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Abstract

Staphylococcus aureus growing in a normal NaGI medium has a specific NaGI tolerance property to grow in the medium contain. ing NaGl in as high a concentration as over 10%. In our comparative study of the cells proliferating in the normal NaGI medium and 10% NaGl medium, we have observed the following differences aside from the changes of lipid composition in the cytoplasmic membrane previously reported. 1. S. aureus grown in high NaGl medium undergoes changes as to increase its size and reduce its surface area. 2. The thickness and weight of cell wall are increased to about 1. 7 times and 1. 32 times, respectively. 3. The protoplast prepared from S. aureus growing in the high NaGI medium shows a weaker resistance to hypotonic condition than that from normal cell.

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ULTRASTRUCTURAL ALTERATION OF THE CELL SURFACE OF STAPHYLOCOCCUS AUREUS CULTURED IN A DIFFERENT SALT CONDITION

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Abstract: *Staphylococcus aureus* growing in a normal NaCl medium has a specific NaCl tolerance property to grow in the medium containing NaCl in as high a concentration as over 10%. In our comparative study of the cells proliferating in the normal NaCl medium and 10% NaCl medium, we have observed the following differences aside from the changes of lipid composition in the cytoplasmic membrane previously reported. 1. *S. aureus* grown in high NaCl medium undergoes changes as to increase its size and reduce its surface area. 2. The thickness and weight of cell wall are increased to about 1.7 times and 1.32 times, respectively. 3. The protoplast prepared from *S. aureus* growing in the high NaCl medium shows a weaker resistance to hypotonic condition than that from normal cell.

It is well known that *Staphylococcus aureus* (to be abbreviated *S. aureus*) shows a strong resistance to a high concentration of NaCl and it can grow in the medium containing NaCl in as high a concentration as 15% (1). Furthermore, we have recognized that *S. aureus*, despite proliferating in a high NaCl medium, has maintained constancy of Na^+ and K^+ concentrations within the cell. Consequently, when *S. aureus* is cultivated in the medium of a high NaCl concentration, it must adapt itself to acquire some special defense mechanism to high salt and high osmotic pressure in order to proliferate. Therefore, it seems only natural to consider that for the adaptation to environments changes in the cell wall as well as in the structures and functional changes of the cell membrane should play an important role. In our observations of changes in the lipids, the main components of the cell membrane, we have observed that cardiolipin contained is only a few per cent of that in normal *S. aureus*, where it is increased to over 50% in the 10% NaCl medium, as we reported elsewhere (1). However, it is difficult to understand why the adaptation to such a great change in NaCl concentration as 14 times can be compensated by this phenomenon alone. In order to elucidate this problem we have studied changes in the cell wall of *S. aureus* mainly from

morphological aspects and give some consideration on its physiological state.

MATERIALS AND METHODS

Microorganism and Culture Method: *S. aureus* 209P was used and the semi-synthetic medium containing a small amount of peptone and yeast extract was used for cultivation (1), occasionally supplemented with 0.05% NaCl (to make its final concentration 145 mM Na^+ equivalent to physiological saline solution when the total medium components are taken into consideration) or 10% NaCl (to make 1,900 mM Na^+). The former medium was designated as normal medium and the latter as 10% NaCl medium. The culture was conducted by gentle shaking with Monod's tube at 37°C, and the checking of bacterial proliferation was done by measuring the absorbance at 650 nm. *S. aureus* grown in normal medium was called as N-Staph and that grown in 10% NaCl medium as 10-Staph.

Preparation of protoplast: *S. aureus* harvested at late log phase was washed thoroughly with 0.8 M sucrose-50mM Tris-HCl buffer (pH 7.2), and was treated with L-11 enzyme (cell wall lytic enzyme purified from *Flabobacterium sp.* which was offered by Prof. KOTANI of Osaka University) for preparation of protoplast (3, 4). The details of procedure are shown in the legend of Fig. 5.

Analysis of Cell Wall: Twenty ml of suspension containing 300 mg late log cells/ml in 50 mM Tris-HCl buffer (pH 7.2) were shaken with 40 ml of glass beads (0.1 mm in diameter) in a Vibrogen Cell Mill (Edmund Bühler) for 30 min. The glass beads and the remaining intact cells were removed by centrifugation at 1,400xg for 5 min. The cell wall fraction was collected by centrifugation at 10,000xg for 25 min. Contaminating cytoplasmic debris was digested with trypsin and RNase. For amino acid analysis of cell wall, 10 mg sample hydrolyzed with 6 N HCl in sealed tube at 110°C for 16 hrs was analyzed on a Nihon Denshi JLC-6AH amino acid analyzer (5). Phosphorus content of cell wall was determined by the method of KATES (6).

Morphological Observation: For the observation of bacteria and protoplasts, a phase-contrast microscope was used. In order to stop the brawnian movement of specimens during the observation, the sample was placed on 0.3% agar containing salts of a suitable concentration for the purpose. For the observation with a scanning electron microscope, the specimen was prepared by TAWARA's method (7) after OsO_4 vapor fixation and was observed with a JSM-UIII scanning electron microscope at 25 KV accelerating voltage. For the observation of thin sections, the sections were prepared by 1% OsO_4 fixation and observation was carried out as it was or after uranyl acetate staining in Hitachi electron microscope Hu-11.

RESULTS

Morphological Observation: In comparing the growth curves between N-Staph and 10-Staph (Fig. 1), the doubling time of 10-Staph was prolonged and

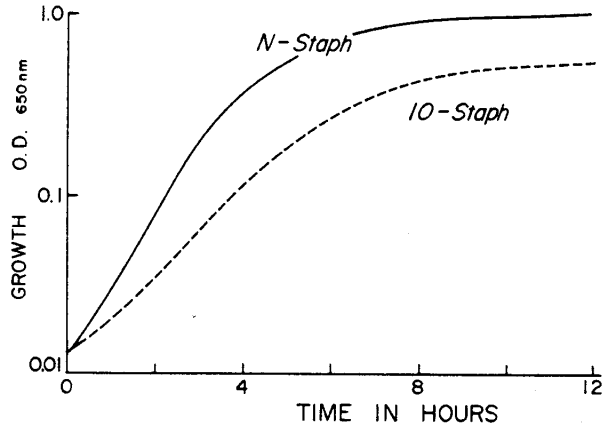

Fig. 1. Comparison of the growth rates in the semi-synthetic media with 0.05% or 10% NaCl. Cultures were carried out with gentle shaking at 37°C. Optical density at 650 nm was followed as a function of culture time.

the growth maximum was somewhat less than that in case of N-Staph. In addition, 10-Staph was somewhat viscous and its orange color was marked as compared with N-Staph.

Fig. 2 shows the results of phase-contrast microscopic observation on the two groups of *S. aureus* just at a late log phase, indicating the difference in

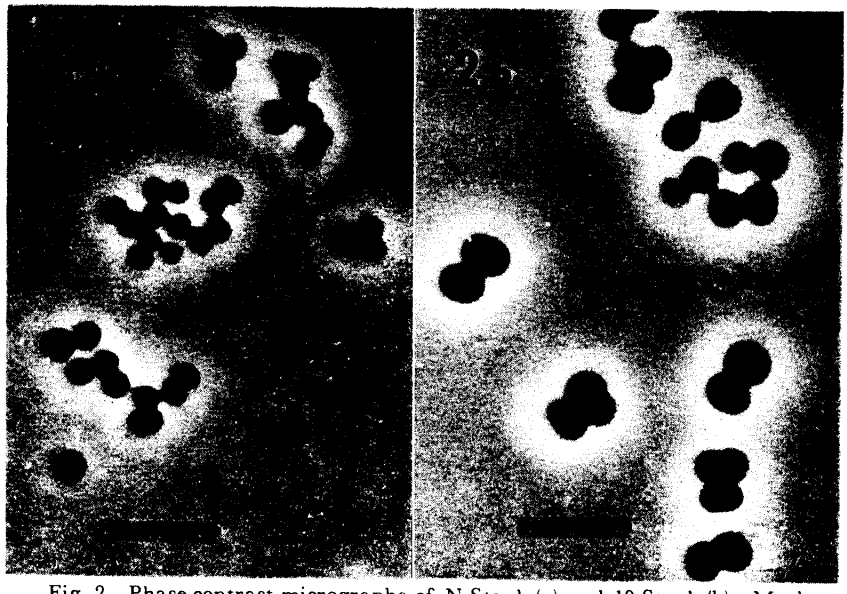

Fig. 2. Phase-contrast micrographs of N-Staph (a) and 10-Staph (b). Markers indicate 5 μm.

their size. Scanning electron microscopic observations were also carried out to see details of their structures as shown in Fig. 3. There could be observed

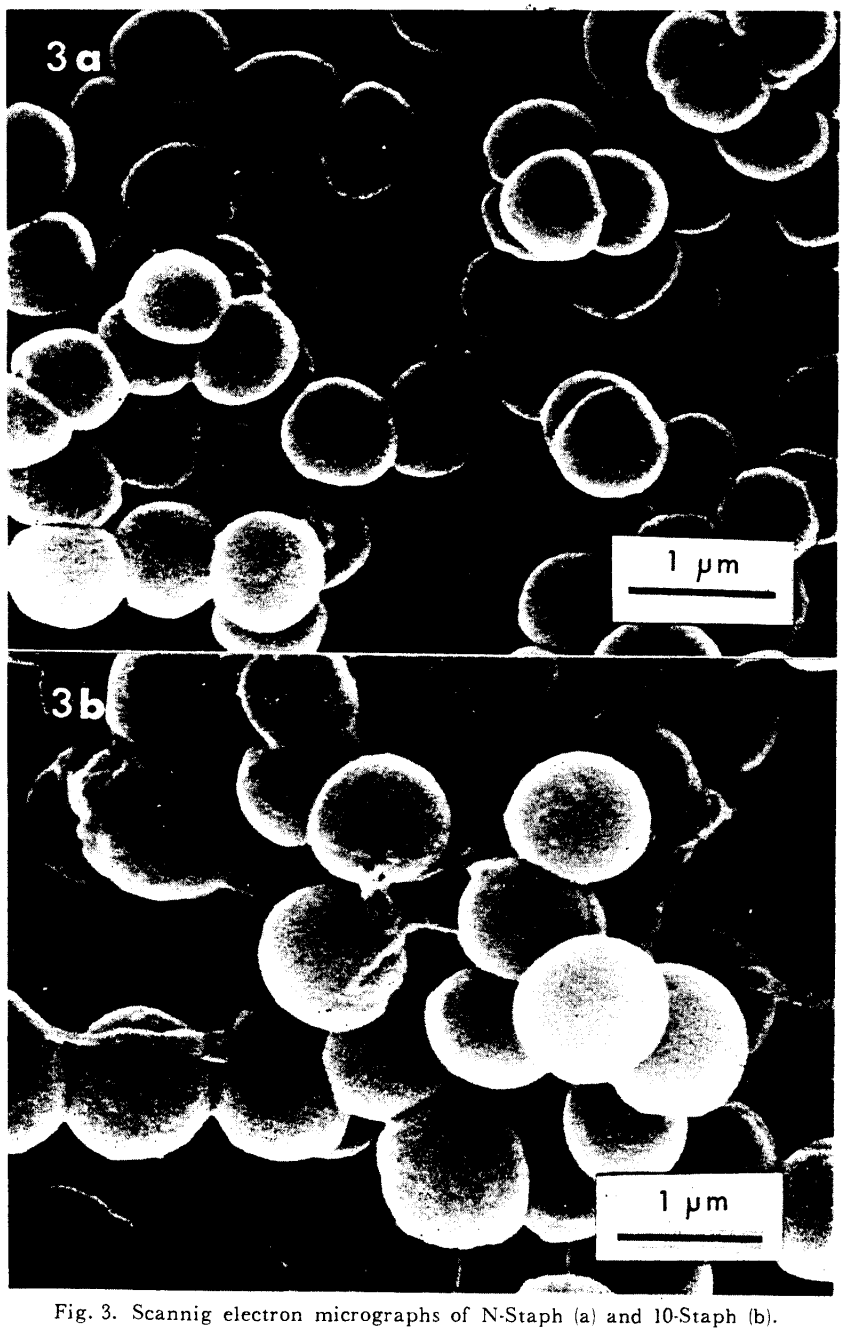

Fig. 3. Scannig electron micrographs of N-Staph (a) and 10-Staph (b).

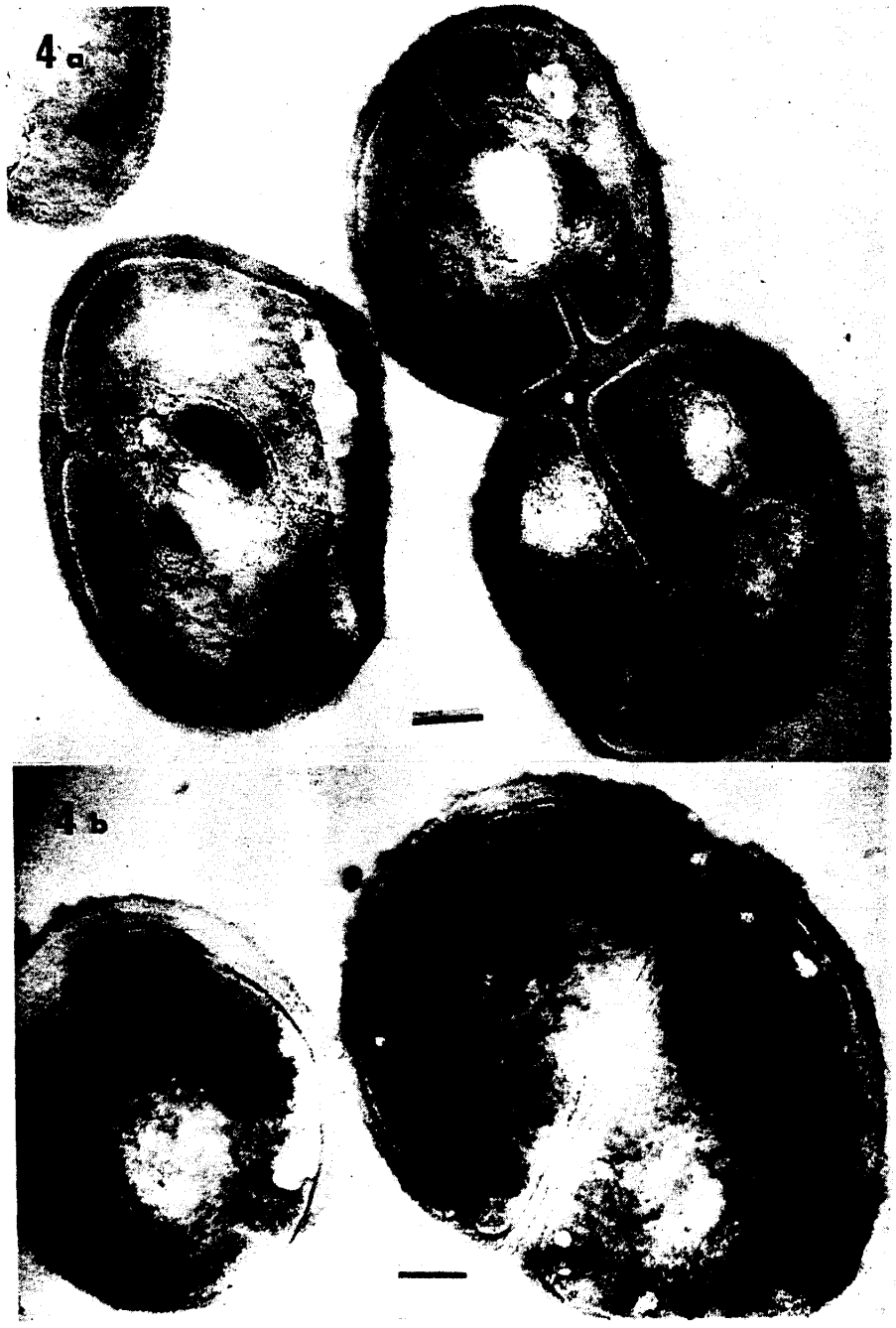

Fig. 4. Thin section of N-Staph (a) and 10-Staph (b). Markers indicate 100 nm.

no difference in the shape, manners of cell division and surface structure. But there was a distinct difference in their size; namely, the diameter of N-Staph was 0.81 μm in average while that of 10-Staph was 1.04 μm. Moreover, in the intercellular space of 10-Staph, there was a slime substance thought to be the source of their viscocity.

Next, intracellular structures of the two groups were compared with thin sections. As shown in Fig. 4, while there was no marked difference in the thickness of cell membrane, the cell wall of N-Staph was about 22 nm thick and that of 10-Staph about 38 nm. In addition, 10-Staph had an irregular cell wall with a slime substance on its surface. As for intracellular structure, cytoplasm of 10-Staph revealed an electron lucent structure on its periphery.

Reactivity of S. aureus to L-11 Enzyme: Fig. 5 illustrates the time course of loss of optical density (OD) by the cell wall lysis of the two groups of *S. aureus* when they were exposed to L-11 enzyme. Despite the fact that the

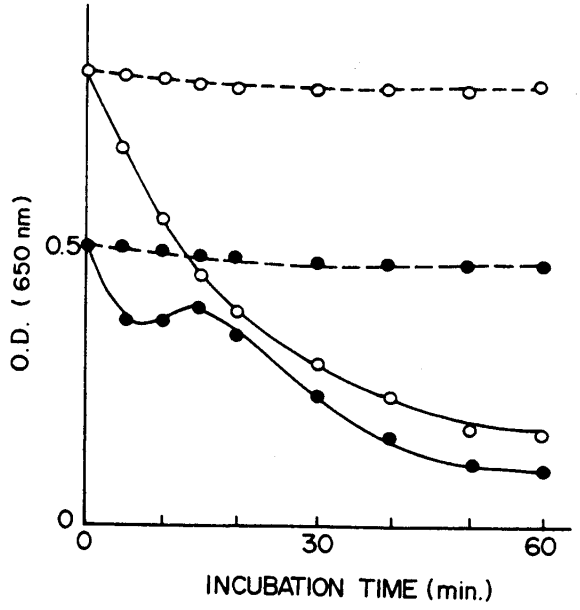

Fig. 5. Change of optical density after incubation with L-11 enzyme. ○---○ N-Staph without enzyme; ○——○ N-Staph with enzyme; ●---● 10-Staph without enzyme; ●——● 10-Staph with enzyme. Cells and enzyme were added to 50 mM Tris-HCl buffer (pH 7.2) containing 0.8 M sucrose, to give 6 mg/ml and 1 unit/ml at final concentration, respectively. The incubation was carried out at 37°C in a shaking water bath. Optical density was measured at 650 nm in a Spectronic 20 (Shimadzu).

experiments were started at the same weight (6 mg wet weight/ml) there was a considerable difference in their OD. This finding can be interpreted as

due to the difference in their cell sizes. When N-Staph was exposed to L-11 enzyme, there occurred a gradual decrease in the OD which reached plateau within about 60 minutes. In the case of 10-Staph, the down-slope had a shoulder which rose in OD once at about 10 minutes after incubation. In other words, there was a difference between the two groups at the time when the cell wall seemed to have undergone half lysis.

Analysis of Cell Wall: When the cell wall weight of N-Staph and 10-Staph was compared with the same dry weight of cell, it was found to be 13.6% and 15.8% respectively (Table). Phosphorous content in cell wall fraction from

TABLE ANALYSIS OF CELL WALL OBTAINED FROM N-STAPH AND 10-STAPH

		N-Staph	10-Staph
Wall % to dry cell (w/w)		13.6	15.8
Phosphorous content (μmoles/mg of dry wall)		1.31-1.33	0.94-1.01
Molar ratio of amino acids	Glycine	4.60	4.28
	Alanine	2.41	2.26
	Lysine	1.05	1.12
	Glutamic acid	1.00	1.00
	Serine	0.29	0.22
	NH_3	24.2	2.40

N-Staph was slightly more that from 10-Staph. The results of amino acid analysis of wall are also shown in Table. Here hardly any significant difference due to culture condition could be seen.

Resistance of Protoplast to Osmotic Shock: The resistance of protoplast prepared with L-11 enzyme to osmotic shock was observed under the sucrose system (Fig. 6). The protoplasts of N-Staph maintained their shape intact in 0.25 M sucrose but they burst below the osmotic pressure of 0.2 M as the limit, while the protoplasts of 10-Staph burst at 0.3 M as the limit, showing the latter to have the cell membrane of a weaker resistance to hypotonicity.

DISCUSSION

In view of the fact that *S. aureus* can proliferate in a high NaCl containing medium (1) and cation content in the growing cell is kept constant (2), barrier mechanism on the cell surface or an accelerated active transport system seems to be involved. In addition, *S. aureus* would require adaptability to maintain its shape in normal state under an abnormally high osmotic pressure. *S. aureus*, cultured under a high NaCl condition, showed a marked change in the membrane lipid, especially an extreme increase of cardiolipin, which would play

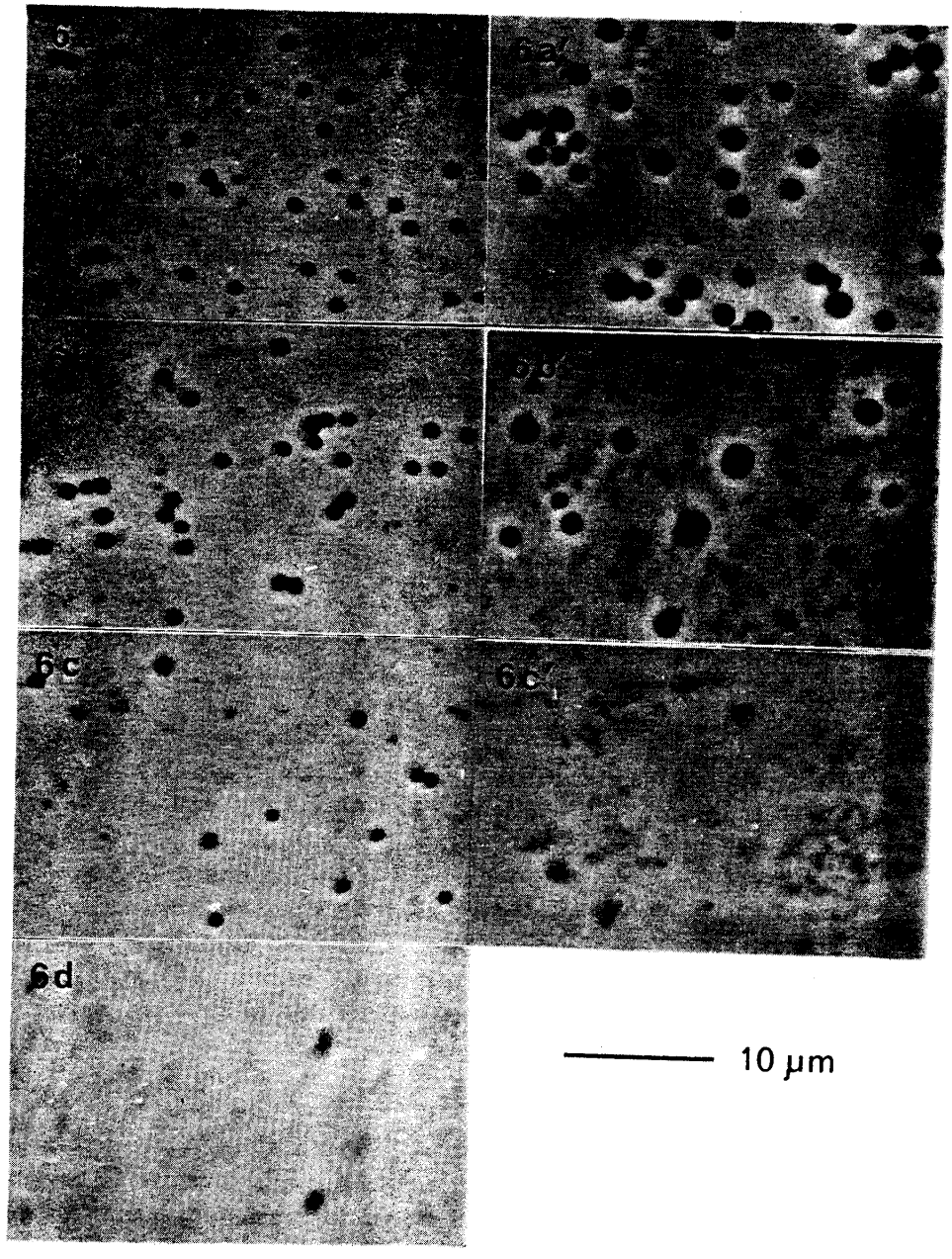

Fig. 6. Osmotical fragility of protoplasts derived from N-Staph (a, b, c, d) and 10-Staph (a', b', c'). The resistance of protoplasts on hypotonicity was checked under sucrose system in phase-contrast microscopy. a and a' show protoplasts in 0.8 M sucrose: b and b' in 0.4 M sucrose: c and c' in 0.25 M sucrose: d in 0.2 M sucrose.

a role in the NaCl tolerance mechanism (1). However, the adaptability to an enormously different environment cannot be met with one phenomenon alone from the general aspect of biological phenomena.

In the present experiment, it has been demonstrated by both the phase-contrast and scanning electron microscopy that *S. aureus* cultured in 10% NaCl medium revealed an increase to 1.28 times in its cell size. Moreover, the micrographs revealed only an increase in the cell size but no distortion of its spherical shape nor any abnormal interruption of cell division. When the bacterial diameter reaches 1.28 times, the volume increases to 2.09 times the original and the relative surface area (the surface area/its volume) reaches 1/1.28. This phenomenon seems to represent a defense mechanism to escape as much from the high NaCl condition as possible. In the gravimetric analysis of the cell wall, the wall quantity of 10-Staph proved to be 1.16 times (15.8%/13.6%) that of N-Staph. This increase coincides approximately with the wall increase to 1.32 times as calculated from the sizes of cell and wall in micrographs. This thickening of the cell wall seems to be adaptive changes as a non-specific barrier to preserve the cell structure against high osmotic pressure. As to the difference in the intracellular structures, there could be observed numerous electron transparent structures in the periphery of the cytoplasm of 10-Staph, but it was impossible to clarify whether these were mesosomes or other structures (8). As for the membrane itself, there was no difference in its thickness by electron microscopic observation.

Cho *et al.* stated that *Halobacterium halobium* growing in high NaCl medium revealed some electron-dense strands inside the cytoplasma running parallel to the cell envelope (9), and Kirk *et al.* reported that *Halobacterium* from the Dead Sea showed hexagonal patterns within layers of the cell envelope (10). But in our experiments of freeze etching, we have observed no pictures comparable to theirs with N-Staph and 10-Staph.

Finally, we present some physiological considerations on the protoplast. We have observed that the protoplast from 10-Staph grown in the hypertonic medium as 1,900 mM of NaCl has a property much weaker to resist hypotonicity than normal cell. The question whether this is due to the difference in the substances contained in protoplast or due to the membrane components has not been clarified. An 0.8 M sucrose solution was used as a protoplast stabilizer in our experiments, but Futai *et al.* (11) reported that this 0.8 M sucrose is slightly hypotonic when considered from the intracellular pressure of *Staphylococcus*. As shown in Fig. 4, there is seen a using phenomenon on the way to decreasing the turbidity of 10-Staph by the wall lysis with L-11 enzyme. This phenomenon might be interpreted that a slightly hypotonic solution has affected especially 10-Staph, and the cell has swollen, making

turbidity temporarily great when the wall has reached half lysis stage (12).

These findings suggest that the NaCl tolerance mechanism of *S. aureus* consists not only of the inhibition of passive transport of Na^+ by changes in the membrane lipid composition but also of the role of a marked thickening of the cell wall serving a nonspecific barrier and a guardian of the cell structure.

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