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The Interaction of Magnesium Ions with Teichoic Acid

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The binding of Mg^{2+} to the wall teichoic acid of *Lactobacillus buchneri* N.C.I.B. 8007 was measured by equilibrium dialysis at controlled ionic concentration and pH. In an aqueous solution containing 10mM-NaCl at pH 5.0 one Mg^{2+} ion was bound for every two phosphate groups of the teichoic acid, with an apparent association constant, $K_{assoc.} = 2.7 \times 10^3 M^{-1}$. On lowering the pH below the pK_a of the phosphate groups the amount of bound Mg^{2+} decreased concomitantly with decreasing ionization of the phosphate groups. Both the amount of Mg^{2+} bound to the teichoic acid and the apparent association constants were similar in the presence of 10mM concentrations of NaCl or KCl but decreased markedly in the presence of 10mM-$CaCl_2$ because of competition between Ca^{2+} and Mg^{2+} for the binding sites. A similar effect was found when the concentration of NaCl was increased from 0 to 50mM. The results are discussed in relation to the function of teichoic acid in the walls of Gram-positive bacteria.

A great deal of information is available about the occurrence and structure of teichoic acids in the cell walls of Gram-positive bacteria (Baddiley, 1972), but comparatively little is known of their function. The suggestion (Archibald *et al.*, 1961) that teichoic acids are involved in ion exchange and in the control of access of ions, particularly Mg^{2+}, to the inner regions of the cell is supported by a number of studies. For example, it has been shown that Mg^{2+} binding by walls containing teichoic acid depends on the presence of the teichoic acid (Heptinstall *et al.*, 1970). Moreover the amount of Mg^{2+} bound is related, although not quantitatively, to the alanine ester content of the teichoic acid (Archibald *et al.*, 1973). Hughes *et al.* (1973) showed that the activity of certain membrane-bound enzymes involved in the biosynthesis of wall polymers in *Bacillus licheniformis* strongly depends on Mg^{2+} and that these ions can be supplied to the cell membrane by wall and membrane teichoic acids. The importance of teichoic acid in the assimilation of Mg^{2+} from the environment has been illustrated by studies on the growth of *Bacillus subtilis* under conditions that impose a constraint on the availability of Mg^{2+}. Thus when grown under conditions of Mg^{2+} limitation (Ellwood, 1970) or in a high concentration of competing Na^+ (Meers & Tempest, 1970; Ellwood, 1971) the cells respond by incorporating increased amounts of teichoic acid into their walls which consequently exhibit greater Mg^{2+}-binding capacity.

All these findings strongly suggest that the binding of Mg^{2+} to teichoic acid in the walls of Gram-positive bacteria is of fundamental importance to their growth and normal function. However, this view has been questioned by some authors (Ou *et al.*, 1973; Doyle *et al.*, 1974). Studies on the physiology of a teichoic acid-deficient mutant of *Staphylococcus aureus* showed that although the cells had a decreased capacity for binding Mg^{2+} compared with the parent strain they did not display increased sensitivity to Mg^{2+} depletion (Ou *et al.*, 1973). It was therefore argued that the teichoic acid in the parent strain did not play a vital role in sequestering Mg^{2+} from the environment. In our view such a conclusion is unjustified since it is clear from the results of Ou *et al.* (1973) that the teichoic acid-deficient mutant bound a measurable amount of Mg^{2+} which could well have been sufficient to meet the requirements of the cells under the conditions studied. Similar studies on a teichoic acid-deficient mutant of *S. aureus* isolated in this laboratory have shown that Mg^{2+} ions are significantly bound to carboxyl groups of an anionic polymer in the cell wall (A. R. Archibald, personal communication). We therefore consider that the ability to bind Mg^{2+} is an important property of the cell wall, which in some mutants or under certain nutritional conditions (Ellwood, 1970) can be conferred on the walls by anionic polymers other than teichoic acids. However, in general the Mg^{2+}-binding ability of the cell walls of Gram-positive bacteria is due chiefly to teichoic acid.

Apart from a recent study by X-ray photoelectron spectroscopy (Baddiley *et al.*, 1973) there is little information available on the nature of the interaction between Mg^{2+} and teichoic acid. In the present paper we present data obtained from studies of the binding of Mg^{2+} to teichoic acid in aqueous solution under controlled conditions of ionic concentration and pH.

From such binding data it is possible to calculate the apparent association constants and to predict the number of binding sites on the teichoic acid, their chemical nature and possibly the manner in which the ions are bound. Information of this kind should be valuable in explaining the various functions of teichoic acid that involve ion binding.

Experimental

Teichoic acid

The wall teichoic acid from *Lactobacillus buchneri* N.C.I.B. 8007 (Shaw & Baddiley, 1964; Archibald *et al.*, 1969) was chosen for studying Mg^{2+} binding. The polymer was extracted and purified as described by Shaw & Baddiley (1964), but an additional purification was necessary to remove a small amount of contaminating nucleic acid. This was achieved by passing the material down a stacked column of Sephadex G-25 and G-75 as described by Slabyj & Panos (1973) except that 0.2M-NaCl was used instead of LiCl as eluent. After dialysis and freeze-drying, analysis of the material by atomic absorption spectrophotometry (a.a.s) in a Unicam SP.90 series 2 instrument (Pye–Unicam Ltd., Cambridge, U.K.) showed that it contained bound Mg^{2+}. This was removed by passing the teichoic acid (50mg/10ml of water) over a column (4cm×1.5cm) of Dowex 50 (Na^+ form) resin (AG 50W-X8; 50–100 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.), which converted the teichoic acid completely into the Na^+ salt. By using the standard analytical methods described by Shaw & Baddiley (1964) the product was shown to have a similar composition to that previously reported (Shaw & Baddiley, 1964; Archibald *et al.*, 1969), although the chain length of the polymer was shorter. The teichoic acid contained eight glycerol phosphate units, about four of which had glucosyl residues on position 2 of glycerol. Most of the remaining glycerol units possessed D-alanine ester residues at position 2.

The Mg^{2+} salt of the teichoic acid was prepared by passing an aqueous solution of the teichoic acid (10mg/5ml) through a column (4cm×0.5cm) of Dowex-50 (Mg^{2+} form) cation-exchange resin.

Other chemicals

3,3′-Dimethylglutaric acid of 'purissima' grade was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. All other chemicals were of A.R. grade and were obtained from BDH Chemicals, Poole, Dorset, U.K.

Measurement of Mg^{2+} binding

Solutions of teichoic acid (Na^+ salt) containing 1μmol of phosphorus/ml were prepared in the required buffer (see below). Portions (1ml) were dispensed into bags made of 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 had been added $MgCl_2$ at concentrations from 0.1 to 1.0mM. In most experiments the buffer contained 3,3′-dimethylglutaric acid (0.11mM) and NaOH (0.1mM) to maintain a pH of 5.0 (Dawson *et al.*, 1969) and thus minimize the hydrolysis of alanyl esters (Archibald *et al.*, 1973). All metal ions were added as the Cl^- salts. In one experiment sufficient aq. 6M-HCl was added to 10mM-NaCl to produce a pH of 1.5, and in another experiment distilled water alone was used (pH5.0–5.3). The bottles were continuously inverted at 20°C for 16h, after 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 by a.a.s. and the binding of Mg^{2+} to the teichoic acid was calculated (Morawetz, 1965). Measurements of the phosphorus (Chen *et al.*, 1956) and alanine (Rosen, 1957) contents of the bags were also made at equilibrium; it was found that even at pH5.0 about 50% of the alanine was released from the teichoic acid during the course of the dialysis. This observation illustrates the extremely labile nature of the alanyl ester linkage, and as a consequence it was not possible by this method to study Mg^{2+} binding to teichoic acid that contained the maximum number of alanyl esters. The amount of teichoic acid (1μg-atom/ml) in the dialysis tube remained constant throughout the experiments.

Presentation and analysis of binding data

The choice of the most suitable means of presenting binding data depends on the nature of the system under investigation (Weber, 1965). The Scatchard model (Scatchard, 1949) treats ion binding in terms of multiple equilibria and is particularly useful where the macromolecule contains only one class of binding sites. This treatment was found to be suitable for our results, which are presented both as binding isotherms ($\bar{r}$ versus A, where $\bar{r}$ = number of Mg atoms bound/phosphate group and A = equilibrium concentration of Mg^{2+} in solution) and as Scatchard plots ($\bar{r}/A$ versus $\bar{r}$).

Results and Discussion

Of the many methods currently available for measuring the binding of small ions to macromolecules (Lawrence, 1972), equilibrium dialysis was selected since it permits conditions to be varied over a wide range of pH and ionic strength. Fig. 1 shows the binding of Mg^{2+} to teichoic acid in the presence of 10mM-Na^+ at pH5.0 and 1.5. The isotherms are of similar shape but there is a marked decrease in the amount of Mg^{2+} bound at pH1.5. The Scatchard plots are linear in each case, indicating that all of the

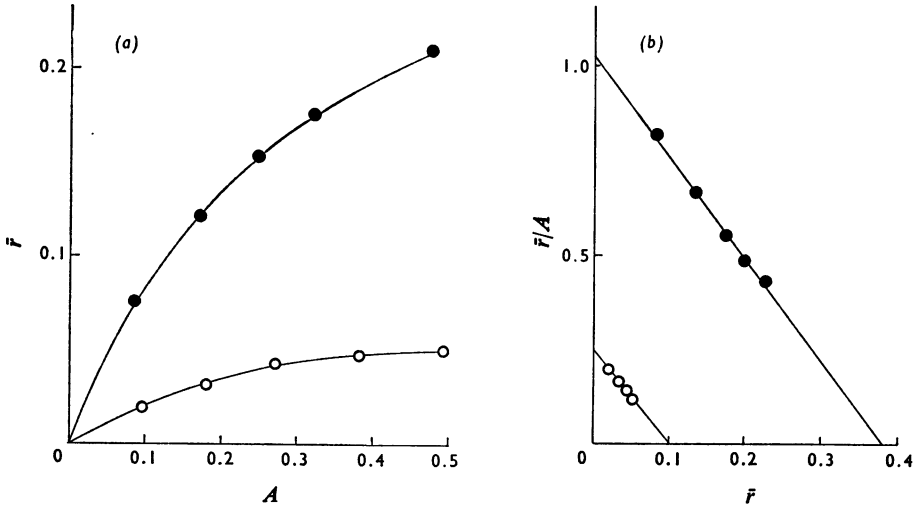

Fig. 1. *Binding of Mg^{2+} to teichoic acid at pH 5.0 and 1.5*

The binding of Mg^{2+} to the wall teichoic acid of *L. buchneri* N.C.I.B. 8007 expressed as isotherms (*a*) and Scatchard plots (*b*), where $\bar{r}$ is the number of Mg atoms bound/phosphate group of the teichoic acid and *A* is the concentration of free Mg^{2+} (mM). Binding was measured by equilibrium dialysis (see the Experimental section) in the presence of 10mM-NaCl at pH 5.0 (●) and at pH 1.5 (○) with a teichoic acid concentration of 1.0μmol of phosphorus/ml.

Mg^{2+}-binding sites on the molecule are equivalent and there is no interaction between adjacent sites. The apparent association constants, $K_{assoc.}$, obtained from the slope of the Scatchard plots are $2.7\times10^3 M^{-1}$ at pH 5.0 and $2.6\times10^3 M^{-1}$ at pH 1.5; hence lowering the pH has little effect on the strength of the Mg^{2+} binding. However, the total number of available sites on the teichoic acid (*n*), the value of which is obtained from the intercept of the Scatchard plot with the $\bar{r}$ axis, shows a decrease of about 75% from 0.38 Mg^{2+}/P at pH 5.0 to 0.1 Mg^{2+}/P at pH 1.5. This is considered to be caused by suppression of ionization of the phosphate groups at low pH. By using a pK_a value of 2.1 (Dawson *et al.*, 1969) it can be shown (Albert, 1968) that the phosphate groups are about 20% ionized at pH 1.5. Thus the decrease in ionization of the phosphate groups can account for the decrease in Mg^{2+} binding and also indicates that these groups provide the binding sites in the molecule. The stoicheiometry of the binding, with a maximum Mg^{2+}/P ratio of approx. 0.4:1, suggests that one Mg^{2+} ion is bound to two phosphate groups. The lack of an exact 0.5:1 ratio may be due to blocking of some of the binding sites by the protonated amino groups of adjacent alanyl ester substituents, as proposed by Archibald *et al.* (1973) and Heptinstall *et al.* (1970). The alanine content of the teichoic acid used in this experiment was 0.21 alanyl esters/phosphate, which makes such a mechanism theoretically possible. The proportions of Mg^{2+}:P:alanine were confirmed by direct analysis of the Mg^{2+} salt of the teichoic acid. The values 0.42 Mg^{2+}:1.0P:0.2alanine found for the Mg^{2+} salt are very close to the values obtained from the Scatchard analysis of the binding data. The construction of space-filling molecular models (Catalin Ltd., Waltham Abbey, Essex, U.K.) shows that a poly(glycerol phosphate) chain with glucosyl and alanyl substituents possesses sufficient flexibility to permit binding of one Mg^{2+} ion to two adjacent phosphate groups. The models also demonstrate that it is feasible for an alanyl group to mask a phosphate group.

The ability of alanine amino groups to compete with cations for phosphate centres of teichoic acids in the wall has been questioned by Ellwood & Tempest (1972). They were unable to observe a correlation between cation binding and alanyl ester content in walls from *Bacillus subtilis*. However, the ester content of their wall preparations was very low and the wide fluctuation in phosphate content of the walls was not taken into account. The very small changes in the amount of alanine ester present would result in only minor differences in the total amount of phosphate available for cation binding. We conclude that the experiments described by Ellwood & Tempest (1972) do not in fact alter the conclusion drawn from the earlier work of Heptinstall *et al.* (1970) and of Archibald *et al.* (1973). Experiments with whole cells (Ellwood & Tempest, 1972) are of little value

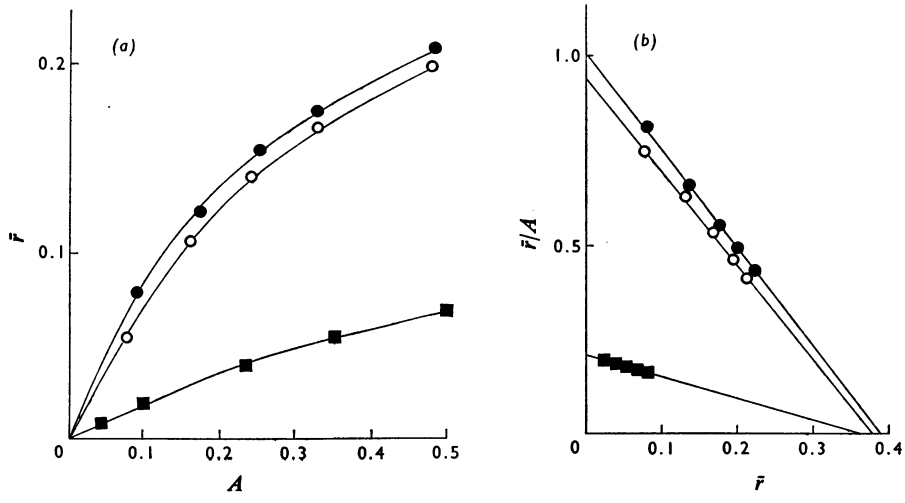

Fig. 2. *Effect of metal ions on the binding of Mg^{2+} to teichoic acid at pH 5.0*

Binding was measured at pH 5.0 in the presence of 10 mM concentrations of NaCl (●), KCl (○), or $CaCl_2$ (■) with a teichoic acid concentration of 1.0 μmol of phosphorus/ml. Results are expressed as isotherms (*a*) and as Scatchard plots (*b*); see legend to Fig. 1 and the Experimental section for details.

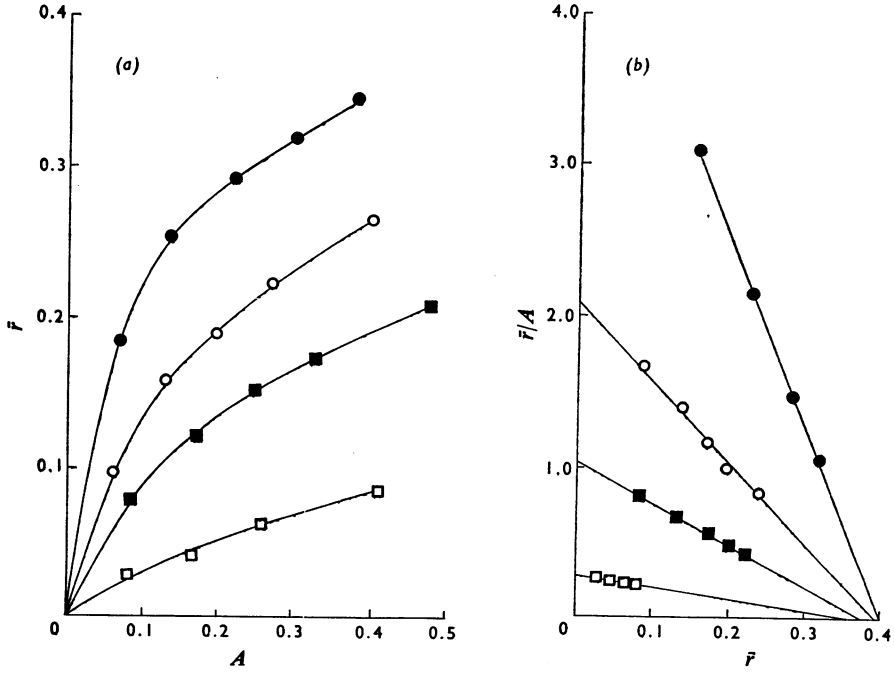

Fig. 3. *Effect of Na^+ concentration on the binding of Mg^{2+} to teichoic acid at pH 5.0*

Binding was measured at pH 5.0 in the presence of NaCl at concentrations of 1 mM (○), 10 mM (■) and 50 mM (□), and in water alone at pH 5.0–5.3 (●). The teichoic acid concentration was 1.0 μmol of phosphorus/ml. Results are expressed as isotherms (*a*) and as Scatchard plots (*b*). See the legend to Fig. 1 and the Experimental section for details.

in studies on binding of cations by teichoic acids, because such cells will contain additional anionic components capable of binding cations.

Fig. 2 shows the binding of Mg^{2+} to teichoic acid at pH 5.0 in the presence of 10mM concentrations of Na^+, K^+ or Ca^{2+} ions. The isotherms show that binding is very similar in the presence of Na^+ or K^+ but is greatly decreased in the presence of Ca^{2+}. The Scatchard plots confirm the similarity of binding in the presence of Na^+ and K^+, but when Ca^{2+} is present the apparent association constant for Mg^{2+} binding is greatly decreased. The decrease in the apparent $K_{assoc.}$ value may be due to competition between the bivalent Ca^{2+} and Mg^{2+} ions for the binding sites (Tanford, 1962). Since the Na^+, K^+ or Ca^{2+} ions were added to the teichoic acid solution before the Mg^{2+} ions, a mixture of Na^+, K^+ and Ca^{2+} ions presumably initially occupy the binding sites. For Mg^{2+} to bind it must not only displace the other ions but must also compete against an excess concentration of them (a 10–100-fold excess in this experiment) for the binding sites. The fact that Mg^{2+} binding can be detected under such conditions therefore reflects the selectivity of teichoic acid for Mg^{2+} over Na^+, K^+ or Ca^{2+} ions. If it can be assumed that Ca^{2+} displaces Na^+ from the teichoic acid as effectively as does Mg^{2+}, then the results in Fig. 2 show that at a given free concentration of Mg^{2+} the ratio of bound Mg^{2+}/bound Ca^{2+} is significantly greater than the ratio of the free ions in solution. The observation that less Mg^{2+} is bound in the presence of Ca^{2+} indicates that the teichoic acid exhibits less selectivity for Mg^{2+} over Ca^{2+} than it does for Mg^{2+} over Na^+ or K^+ ions.

The effect of competition of Na^+ for the binding sites was further investigated by studying the effect of Na^+ concentration on Mg^{2+} binding. Fig. 3 shows that the amount of Mg^{2+} bound to the teichoic acid decreases as the concentration of Na^+ is increased. The Scatchard plots show that the apparent association constant decreases rapidly as the Na^+ concentration is raised but the total number of available sites remains approximately the same. The response of *B. subtilis* to an increase in the concentration of NaCl in the growth medium is to increase the amount of teichoic acid in the wall (Meers & Tempest, 1970; Ellwood, 1971) and thereby maintain an adequate supply of Mg^{2+}. Our results confirm that teichoic acid does bind less Mg^{2+} in the presence of increasing concentrations of NaCl and that the Mg^{2+} is less tightly bound. There is about a 20-fold decrease in the apparent association constant as the concentration of the competing Na^+ is raised from 0 to 50mM. A similar decrease was produced by a 10mM concentration of Ca^{2+} and this illustrates the relative competitive effects of univalent and bivalent ions on Mg^{2+} binding.

The experiments we have reported demonstrate the ability of isolated teichoic acid to bind selectively Mg^{2+}. This property is likely to be of significance to bacteria that contain teichoic acid in their walls. Hughes *et al.* (1973) showed that wall and membrane teichoic acids combine in providing an adequate supply of Mg^{2+} ions to the cell membrane, where they are required by various enzyme systems, to maintain membrane stability and for transport across the membrane to fulfil many functions at subcellular sites. It follows that the wall teichoic acid must possess sufficient affinity and selectivity for Mg^{2+} to be able to scavenge it from an excess of competing ions in the surrounding medium. However, the Mg^{2+} must not be so tightly bound that it cannot be transferred to the membrane teichoic acid and/or to other membrane components. Presumably the apparent association constants reported in the present paper reflect an optimum balance between these two requirements for the cells growing under the particular conditions of this experiment.

The transfer of Mg^{2+} across the wall from the medium to the membrane is unlikely to be controlled exclusively by the association constants of the wall components for Mg^{2+}. Steric and electrostatic factors arising from the architecture of the wall will influence the passage of ions. It is possible that configurational changes in the teichoic acid molecules resulting from changes in the ionic environment could affect Mg^{2+} uptake. Increasing concentrations of NaCl have been shown to change the configuration of isolated teichoic acid from an extended rod form at low concentrations to a random coil at high concentrations (Doyle & Birdsell, 1972; Doyle *et al.*, 1974). Increasing concentrations of NaCl also produce a contraction of the cell walls of Gram-positive bacteria (Ou & Marquis, 1970). The alanyl esters may be functionally involved, since their removal increases Mg^{2+} binding by the walls (Archibald *et al.*, 1973) and also causes the walls to swell (Ou & Marquis, 1970). It is clear that teichoic acid is intimately involved in the assimilation of Mg^{2+}, but the means by which regulation of uptake is achieved has yet to be established. The possibility that cation transfer between phosphate centres along the teichoic acid chain and also between teichoic acid and membrane-bound enzymes involves incompletely hydrated cations has been discussed by Hughes *et al.* (1973).

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