.

In order to confirm the above results using a non-laboratory strain of P. aeruginosa, cells of a clinical isolate were plasmolyzed with 600 mosM NaCl and the penetration of the above described saccharides into the NaCl-expanded periplasm was examined. The result demonstrated that only ribose and α-methylmannoside are diffusible across the outer membrane (Fig 3). Di-, tri- and tetrasaccharides stayed out of the cells. The



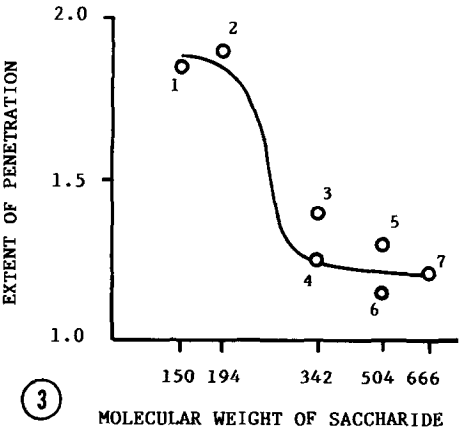


Fig. 2. Penetration of saccharides into the periplasm of the cells treated with the hypertonic saccharides. Cells of P. aeruginosa PAO1 were treated with various sizes of saccharides as described in Materials and Methods. The saccharide-treated cells were centrifuged at 58,000 xg for 26, 28, 36, 58 and 180 sec for pentose-, hexose-, disaccharide-, trisaccharide- and tetrasaccharide-treated cells after standing at 23° C for 6 min. The different time of centrifugation is an attempt to adjust the buoyant density of the saccharide solution, though centrifuge force during acceleration and deceleration of the rotor was not corrected. The pellet was resuspended in 100 µl of Na-Mg-PO_4 and was centrifuged again at 58,000 xg for 1 min after standing at 23°C for 3 min. Saccharides and G6PDH in the 1st and the 2nd centrifuged-supernatants were quantified as described above and the extent of the solute penetration was calculated as

$$[(\text{saccharide/G6PDH})_{2\text{nd sup}} / (\text{saccharide/G6PDH})_{1\text{st sup}}] \times 100.$$

The impermeable and permeable solutes show these values as 1 and over 1, respectively. G6PDH was neither permeable across the outer membrane nor inactivated during the experiment as was confirmed by the corecovery of G6PDH and Ficoll 400 (M_r, 400,000) in the mixed experiment. Saccharides used were ribose (1), α-methylmannoside (2), sucrose (3), cellobiose (4), raffinose (5), maltotriose (6), and stachyose (7).

Fig. 3. Penetration of saccharides into the periplasm of the cells plasmolyzed with a hypertonic sodium chloride. Cells of P. aeruginosa BMHno.1 were mixed with 30 µl of 0.8 M NaCl in Na-Mg-PO_4 and were kept at 23°C for 3 min. To this was added 30 µl of 150 mM saccharide-0.22M NaCl-0.5 units of G6PDH in Na-Mg-PO_4 and the penetration experiment was carried out after 3 min as described in the legend to Fig 2, except that the cells were centrifuged for 30 and 60 sec for the 1st and the 2nd centrifugations, respectively. Saccharides used were the same to Fig 2.

results obtained in the penetration experiments by using the different plasmolyzing agents and two strains of P. aeruginosa consistently showed that only pentose and hexose among the saccharides tested were penetrable across the outer membrane of P. aeruginosa. These results fully support the findings in the electron micrographs of the saccharide-treated cells. Thus, it became evident that the outer membrane of P. aeruginosa allows

the penetration of pentose and hexose but the membrane does not allow the penetration of saccharides larger than disaccharides.

Decad and Nikaido (5) showed that the outer membrane of P. aeruginosa is highly permeable upto the polysaccharides of M_r 3,000 to 7,000. We are unable to confirm their result. Instead, we found the exclusion limit of the membrane as the size of saccharide larger than M_r, 194 but less than M_r, 342. The presence of the small pore is not limited to a laboratory strain but is also confirmed in a clinical isolate of P. aeruginosa.

Caulcott et al reported (6) that the outer membrane of P. aeruginosa is only permeable to the size of sucrose by the efflux experiment. Though the conclusion derived from this experiment was consistent with that of the present study, this experiment conceive the following difficulties. (i) Sucrose was used as a permeable reference solute. (ii) The experimental procedure required a centrifugation of the isotope-entrapped cells in the absence of the labeled solutes before the efflux experiment. A time required for this process is long enough to allow the exit of the permeable solute across the outer membrane (Yoneyama, H., and Nakae, T., unpublished data).

The present study visualized the permeability of the outer membrane of P. aeruginosa and found that the exclusion limit of the membrane is less than the size of disaccharide but more than the size of hexose. This conclusion is consistent with a poor permeability of this membrane to antibiotics (1). We believe that this information is of clinical and pharmaceutical interests.

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