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Influence of Alanyl Ester Residues on the Binding of Magnesium Ions to Teichoic Acids

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The binding of Mg^{2+} to the ribitol teichoic acid of *Staphylococcus aureus* H walls was examined by equilibrium dialysis in solution and in the intact wall; the influence of alanyl ester groups on binding was determined. In solution the ribitol polymer had a lower affinity than did a glycerol teichoic acid and bound Mg^{2+} in the ratio Mg^{2+}/P of 1:1. The presence of alanyl ester residues caused a decrease in the amount of cations bound in stoicheiometric proportion to the ratio Ala/P, but the affinity constant was unaltered. It is concluded that in solution the ribitol teichoic acid binds Mg^{2+} univalently to phosphate groups and univalently to a counter-ion. In the intact wall the binding of Mg^{2+} was different. The affinity constant was higher and resembled that of a glycerol teichoic acid. It is concluded that Mg^{2+} forms bridges across phosphate groups in teichoic acid chains lying adjacent to each other in the wall. The effect of alanyl esters was similar to that in solution, but Scatchard plots were not linear at low concentrations of Mg^{2+} where it was shown that the difference in affinities between walls with and without alanyl ester residues was much greater than it was at higher concentrations of Mg^{2+}. Thus at very low concentrations of Mg^{2+} effective binding to the wall is markedly improved by loss of alanyl ester residues.

There is evidence that teichoic acids participate in the exchange of ions between the cell walls of Gram-positive bacteria and the surrounding medium, and thereby maintain a high concentration of bivalent ions, particularly Mg^{2+}, in the region of the cell membrane (Baddiley, 1972; Archibald, 1974). Hughes *et al.* (1973) have shown that in a cell-free extract from *Bacillus licheniformis* certain Mg^{2+}-dependent membrane-bound enzyme systems preferentially utilize ions that are associated with teichoic acids rather than externally added ions. It was therefore suggested that in intact cells the wall and membrane teichoic acids form an integrated system for supplying cations to the membrane; the wall teichoic acid providing a reservoir of bound Mg^{2+} that can be transferred to the membrane teichoic acid and hence to the cell membrane and utilized as required (Hughes *et al.*, 1973). The details of the nature of binding of such cations and the mechanism of their passage across the wall are unknown. Hughes *et al.* (1973) have suggested that bivalent cations might be transferred directly from ligand to ligand in the cell wall without intermediate solvation.

The amount of teichoic acid in the walls of bacteria clearly affects the amount of Mg^{2+} bound by the walls (Heptinstall *et al.*, 1970), and cells can regulate their teichoic acid content according to the availability of Mg^{2+} in the medium. For example, under conditions of restricted supply of Mg^{2+}, or in high concentrations of Na^{+}, cells of *Bacillus subtilis* incorporate increased amounts of teichoic acid into their walls, presumably to secure an adequate supply of Mg^{2+} (Meers & Tempest, 1970).

However, the amount of Mg^{2+} bound by cell walls is also influenced by the alanyl ester content of the teichoic acid. The amount of Mg^{2+} bound by walls of staphylococci is markedly decreased by the presence of alanyl ester residues (Heptinstall *et al.*, 1970; Archibald *et al.*, 1973), although a proportional relationship was not observed. When staphylococci are grown in the presence of NaCl the amount of alanyl ester in their walls is greatly decreased and the walls have an increased capacity to bind Mg^{2+} (Heptinstall *et al.*, 1970). It has been suggested that the free amino groups of the alanyl ester residues on the teichoic acid might regulate cation binding by direct competition for the negatively charged phosphate groups; one possible mechanism has been discussed by Baddiley *et al.* (1973). Moreover, information obtained by X-ray photoelectron spectroscopy also suggests that the alanyl ester residues might affect the way in which Mg^{2+} binds to the wall (Baddiley *et al.*, 1973).

It is clear that the alanyl ester residues have some functional importance since they are incorporated into walls of *B. subtilis* var. *niger* grown at low pH

values under conditions of N limitation when presumably only those N-containing components essential for growth would be synthesized (Ellwood & Tempest, 1972). The pH of the growth medium has a major influence on the alanyl ester content of the walls. Archibald *et al.* (1973) showed that walls of *Staphylococcus aureus* H grown at pH 5.0 contain more alanyl ester and bind less Mg^{2+} than do walls grown under similar nutritional conditions at pH 6.0 or 7.0. The differences in alanyl ester content were due largely to removal of these residues by base-catalysed hydrolysis in media at pH 6 and 7, and the incorporation of alanine into the wall did not appear to be influenced by the pH of the medium. Ellwood & Tempest (1972) demonstrated a similar effect of pH on alanyl ester content with *B. subtilis*, but the variation in alanine content with pH was too small to affect the Mg^{2+}-binding capacity of the walls.

The growth temperature can also affect the alanyl ester content of walls (Novitsky *et al.*, 1974). In *Bacillus coagulans*, cells grown at 55°C had a lower ratio of alanine/phosphorus in their wall teichoic acid than did those grown at 37°C. Moreover, the walls of cells grown at the higher temperature, although containing rather less teichoic acid than those grown at the lower temperature, bound more Mg^{2+}, presumably due mainly to the increased ability of the teichoic acid lacking alanine to bind cations.

Lambert *et al.* (1975) presented data on the binding of Mg^{2+} to the isolated poly(glycerol phosphate) wall teichoic acid of *Lactobacillus buchneri*. The binding was measured by equilibrium dialysis under controlled conditions of pH and ionic strength. The apparent association constant for Mg^{2+} and the number of binding sites on the teichoic acid was determined from Scatchard plots. Thus it was shown that one Mg^{2+} ion binds to two phosphate groups with an apparent association constant of $2.8 \times 10^3 M^{-1}$ in 10mM-NaCl at pH 5.0. However, the alanyl ester content of the teichoic acid was low and it was not possible to assess the influence of the alanine residues on the cation-binding process. We have now used the same technique to measure the binding of Mg^{2+} to the poly(ribitol phosphate) wall teichoic acid from *S. aureus* H, in which the alanyl ester content is high, and we have studied the effect on the ion binding of removal of the alanine. We have also used the method to investigate the binding of Mg^{2+} to isolated walls of *S. aureus* H before and after removal of the alanyl esters. The findings help to explain the interaction between Mg^{2+} and teichoic acid in the cell wall and the influence of the alanyl esters on the binding.

Experimental

Preparation of cell walls of S. aureus H

S. aureus H was grown in nutrient broth for 16h under forced aeration at 37°C as described by Baddiley *et al.* (1962). The pH of the medium was 5.2 when the cells were harvested. The cells were washed once with 0.9% NaCl and immediately disrupted with no. 11 Ballotini beads in a Braun cell disintegrator. The walls were recovered by centrifugation, washed once with 0.9% NaCl and then treated with 2% (w/v) sodium dodecyl sulphate (specially pure, BDH Chemicals Ltd., Poole, Dorset, U.K.), at 100°C for 2min to remove residual membrane material and to inactivate autolytic enzymes. The purified walls were then washed successively with distilled water, 0.1M-EDTA at pH 5.0 to remove Mg^{2+}, distilled water again and finally exhaustively dialysed against distilled water to remove traces of EDTA. The walls were analysed for Mg by atomic absorption spectroscopy using a Pye–Unicam SP.90 series 2 spectrometer (Pye–Unicam Ltd., Cambridge, U.K.) after wet-ashing by the method described by Eagon (1969). No Mg was detected in the walls.

Extraction and analysis of teichoic acid from walls

Teichoic acid was extracted from the purified walls with trichloroacetic acid as described by Baddiley *et al.* (1962), and purified by passage through a stacked column of Sephadex G-25 and G-75 (Slabyj & Panos, 1973) with 0.2M-NaCl as eluent. After dialysis and freeze-drying, analysis of the material by the methods described by Baddiley *et al.* (1962) showed that it had a similar composition to that reported by Baddiley *et al.* (1962). The material was not contaminated with either nucleic acid or membrane teichoic acid. Analysis showed that Mg^{2+} was absent and that Na^+ was present as counter-ion to the phosphate. The alanine content of the teichoic acid was measured after acid hydrolysis (4M-HCl, 100°C, 15h) by using a Jeol amino acid analyser (model JLC-6AH, Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan). Alanine was the only amino acid detected and the molar ratio of alanine/phosphorus (total phosphorus measured by the method of Chen *et al.*, 1956) was 0.67:1. This ratio was confirmed by direct measurement of the free amino groups in the untreated teichoic acid by the method of Rosen (1957).

Removal of alanyl esters from teichoic acid

Alanyl esters were removed by incubating a solution of teichoic acid (20mg) in 10mM-Tris–HCl buffer (5ml) at pH 9.0 for 3h at 37°C. The solution was then dialysed against distilled water for 24h at 5°C to remove the alanine produced by hydrolysis and the buffer ions. No phosphorus was lost, but the alanyl ester content had decreased to 0.01 mol of alanine/mol of phosphorus.

Removal of alanyl esters from the walls

Alanyl ester residues were removed from the teichoic acid in the purified walls by suspending them

(200mg) in 10mM-Tris–HCl buffer (100ml) at pH9.0 for 3h at 37°C. The walls were removed from the suspension by centrifugation and washed twice with water. The phosphorus and alanyl ester content of the walls were measured before and after treatment at pH9.0 as described by Archibald *et al.* (1973). A negligible amount of phosphorus was lost from the walls by the mild alkali treatment, but the alanyl ester content substantially decreased to 0.04mol of alanine/mol of phosphorus. Teichoic acid was also extracted from the treated walls and purified as described above; nearly all of the alanyl ester residues had been removed from the teichoic acid without degrading the teichoic acid chain.

Measurement of Mg^{2+} binding

Binding of Mg^{2+} to purified teichoic acid and to cell walls was measured as described by Lambert *et al.* (1975). Solutions of teichoic acid or suspensions of cell walls, each containing 1 μmol of phosphorus/ml were prepared in buffer. The buffer solution contained 10mM-NaCl and sufficient 3,3'-dimethylglutaric acid (0.11mM) and NaOH (0.1mM) to maintain a pH of 5.0 and thus minimize the hydrolysis of alanyl esters. Portions (1ml) of the teichoic acid solution or wall suspension were placed in bags made from 20/32 Visking dialysis tubing (Visking Co., Chicago, Ill., U.S.A.); the bags were placed in Universal (25ml) screw-top bottles containing 10ml of similar buffer to which was added $MgCl_2$ at concentrations from 0.1 to 1.0mM. The bottles were continuously inverted at 20°C for 16h, during which time equilibrium was attained between the bag contents and the dialysing medium. Mg^{2+} concentrations were then measured inside and outside of the bags. The concentration in the dialysing medium was measured directly after suitable dilution; the bag contents (0.5ml) were first wet-ashed as described by Eagon (1969) then diluted and analysed for Mg. The remaining 0.5ml of material from each bag was analysed for phosphorus and alanyl ester to establish whether loss of teichoic acid or hydrolysis of alanyl ester had occurred during dialysis. In the experiments with teichoic acid in solution, phosphorus was measured by the method of Chen *et al.* (1956) and alanyl ester was determined directly by the method of Rosen (1957) without hydrolysing; for experiments with cell walls, phosphorus and alanyl ester were determined as described by Archibald *et al.* (1973). There was a negligible decrease in the amounts of phosphorus or alanyl ester of either the teichoic acid solutions or the cell-wall suspensions during the course of the dialysis.

The Mg^{2+} binding was calculated (Morawetz, 1965) in terms of mol of Mg bound/mol of phosphorus. The results are expressed as binding isotherms, i.e. $\bar{r}$ versus A, where $\bar{r}$ = number of Mg^{2+} ions bound/phosphate group and A = equilibrium concentration of Mg^{2+} in solution (mM) and as Scatchard (1949) plots, i.e. $\bar{r}/A$ versus $\bar{r}$.

Results and Discussion

Fig. 1(*a*) shows the effect of removal of alanyl esters on the binding of Mg^{2+} to isolated teichoic acid from *S. aureus* walls. The isotherm shows that at all concentrations of Mg^{2+} studied the teichoic acid from which alanyl esters had been removed (0.01 mol of alanyl ester/mol of phosphorus) bound more than twice as much Mg^{2+} as did the teichoic acid with intact alanyl esters (0.67mol of alanyl ester/mol of phosphorus). The Scatchard plots (Fig. 1*b*) are linear

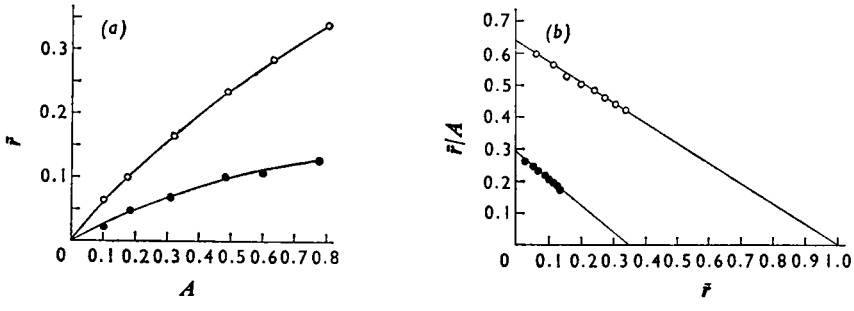

Fig. 1. *Effect of alanyl esters on binding of Mg^{2+} to isolated teichoic acid*

The binding of Mg^{2+} to the wall teichoic acid of *S. aureus* H expressed as isotherms (*a*) and as Scatchard plots (*b*), where $\bar{r}$ is the number of Mg^{2+} ions bound/phosphate group of the teichoic acid and A is the concentration of free Mg^{2+} ions (mM). Binding was measured by equilibrium dialysis (see the Experimental Section) at 20°C in the presence of 10mM-NaCl at pH5.0. The teichoic acid solutions contained 1.0 μmol of phosphorus/ml; ●, teichoic acid containing 0.67mol of alanyl ester/mol of phosphorus; ○, teichoic acid treated at pH9.0 (see the Experimental section) to decrease the alanine content to 0.01 mol of alanyl ester/mol of phosphorus.

in each case and thus indicate that all the Mg^{2+}-binding sites are identical and that there is no interaction between the sites (Scatchard, 1949). The apparent association constants, $K_{assoc.}$, obtained from the slopes of the Scatchard plots (Fig. 1*b*) are 0.83×10^3 M^{-1} for teichoic acid containing alanyl ester residues, and 0.63×10^3 M^{-1} for teichoic acid with these residues removed. The difference between these values is probably not significant and is due to errors in measuring the Mg^{2+} concentration and in extrapolating the Scatchard plots (Madsen & Robertson, 1974). Both values are considerably lower than the value of 2.7×10^3 M^{-1} obtained under identical conditions for the binding of Mg^{2+} to the poly(glycerol phosphate) wall teichoic acid of *L. buchneri* (Lambert *et al.*, 1975). The low apparent association constant indicates that Mg^{2+} is less strongly bound to the poly(ribitol phosphate) teichoic acid of *S. aureus* H than to the poly(glycerol phosphate) teichoic acid, and consequently a different mechanism of binding might be involved. The total number of available sites (n) on the teichoic acid, the value of which is obtained from the intercept of the Scatchard plot with the $\bar{r}$ axis, is 0.35 Mg^{2+}-binding site/atom of phosphorus when alanyl esters are present and 1.0 site/phosphorus atom when alanyl esters are absent. In both cases these values imply a 1:1 stoicheiometry of binding between Mg^{2+} and phosphate groups if it is assumed that each alanyl ester interacts electrostatically with its adjacent phosphate group and blocks the site for Mg^{2+} binding. The alanyl ester content of 0.67 alanine residue/phosphate group almost exactly meets the stoicheiometric requirement for such a mechanism.

If one Mg^{2+} is bound for every phosphate group then it follows that another anion must be associated with each bound Mg^{2+}; under the conditions of these experiments the anions involved could be Cl^- or dimethylglutarate. In the case of the poly(glycerol phosphate) teichoic acid it was shown that one Mg^{2+} is bound for every two phosphate groups and the construction of space-filling molecular models showed that it is sterically possible for one Mg^{2+} to bind to two adjacent phosphate groups in the chain (Lambert *et al.*, 1975). For the poly(ribitol phosphate) teichoic acid studied here similar attempts to construct molecular models (Catalin Ltd., Waltham Abbey, Essex, U.K.) showed that binding of one Mg^{2+} to two adjacent phosphates would involve considerable steric strain due to the greater distance between phosphate groups in the chain. Further, the convolution of the molecule necessary to bring adjacent phosphate groups sufficiently close together to bind one Mg^{2+} is hindered by the bulky *N*-acetylglucosamine substituents on each of the ribitol residues (Baddiley *et al.*, 1962). The condition where one Mg^{2+} is bound to one phosphate group with one associated mobile anion involves no steric strain in the molecule. We therefore suggest that under the conditions of teichoic acid concentration and ionic strength studied here one Mg^{2+} binds to every phosphate group that does not have an adjacent alanyl ester. The alanyl ester content therefore controls the amount of Mg^{2+} bound to the teichoic

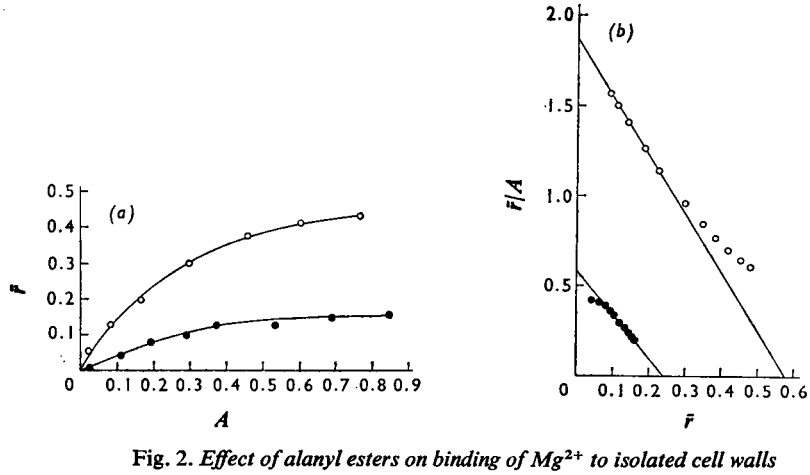

Fig. 2. *Effect of alanyl esters on binding of Mg^{2+} to isolated cell walls*

The binding of Mg^{2+} to isolated cell walls of *S. aureus* H expressed as isotherms (*a*) and as Scatchard plots (*b*), see legend to Fig. 1 for explanation of axes. Binding was measured by equilibrium dialysis (see the Experimental section) at 20°C in the presence of 10 mM-NaCl at pH 5.0. The cell-wall suspensions contained 1.0 μmol of phosphorus/ml; ●, walls in which the teichoic acid contained 0.67 mol of alanyl ester/mol of phosphorus; ○, walls treated at pH 9.0 (see the Experimental section) to decrease the alanyl ester content of the teichoic acid to 0.04 mol of alanyl ester/mol of phosphorus.

acid but does not affect the binding affinity as expressed by the association constant. The low apparent association constants reflect the weaker nature of the univalent Mg^{2+} binding to the ribitol teichoic acid compared with the bivalent binding to the glycerol teichoic acid.

Fig. 2(*a*) shows the binding of Mg^{2+} to purified cell walls measured under similar conditions to those used for isolated teichoic acid. The alanyl ester content has a marked effect on the amount of Mg^{2+} bound; removal of the esters results in a two- to three-fold increase in the amount of Mg^{2+} bound over the range of concentrations studied. Both of the Scatchard plots (Fig. 2*b*) for the cell walls are curved, from which it is concluded that the conditions of identical and independent binding sites are not fulfilled. Nevertheless the plots yield useful information about the mechanism of Mg^{2+} binding to the walls. Considering first the walls from which alanyl esters had been removed, the Scatchard plot has the greatest slope at low values of Mg^{2+} binding, and the extrapolated values of $K_{assoc.}$ and n from this region of the plot are approx. $3.2\times10^3\,M^{-1}$ and 0.57 Mg^{2+} sites/phosphate respectively. These values suggest that in the walls one Mg^{2+} is bivalently bound to two phosphate groups. However, we have already shown that binding of one Mg^{2+} to two adjacent phosphate groups in the same poly(ribitol phosphate) chain is proscribed by the distance between the phosphate centres and does not occur in solution. Bivalent binding in the walls could therefore only occur between phosphate groups in different teichoic acid molecules. Although little is known of the structural organization of teichoic acid molecules in the cell wall, on the basis of current knowledge (Archibald, 1974; Birdsell *et al.*, 1975; Doyle *et al.*, 1975) we can assume that some of the teichoic acid molecules are sufficiently close together to permit Mg^{2+} to bind between them. As the amount of Mg^{2+} bound to the walls increases the slope of the Scatchard plot decreases; hence the affinity of the walls for Mg^{2+} decreases as the binding sites become occupied. This effect might be caused by the bound ions restricting the access of other ions to the remaining unfilled binding sites. Possibly the binding of Mg^{2+} between the teichoic acid chains alters their conformation sufficiently to restrict further binding.

The Scatchard plot for Mg^{2+} binding to walls containing alanyl esters is also curved, but in this case at low Mg^{2+} binding the slope decreases. The average slope of the Scatchard plot over the whole range of binding gives values of $K_{assoc.} = 2.7\times10^3\,M^{-1}$ and $n = 0.23$ Mg^{2+} sites/phosphate. The high association constant again indicates that Mg^{2+} is bivalently bound to the walls and the number of available sites is approximately the number that would be expected if each of the alanyl esters (0.67 alanyl ester residue/phosphate) blocks a phosphate group in the manner discussed above for the isolated teichoic acid. A possible explanation of the decrease in the slope of the Scatchard plot at low Mg^{2+} binding is that the alanyl esters might interfere with the binding of Mg^{2+} between the chains in the wall. If the teichoic acid chains are arranged in sufficiently close proximity to permit Mg^{2+} to bind between them, then, in the absence of these or other bivalent cations, the alanyl esters could bind the chains together by interacting electrostatically with phosphate groups in adjacent chains rather than in the same chain. Consequently for Mg^{2+} to bind between the chains the alanyl esters must first be displaced, and this is reflected in the lower affinity of the walls for Mg^{2+} at low Mg^{2+} concentrations. Once the alanyl esters have been displaced from interacting between the chains Mg^{2+} ions are free to bind between any phosphate centres that are not blocked by adjacent alanyl esters from their own chains.

Whatever the causes of the curved Scatchard plots, it is clear that at low concentrations of Mg^{2+} the difference between the affinities of the walls containing alanyl esters and those without alanyl esters is greater than it is at higher concentrations. This observation could be of considerable significance, since it implies that cells growing under conditions of low Mg^{2+} concentration exhibit much greater affinity for Mg^{2+} when there are no alanyl esters in the walls.

The differences between the binding of Mg^{2+} to isolated teichoic acid and to cell walls emphasize the importance of the architectural arrangement of the polymers in the cell wall in controlling the manner in which ions are bound. The results reported here confirm those of Archibald *et al.* (1973) that the alanyl ester content controls the amount of Mg^{2+} bound to the walls. The lack of a precise stoicheiometric relationship between alanyl ester content and Mg^{2+} binding in walls of *S. aureus* H reported by Archibald *et al.* (1973) is probably a consequence of the complexity of the neutralization reactions between the alanyl esters and the phosphate groups. That Ellwood & Tempest (1972) were unable to demonstrate a similar effect of alanyl ester content on Mg^{2+}-binding capacity in *B. subtilis* is probably due to the low alanyl ester content of the walls they studied.

Removal of alanyl esters from cell walls may have a profound effect on the viability of the organisms under certain environmental conditions. Hurst *et al.* (1974, 1975) have shown that sublethal heating of *S. aureus* at 52°C results in loss of 40% of the cellular Mg^{2+} and 65% of the ester-bound alanine from the teichoic acid. Cells treated in this way lose their ability to grow in the presence of 7.5% (w/v) NaCl. During recovery from heat injury the cells rapidly synthesize D-alanine, and the Mg^{2+} content of the whole cells returns to normal, even when Mg^{2+} is present in the medium at very low concentrations

(3 μM) or in the presence of EDTA. Hurst *et al.* (1975) suggest that soon after heat injury, in the absence of alanyl esters, Mg^{2+} is strongly bound to the teichoic acid. This theory is in accord with the present findings that walls containing no alanyl esters have a greater capacity for binding Mg^{2+} and that at low concentrations of Mg^{2+} the affinity of the walls for these cations is greater than when alanyl esters are present. However, it is clear that at Mg^{2+} concentrations greater than about 0.2 mM the alanyl ester content of the walls controls only the amount of Mg^{2+} bound and not the affinity with which the ions are bound.

We have assumed in this discussion that teichoic acid is the major component of the walls responsible for binding Mg^{2+} (Heptinstall *et al.*, 1970). Other wall components will undoubtedly influence the ion-binding to some extent. For example, Rayman & MacLeod (1975) have shown that Mg^{2+} interacts with the un-cross-linked peptide residues of the peptidoglycan in a marine pseudomonad. However, the peptidoglycan of *S. aureus* is highly cross-linked (cf. Archibald, 1972) and any similar contribution to the ion binding made by the peptidoglycan is likely to be small.

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