THE EFFECTS OF WASHING TREATMENTS ON THE COMPOSITION OF *SALMONELLA ORANIENBURG*

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Summary

Cells of *Salmonella oranienburg* grown in liquid media of water activity (a_w) 0·993 were washed in water and in solutions of sucrose, glycerol, NaCl, KCl, NH_4Cl, and $MgSO_4$ at a range of a_w. Retention of sodium and potassium by the cells and loss of compounds absorbing in the ultraviolet were measured. At pH 7·5, cells retained most sodium after washing in water, while retention of potassium and ultraviolet-absorbing compounds was greatest after washing in isotonic sucrose and electrolyte solutions. Changes in the pH of washing solutions markedly affected retention of cell constituents. When washed in solutions of a_w from 1·000 to 0·990, the contents of potassium and ultraviolet-absorbing compounds in the cells were a function of a_w while the sodium content was largely dependent on the nature of the washing solute. These results are discussed and recommendations made concerning the choice of solutions for washing and suspending cells of this organism.

I. Introduction

The preparation of microorganisms for experimental purposes frequently involves a washing procedure to free the cells from contamination by constituents of their previous environment. Washing is usually performed with water, buffers, or salt solutions. For washing other particulate fractions (e.g. mitochondria) sucrose solutions are generally preferred. The effects of these treatments on the composition of the cell have not been widely considered, especially with bacteria.

A previous investigation (Christian 1955) showed that the amount of potassium accumulated by respiring cells of *Salmonella oranienburg* was related to the water activity (a_w) of the medium but that the sodium content of the cells was not. The present communication concerns the ability of cells to retain their internal sodium and potassium during washing in solutions of electrolytes and non-electrolytes. For comparison, the leakage of some compounds absorbing in ultraviolet light has also been followed. The effects of the composition of the growth medium, the nature of the washing solute, a_w, and the pH of the washing solution have been investigated. Large differences have been found in their effects upon leakage of sodium, potassium, and compounds absorbing at 256 mμ.

II. Methods

The test organism was a strain of *S. oranienburg* whose water relations had been studied previously (Christian 1955). Cells were grown in brain heart broth, or nutrient broth plus KCl (both media 0·993 a_w) for 16 hr at 30°C and aerated by

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shaking. The stationary phase cells were harvested by centrifugation, and suspensions prepared in the spent medium at concentrations of about 60 and 120 mg wet cells per ml for cells grown in nutrient broth and brain heart media respectively.

Replicate 5-ml samples were dispensed in 7-ml glass centrifuge tubes, centrifuged, drained, and the tube walls blotted dry. After weighing, various pellets were used in washing experiments, for dry weight estimations after 20 hr at 98°C, and for determination of intercellular space.

Intercellular space was determined with cells grown in brain heart broth by the method of Conway and Downey (1950), using sucrose as the test solute. A small known volume of sucrose solution (0·993 a_w) was added to the centrifuge tube and the cells resuspended in it. After centrifugation the sucrose concentration in the supernatant was determined using the benzidine reagent of Jones and Pridham (1954). From the degree of dilution of sucrose by interstitial liquid an intercellular space of $2 \cdot 27 \pm 0 \cdot 21$ ml per g dry weight was calculated. A similar value is obtained from the data of Mitchell and Moyle (1956) on the phosphate-impermeable space in pellets of *Bacterium coli* at the same a_w. Experiments with *S. oranienburg* in which centrifugation was delayed until 1 hr after suspension showed slow penetration of the cells by sucrose. Hence the value found here is believed to be a reasonable estimate of the interstitial space.

Cells were washed in water and in solutions of sucrose, glycerol, NaCl, KCl, NH_4Cl, and $MgSO_4$ at 0·993 a_w and in solutions of sucrose, NH_4Cl, and $MgSO_4$ at a_w ranging from 1·000 to 0·990. 5-ml aliquots of the solutions were added to the wet pellets, the cells suspended with a capillary pipette, and the suspensions centrifuged at ambient temperature. The supernatants, suitably diluted, were retained for measurement of absorption at a wavelength of 256 mμ in a Beckman DU spectrophotometer. The centrifugates were treated with 0·25 ml 40 per cent. trichloroacetic acid, suspended in glass-distilled water, and centrifuged. The supernatants were analysed for sodium and potassium in an E.E.L. flame photometer. Sodium was not measured in NaCl-washed cells nor potassium in KCl-washed cells. The concentrations of ultraviolet-absorbing compounds in the washings were expressed as optical density per 100 mg dry wt. of cells, and the sodium and potassium in the cell pellets as μ-equiv. per 100 mg dry wt. of cells.

The washing solutions were not buffered. The pH of the suspension during washing was controlled by the pH and buffering capacity of the cells and the interstitial growth medium. To regulate the pH during washing, the pH of the stock suspension was adjusted to the desired value with HCl. The pH of unadjusted suspensions from both media was 7·5–8·0, and washing experiments were performed at pH 4·5, 6·0, and 7·5 with cells from brain heart broth.

III. Results

(a) *Sodium and Potassium Content of Unwashed Cells*

Unwashed cell pellets from brain heart broth contained 2·27 ml of intercellular medium per g dry wt. and the concentrations of sodium and potassium in this medium were 146 m-equiv./l and 24 m-equiv./l respectively. When the results of whole-

pellet analyses were corrected for these extracellular contributions the cells contained 15·4 μ-equiv. sodium and 35·4 μ-equiv. potassium per 100 mg dry wt.

On resuspending the cells in washing solutions, the interstitial medium was diluted by a factor of 20. If the same degree of packing is assumed in the pellet before and after washing, the contribution of the intercellular space of the washed pellet was 1·6 μ-equiv. sodium and 0·3 μ-equiv. potassium per 100 mg dry wt. Hence if there were no leakage of sodium or potassium from the cells during washing, the maximum recoverable value in the pellet would be 17 μ-equiv. sodium and 35·7 μ-equiv. potassium per 100 mg dry wt.

The internal water content of the cell was obtained by subtracting the water content of the intercellular medium from that of the whole pellet. On this basis it was calculated that the concentrations of sodium and potassium within the cell, assuming them to be free in the aqueous phase, were 94 and 216 m-equiv./l respectively.

(*b*) *Influence of the Washing Solute on Contents of Cells Grown in Brain Heart Broth*

Brain heart broth (37 g dry matter/l) contained sodium and potassium at concentrations of 146 and 24 m-equiv./l, a sodium : potassium ratio of 6·08. The yield of cells was about 2 g dry wt./l. Cells were washed in various solutions of 0·993 a_w and in water (1·000 a_w).

The amounts of sodium retained in the cell pellet after one washing at pH 7·5 in various solutions and in water are shown in Figure 1(*a*). The sodium values in glycerol- and in water-washed pellets of 17·0 and 18·4 μ-equiv. per 100 mg dry wt. respectively are close enough to the calculated content of 17·0 μ-equiv. to conclude that none of the sodium within the cell was lost during washing in either of these media at pH 7·5.

Figure 1(*b*) shows the effects of washing treatments at pH 7·5 on the potassium content of cell pellets. Under the best conditions the recovery of potassium after washing was about 31 μ-equiv. per 100 mg dry wt. Since the theoretical content was 35·7 μ-equiv., none of these treatments completely prevented the loss of intracellular potassium.

The ultraviolet absorption spectrum of supernatants from cells washed in water showed a marked maximum at 256 mμ, and the absorption of all washings from these experiments was measured at this wavelength. Figure 1(*c*) shows values obtained after washing at pH 7·5. It is apparent that none of these treatments prevented the loss of these substances, presumably largely purines and pyrimidines, from the bacterial cell.

(*c*) *Influence of the Washing Solute on Contents of Cells Grown in Nutrient Broth plus KCl*

Nutrient broth, 0·999 a_w, was adjusted to 0·993 a_w by addition of KCl. The medium then contained 10 m-equiv. sodium and 193 m-equiv. potassium/l, a sodium: potassium ratio of 0·052. This contrasts with the ratio of 6·08 for brain heart broth.

The yield of cells was about 0·5 g dry wt./l. Washing experiments were performed at pH 7·5 and the results are shown in Figure 2.

The trends were broadly similar to those observed with cells grown in brain hearth broth, but there were some differences. Relative to the other treatments, washing in $MgSO_4$ and glycerol solutions retained less sodium than with cells grown

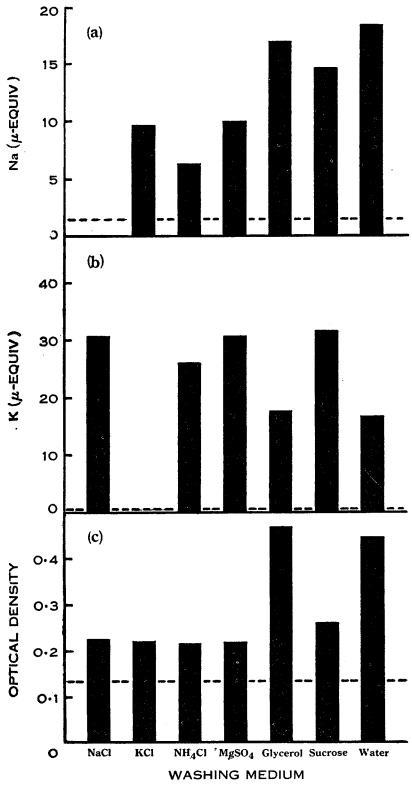

Fig. 1

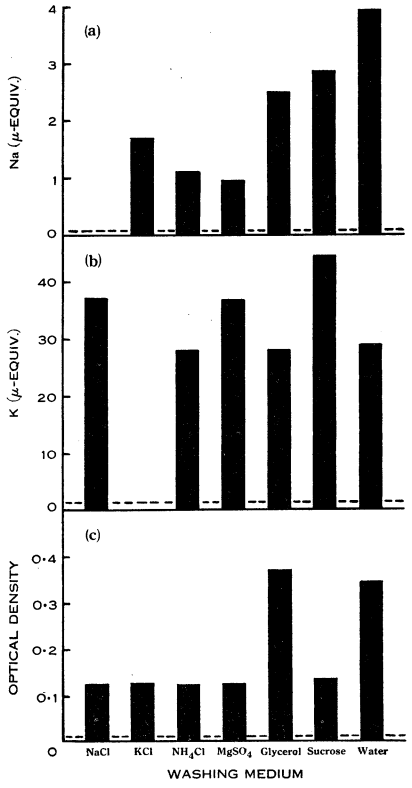

Fig. 2

Figs. 1 and 2.—Effect of washing at pH 7·5 in solutions of 0·993 a_w and in water on the composition of *S. oranienburg*. Cells grown in brain heart broth (0·993 a_w) (Fig. 1) and nutrient broth plus KCl (0·993 a_w) (Fig. 2). Broken lines indicate the contribution of contaminating growth medium. (*a*) Sodium content of cell pellet. (*b*) Potassium content of cell pellet. (*c*) Optical density at 256 mμ of washings after dilution to one-tenth. Values are per 100 mg dry wt. of cells.

in brain heart broth (Fig. 2(*a*)). Sucrose was clearly superior to all other solutes tested in preventing leakage of potassium (Fig. 2(*b*)) while NH_4Cl was no better than glycerol or water. Figure 2(*c*) indicates that the leakage of compounds absorbing in the ultraviolet followed the same pattern as was found for cells from brain heart broth.

(*d*) *Effect of pH*

Cells grown in brain heart broth were washed in various solutions of 0 993 a_w and in water at pH's of 4·5, 6·0, and 7·5. Figure 3 shows that, in general, sodium retention was lowest at about pH 6·0 and that differences between treatments were

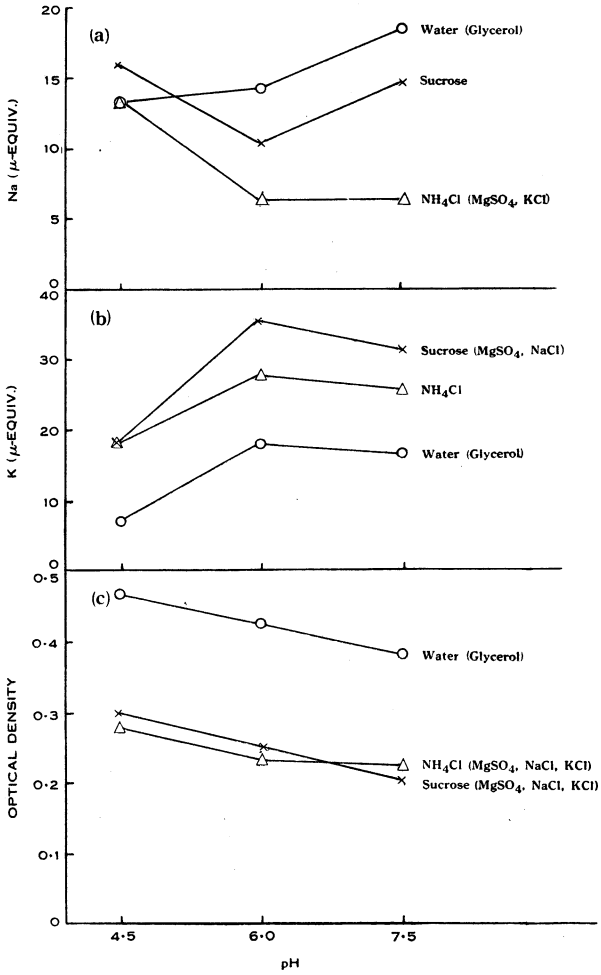

Fig. 3.—Effect of pH during washing in solutions of 0·993 a_w and in water on composition of *S. oranienburg*. Cells were grown in brain heart broth (0·993 a_w) and washed in sucrose (×), NH_4Cl (△), and water (○). Solutes in parenthesis gave results similar to the adjacent curves. (*a*), (*b*), and (*c*) as in Figures 1 and 2.

least at pH 4·5. The potassium content of cells was greatest at pH 6·0 and cells washed at this pH in all three solutions contained amounts of potassium within 5 per cent. of the corrected content of unwashed cells. Thus if unwashed cells in a medium of pH 6·0 have the same potassium content as those at pH 7·5, these three treatments at pH 6·0 prevent its loss from the cells. Change in pH did not affect the relative

efficiencies of the three solutions in preventing loss of ultraviolet-absorbing compounds but losses increased as the pH fell from 7·5 to 4·5.

(e) *Effect of Repeated Washings*

In experiments so far reported, cells were washed in the presence of medium carried over in the interstices of the cell pellet. To determine whether the results were affected by the small volume of solution used, cell pellets were washed three times in sucrose solutions or in water and analysed for sodium and potassium after each washing. The results are given in Figure 4.

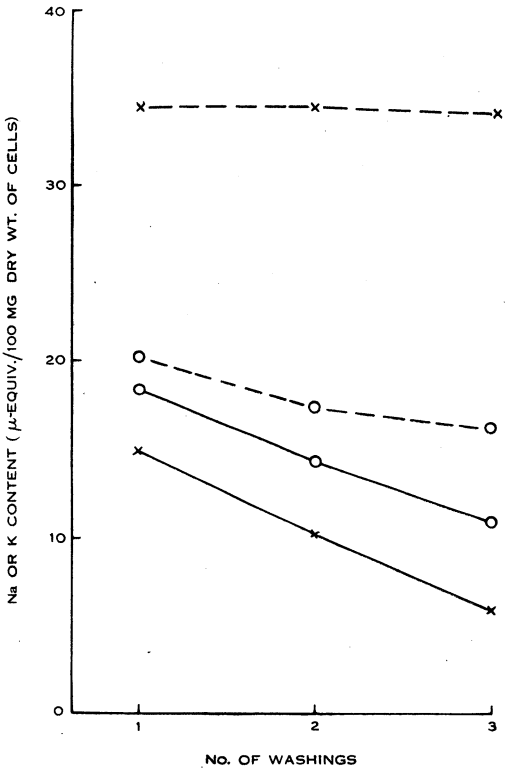

Fig. 4.—Effect of repeated washing at pH 7·5 in sucrose solution of 0·993 a_w and in water on the sodium and potassium content of *S. oranienburg*. Cells grown in brain heart broth (0·993 a_w). ——Sodium content. – – – Potassium content. × Sucrose-washed cells. ○ Water-washed cells.

These data confirm the validity of experiments on potassium leakage, since very little potassium was lost in the second and third washings in either sucrose solution or water. When cells were washed once in sucrose and then once in water the amounts of sodium and potassium retained were consistent with the results of Figure 4. These facts suggest that the degree of dilution of the growth medium was not an important factor in potassium retention. The steep fall in sodium content during the second wash in water may have been due to dilution of the contaminating medium, or to the leakage of some other substance not estimated. The same factors

may affect the sodium status of sucrose-washed cells, but it remains true that cells retain much more sodium after repeated washings in water than after identical treatment in sucrose solutions.

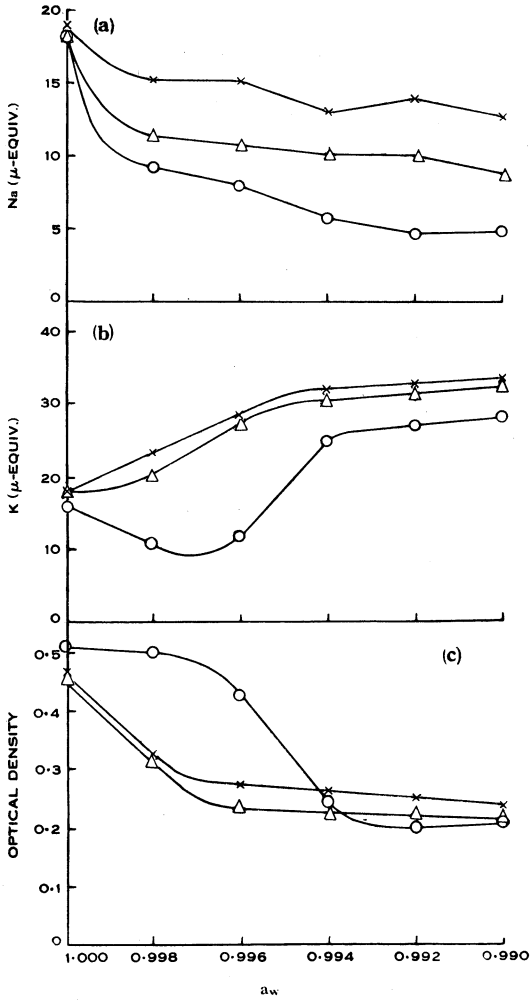

Fig. 5.—Effect of a_w of the washing solution on composition of *S. oranienburg*. Cells were grown in brain heart broth (0·993 a_w) and washed at pH 7·5 in solutions of sucrose (×), $MgSO_4$ (△), and NH_4Cl (○). (*a*), (*b*), and (*c*) as for Figures 1 and 2

(*f*) *Effect of* a_w *of the Washing Solution*

In view of the large differences in the composition of cells after washing in water and in most solutions at 0·993 a_w, the effect of a_w of the washing solution was studied. Cells grown in brain heart broth were washed in solutions of sucrose, NH_4Cl, and $MgSO_4$ at several a_w between 1·000 and 0·990. Sucrose was chosen for its

overall efficiency in preventing leakage, NH_4Cl for its inefficiency, and $MgSO_4$ for its similarity to NaCl and KCl in experiments at 0·993 a_w. The results are shown in Figure 5.

The differences in sodium content found previously for cells washed in the three solutions at 0·993 a_w were largely maintained over the range 0·990–0·998 a_w (Fig. 5(*a*)). The differences were not eliminated until the a_w was very close to 1·000, suggesting that retention of sodium by the cell depends much more on the nature of the solute in the suspending medium than on its concentration.

In all three washing solutions, gross leakage of potassium commenced when a_w exceeded 0·994 (Fig. 5(*b*)). As with sodium, NH_4Cl retained less potassium at all a_w than the other solutions, but in this case the a_w of the solution was more important than with sodium leakage.

The optical density of supernatants was not affected by hypertonic solutions, but increased rapidly when a_w of washing solutions exceeded 0·994 for NH_4Cl and 0·997 for sucrose and $MgSO_4$ (Fig. 5(*c*)). This points to another striking difference between the protection afforded by NH_4Cl on the one hand and sucrose and $MgSO_4$ on the other, for while potassium and ultraviolet-absorbing compounds commenced to leak from cells at the same a_w in NH_4Cl, they leaked at very different a_w in the other two solutions.

IV. Discussion

It is apparent from the results presented that none of the washing treatments tested is capable of retaining completely both the sodium and potassium content of cells of *S. oranienburg*. The similarity between the effects of water and isotonic glycerol solution on leakage of solutes is in accord with the well-known penetrating powers of glycerol and might have been predicted from earlier studies with this organism (Christian 1955). Here it was found that the effect of high concentrations of glycerol on respiration and potassium accumulation was negligible, while solutions of salts and sugars of the same a_w were inhibitory.

Loss of sodium and potassium during washing with electrolytes is probably the result of an ionic replacement, but at 0·993 a_w the effect is much larger with cellular sodium than potassium. The slight loss of both ions when cells are washed in sucrose is unlikely to be due to replacement by sucrose but rather to the presence of electrolytes carried over from the initial interstitial medium. The same carry-over is present in cells washed in water or glycerol which apparently lose no sodium. Thus a requirement for this type of replacement may be maintenance of a high effective osmotic pressure.

Since the differences in the effects of various solutes on leakage of electrolytes largely disappear when cells are washed at pH 4·5, it is probable that the pH becomes more important than the solute of the washing solution. However, the pH affects sodium and potassium leakage in opposite ways, and at low pH (4·5) loss of potassium is at a maximum and of sodium at a minimum. This complementary response may be related to the linkage between sodium excretion and potassium accumulation which has been postulated for many other biological systems.

It has been claimed that cells of *Escherichia coli* are freely permeable to sodium chloride (Roberts *et al.* 1955). The sodium content of the cell might therefore be expected to fall rapidly on washing in water. Although it did fall steeply on the second and third washings, there was no loss in the first wash. During the first washing in water, the external sodium from brain heart medium is present at about 7·3 m-equiv./l. while the internal sodium concentration is about 94 m-equiv./l. The absence of leakage at a concentration gradient of 13 : 1 suggests that the cell does not allow free outward diffusion of the sodium which enters it during growth.

The two media on which cells were grown differed greatly in sodium and potassium content but were of the same a_w. Although no striking differences were observed in the response of the two types of cells to the washing treatments, the differences in their sodium and potassium contents deserve comment. The sodium content, after cells had been washed in water, was much higher in cells grown in the high-sodium medium. However, potassium contents, determined after sucrose washing, were little affected by the potassium content of the medium, viz. 31·6 and 44·5 m-equiv. potassium per 100 mg dry wt. for cells from media containing 24 and 193 m-equiv. potassium/l respectively. Hence the sodium content of cells is probably a function of the sodium content of the growth medium while the potassium content of cells is largely independent of the external potassium concentration. In the light of earlier experiments (Christian 1955) it is likely that the content of cellular potassium is largely a function of the a_w of the growth medium.

The suggestion was made in this previous paper that the increase in potassium content of cells during respiration at low a_w might be a major change by which the cell equilibrated to the more concentrated environment. Calculations made at that time based on internal water content equal to three times the dry weight showed that the lowering of internal a_w by potassium, coupled with a univalent anion, would account for only two-thirds of the a_w lowering of the external medium. Experiments have now shown that under these conditions of growth and centrifugation the internal water is only about twice the dry weight, and hence the internal concentration of potassium salts may be sufficient to balance the external a_w.

There is no evidence that all of the substances lost from the cells during washing were originally situated within the plasma membrane. With the compounds absorbing in the ultraviolet it is possible that the optical density found in excess of that due to carry-over from the growth medium was the result of desorbed purine and pyrimidine compounds from the cell surface. However, it seems likely, at least in the case of potassium and optical density measurements, that the compounds lost from cells in some hypotonic solutions were of intracellular origin.

Earlier experiments (Christian and Scott 1953) showed that the motile salmonellae are a homogeneous group in regard to their water relations. Thus it may be assumed that the results obtained with *S. oranienburg* apply to the whole genus and to the Enterobacteriaceae generally. However, some Gram-positive species behave very differently. Preliminary experiments with *Staphylococcus aureus* showed that little potassium was lost on washing in any of the isotonic solutions or in water, while with *Bacillus megaterium* the leakage of compounds absorbing in the ultraviolet was similar in all the solutions tested.

It is concluded that no one set of conditions tested completely prevented leakage of all the constituents studied from cells of *S. oranienburg*. However, the following suggestions may be made concerning the choice of solutions in which to wash or dilute suspensions of such cells: (1) concentrations hypotonic to the growth medium may be detrimental; (2) sucrose is probably preferable to electrolytes; (3) if electrolytes are used, those with cations of smaller hydrated ionic radii (e.g. potassium and ammonium) should be avoided; (4) a pH close to 7 is preferable to more acid conditions.

V. References

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