THE EFFECTS OF WASHING TREATMENTS ON THE COMPOSITION OF STAPHYLOCOCCUS AUREUS

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Summary

Cells of Staphylococcus aureus grown in a medium of water activity ($a_w$) 0·993 were washed in water and in solutions of sucrose, glycerol, NaCl, KCl, NH₄Cl, and MgSO₄ of 0·993 $a_w$. Retention of sodium and potassium and loss of ultraviolet-absorbing compounds were measured. Retention of sodium was greatest after washing in water or in glycerol solution, while potassium and ultraviolet-absorbing compounds were largely retained in all of the washing treatments. With cells grown in a medium of 0·92 $a_w$, washing in solutions of sucrose or NaCl resulted in leakage of potassium and ultraviolet-absorbing substances if the $a_w$ of the washing solution exceeded 0·94. Butanol treatment revealed fixed anions within the cell with equal affinity for sodium and potassium ions. The negative changes in crude cell wall preparations also showed no differentiation between these cations. These results are discussed and compared with data obtained previously for Salmonella oranienburg.

I. INTRODUCTION

It has been shown (Christian 1958) that washing cells of the Gram-negative Salmonella oranienburg in hypotonic solutions of both electrolytes and non-electrolytes removed much of the cell potassium and ultraviolet-absorbing compounds. Sodium also was removed by washing, but the amount lost was determined less by the tonicity than by the nature of the solute, the loss being greatest in electrolyte solutions and least in water and in glycerol solution. Washing in solutions of low pH increased loss of potassium and ultraviolet-absorbing compounds but decreased loss of sodium.

This paper reports the effects of washing on the composition of a Gram-positive organism, Staphylococcus aureus. Marked differences were again found between sodium and potassium retention. The extent and specificity of adsorption of these ions to sites within the cell and in the cell wall were determined. It is concluded that most of the sodium and potassium in cells of Staph. aureus are not adsorbed to fixed ions but are retained by a structural barrier with very different permeabilities to sodium and potassium.

II. METHODS

The test organism was Staph. aureus 49/1974, which was one of the strains whose water requirements for growth were reported by Scott (1953). Cells were grown at 30°C in brain–heart infusion broth of water activity ($a_w$) 0·993. Aeration was by shaking. Cultures in the stationary phase were centrifuged and the cells resuspended in the spent medium at a concentration of about 100 mg wet cells per millilitre. Replicate 5-ml samples were again centrifuged, the pellets drained, and the tube walls blotted dry. These pellets were used in subsequent experiments.

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EFFECTS OF WASHING ON A STAPHYLOCOCCUS 325

Dry weight was determined after drying for 20 hr at 100°C. Phosphate was used to estimate intercellular space by the method of Conway and Downey (1950), and the cell water was calculated by subtracting intercellular from total water. Knowledge of the intercellular space also allowed the correction of unwashed pellet analyses for contaminating growth medium.

In washing experiments, cells were resuspended in 5 ml of water or of 0.993 \( a_w \) solutions of sucrose, glycerol, NaCl, KCl, NH\(_4\)Cl, or MgSO\(_4\). After centrifugation, the absorption of suitable dilutions of the supernatants were measured at a wavelength of 256 m\( \mu \). The centrifugates were extracted with cold trichloroacetic acid and the extracts analysed for sodium and potassium by flame photometry. Phosphate was estimated by the method of Fiske and Subbarow (1925). The results are expressed in terms of the initial dry weight of cells.

The pH of a suspension of cells in spent growth medium was close to 7.0. The effects of washing in various solutions at pH 4.5, 6.0, and 7.0 were determined by the methods described previously (Christian 1958).

Butanol treatment consisted of washing cells in water, then in 5 ml 5% (v/v) aqueous n-butanol solution, and again in water.

Isolated cell walls were prepared by shaking aqueous cell suspensions with No. 12 ballotini beads at 1°C. Changes in viable count, turbidity, and Gram stain were followed. Appreciable loss of Gram-positive reaction was observed after 1 hr when the light scattering and viable count of the suspension had fallen to 30 and 28% respectively. After shaking for 4 hr (turbidity and viable count 8 and 0·04% respectively) the residue was centrifuged and washed twice in distilled water. The yield of crude cell wall material was 12·9% of the initial cell dry weight.

III. Results

(a) Sodium and Potassium Content of Unwashed Cells

The intercellular medium in unwashed cell pellets contained 154 m-equiv. sodium and 25 m-equiv. potassium per litre. The volume of intercellular medium was 1·55 ml/g dry wt. When whole pellet analyses were corrected for these intercellular contributions, the cells contained 9·6 \( \mu \)-equiv. sodium and 108 \( \mu \)-equiv. potassium per 100 mg dry weight.

During a single washing in 5 ml solution, the intercellular medium was diluted by a factor of about 20. The carry-over of intercellular medium was calculated as 1·4 \( \mu \)-equiv. sodium and 0·2 \( \mu \)-equiv. potassium per 100 mg dry weight. Hence, if no sodium or potassium was lost from the cells during a single washing the washed pellet would contain 11·0 \( \mu \)-equiv. sodium and 108·2 \( \mu \)-equiv. potassium per 100 mg dry weight.

The intracellular water was 1·61 ml/g dry weight, within the range quoted for this organism by Mitchell and Moyle (1956). Thus the internal sodium and potassium represent concentrations of 60 and 670 m-equiv/l cell water respectively, assuming that these ions are in a homogeneous solution. The ratios of internal to external concentrations were 27 for potassium and 0·4 for sodium, showing that the
cells maintain appreciable gradients across their boundaries, accumulating potassium and excluding sodium.

(b) Influence of the Washing Solute on Contents of Cells

The amounts of sodium retained in the cell pellet after one washing at pH 7.0 in various solutions (0.993 \(a_w\)) and in water are shown in Figure 1(a). Cells washed in water or in glycerol solution lost little of their initial sodium, but some 60% was removed by one washing in electrolyte solution.

Fig. 1.—Effect of washing at pH 7.0 in solutions of 0.993 \(a_w\) and in water on the sodium (a) and potassium (b) content of cells of Staph. aureus grown in brain-heart broth (0.993 \(a_w\)). Values are per 100 mg dry weight of cells.

(c) Optical density at 256 m\(\mu\) of washings after dilution to one-tenth.

Figure 1(b) shows that little or no potassium was lost from cells during any treatment. The largest loss, in NH\(_4\)Cl solution, was less than 10%. Loss of ultraviolet-absorbing compounds was similar in all treatments (Fig. 1(c)). The greatest leakage was found in MgSO\(_4\) solution, which also allowed greatest loss of sodium.

(c) Effect of pH

After washing in various solutions of pH 4.5, 6.0, and 7.0, retention of sodium was lowest at 6.0 in water and non-electrolyte solutions, but showed a slight increase in electrolyte solutions as the pH was reduced from 7.0 to 4.5. On the other hand, potassium retention was highest at pH 6.0 in all solutions and fell at pH 4.5. Loss of ultraviolet-absorbing compounds was not markedly affected by pH.
EFFECTS OF WASHING ON A STAPHYLOCOCCUS

Effect of Repeated Washings

(d) Effect of Repeated Washings

When cell pellets were washed only once, the result may have been influenced by the carry-over of constituents from the growth medium. Several pellets were, therefore, washed three times in water or in 0·993 aw sucrose solution, and pellets taken after each washing were analysed for sodium and potassium. The results are shown in Figure 2. Some sodium was lost during each washing, but at any stage much less sodium was retained by sucrose-washed than by water-washed cells. While the potassium content was little affected by water washing, the second and third sucrose treatments led to some loss. When the logarithms of sodium contents of cells washed up to four times in water or in solutions of sucrose, KCl, or NH₄Cl were plotted against the number of washings the curves were linear, and extrapolated to values between 9·9 and 11·2 μ-equiv/100 mg. These values are close to the calculated initial sodium content.

(e) Effect of aw of the Washing Solution

The results of Figure 1 showed that cells grown at 0·993 aw did not lose potassium when washed in water. To determine whether a greater osmotic shock would release cell constituents, cells grown at 0·92 aw in brain–heart broth plus NaCl were washed in NaCl solutions over the range 0·90–1·00 aw. Figure 3 shows that loss of both potassium and ultraviolet-absorbing compounds commenced when the aw exceeded 0·94. Similar curves were obtained when NaCl was replaced by sucrose in the washing solutions. Thus these cell constituents were retained by a barrier whose properties could be altered by osmotic stress.
Effect of Butanol on Retention of Solutes

Since it appeared that an osmotically sensitive barrier was concerned in retention of potassium and ultraviolet-absorbing compounds, it was of interest to determine how destruction of this barrier affected retention of cell solutes. Cells were washed in aqueous solutions of n-butanol of concentrations from 0 to 5% v/v. The ultraviolet absorption of the supernatants were estimated and after washing in 5 ml of water the amounts of sodium, potassium, and phosphate remaining in the cells were determined. The results are shown in Figure 4. Gross leakage of all four constituents commenced when the butanol concentration approached 4%. Hence the same or a very similar structure was responsible for the retention of these substances. This structure is presumed to be the plasma membrane (Mitchell and Moyle 1956). However, the proportions of sodium, potassium, and phosphate lost on treatment with 5% butanol differed widely, being 51, 69, and 91% respectively.

The data of Figure 1 show that much more sodium was lost from cells washed in electrolyte solutions than from those washed in water, suggesting an exchange of cations. This effect of electrolytes was examined further in a washing experiment using cells with undamaged and damaged membranes. Replicate cell pellets were washed in water or in 5% butanol, then in 0·993 a_w solutions of sucrose or NH_4Cl. The amounts of sodium and potassium retained are shown in Table 1. The sodium content of cells used in this experiment was higher and the potassium content lower than usual. In respect of sodium, three components were apparent: about 3 μ-equiv.
which was extracted by NH$_4$Cl in excess of that extracted in sucrose, and which was not related to the integrity of the membrane, about 7 $\mu$-equiv. which was removed by either solution when the membrane had been damaged, and a small residual. These data suggest that about 3 $\mu$-equiv. sodium existed outside the membrane, adsorbed to negative charges, and that the remainder was within the membrane. After butanol treatment, 89 $\mu$-equiv. potassium was removed by NH$_4$Cl, and only 69 $\mu$-equiv. by sucrose solution. This suggested that while all of the cell potassium was within the plasma membrane, 23–25% of this potassium was adsorbed to negatively charged sites from which it could be displaced by other cations.

![Graph](image)

Fig. 4.—Effect of washing in aqueous n-butanol solutions on the sodium (○), potassium (×), and phosphate (△) content of cells grown in brain–heart broth (0·993 g$\cdot$w$^{-1}$). Values are per 100 mg dry weight of cells. ○ Optical density at 256 m$\mu$ of washings after dilution to one-tenth.

As it seemed likely that some sodium was adsorbed to the cell wall, the cation-adsorbing capacity of isolated cell walls was determined. Wall preparations were washed once in a solution containing 0·1m NaCl and 0·1m KCl, followed by a washing in distilled water. The preparation retained 2·3 $\mu$-equiv. sodium and 2·2 $\mu$-equiv. potassium per 100 mg dry weight of original cells. Thus there was no discrimination between these ions. After allowing for carry-over from the salt solution, about 4 $\mu$-equiv. total cation was adsorbed to the cell wall. For cells in brain–heart broth, most of these sites would be occupied by sodium.

The large differences in sodium and potassium contents of whole cells and the high concentration gradients of these ions suggested a preferential adsorption of potassium over sodium. To examine the specificity of adsorption, butanol-treated cells (see Section II) were washed with solutions containing equimolar concentrations of NaCl and KCl, washed again with water, and the residual sodium and
potassium determined. The results, corrected for carry-over from the salt solutions, are shown in Figure 5. The high initial potassium content was reduced by sodium replacement as the concentration of the salt solution increased, and in 0·2M solution the adsorption sites appeared saturated with sodium and potassium in the molar ratio 1 : 1. The total cation then held was 27 μ-equiv. per 100 mg dry weight of whole cells. This agrees with the differences between amounts found for sucrose- and NH₄Cl-washed cells after butanol treatment of 22 μ-equiv. potassium and 3·1 μ-equiv. sodium, giving 25·1 μ-equiv. total cation. If butanol did not cause an appreciable change in the number of cation adsorption sites in the living cell, cells of dry weight 100 mg were capable of adsorbing about 27 μ-equiv. univalent cations, with about 15% of this capacity probably residing in the cell wall.

**Table 1**

**Sodium and Potassium Contents of Butanol-Treated and Control Cells of Staphylococcus aureus After Washing in Sucrose and Ammonium Chloride Solutions of 0·993 aₜw**

<table>
<thead>
<tr>
<th>Washing Solution</th>
<th>Sodium Content (μ-equiv/100 mg dry wt.)</th>
<th>Potassium Content (μ-equiv/100 mg dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Cells</td>
<td>Butanol-treated Cells</td>
</tr>
<tr>
<td>Sucrose</td>
<td>13·5</td>
<td>6·3</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>10·5</td>
<td>3·2</td>
</tr>
<tr>
<td>Difference</td>
<td>3·0</td>
<td>3·1</td>
</tr>
</tbody>
</table>

**IV. Discussion**

The results obtained with *Staph. aureus* may be compared with data reported earlier for *Salmonella oranienburg* (Christian 1958). During washing, both organisms lost sodium most readily to sodium-free solutions of electrolytes and least readily to water. The amount of sodium leaving the cells was influenced more by the nature of the washing solute than by the aₜw. Both organisms lost potassium and ultraviolet-absorbing compounds when the external aₜw was increased, but a much greater change in aₜw was required to induce leakage from staphylococci than from salmonellae. This difference was probably due to the greater resistance of cocci to osmotic swelling and hence deformation of permeability barriers. These bacteria also differed in Gram reaction. This does not appear to be an important factor since loss of potassium by the Gram-positive rod *Bacillus megaterium* during the various washing treatments was qualitatively the same as for *S. oranienburg* (unpublished observations).

Changes in pH values of washing solutions affected retention of sodium and potassium in opposite ways, as was found with *S. oranienburg*. Lowering the pH to 4·5 caused increased sodium and decreased potassium retention. No explanation
EFFECTS OF WASHING ON A STAPHYLOCOCCUS

can be offered for this effect. Increased hydrogen ion concentration might be expected to reduce the cell sodium content, as occurred when the potassium and ammonium ion concentrations of the washing solutions were increased.

While cells of *Staph. aureus* grown at 0.993 aw contained 106 μ-equiv. potassium per 100 mg (Fig. 1(b)), those grown in media of 0.92 aw contained 60 μ-equiv. (Fig. 3). This fall in potassium content was offset by a decrease in the water content of cells grown at the lower aw, so that the concentration of potassium in the cell water increased from 0.67m at 0.993 aw to 1.08m at 0.92 aw. The effects of the aw of the growth medium on the composition of *Staph. aureus* will be reported later.

Cells of *Staph. aureus* contain large amounts of potassium and small amounts of sodium in comparison with many other bacteria (Christian and Waltho 1961). The present data show that the cells possessed some 27 μ-equiv. fixed negative charges per 100 mg dry weight which could adsorb univalent cations, but the sodium and potassium of the cell together far exceed this concentration. Further, these anionic sites showed no selective affinity for potassium ions and could not be responsible for the steep gradient in potassium concentration that exists across the cell boundaries. Therefore, most of the cell cations (and other solutes) must have
been retained by a mechanism other than adsorption. The data agree with the conclusion of Mitchell and Moyle (1956) that a butanol-labile barrier is the structure involved.

This barrier must differ greatly in its permeability properties towards sodium and potassium. The washing technique employed probably measured only those changes in composition resulting from passive efflux, since (1) no substrate was added; (2) endogenous activity was relatively low \((Q_{O2} = 5\) (unpublished data)) and the interval between suspension and centrifugation was short; (3) external concentrations of the effluxing ions were too low to permit appreciable influx. Thus the passive efflux of potassium was very much slower than that of sodium. Further, sodium efflux was greatly accelerated by the presence of other electrolytes which had little effect on efflux of potassium. It is reasonable to assume that exchange of sodium with other cations was involved here.

Most of the cell sodium was initially within the cell membrane, and the acceleration of sodium efflux by potassium and ammonium ions suggests that these ions penetrated the membrane. However, cell potassium did not readily leave the cell nor was it appreciably replaced by ammonium ions. It is proposed, therefore, that other cations increased sodium efflux, not by displacing it from within the osmotic barrier, but by displacing it from adsorption sites outside this barrier, i.e. on the surface of the membrane or in the cell wall.

V. Acknowledgment

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VI. References


