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The Function of Teichoic Acids in Cation Control in Bacterial Membranes

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1. The effects of teichoic acids on the Mg^{2+}-requirement of some membrane-bound enzymes in cell preparations from *Bacillus licheniformis* A.T.C.C. 9945 were examined. 2. The biosynthesis of the wall polymers poly(glycerol phosphate glucose) and poly(glycerol phosphate) by membrane-bound enzymes is strongly dependent on Mg^{2+}, showing maximum activity at 10–15mM-Mg^{2+}. 3. When the membrane is in close contact with the cell wall and membrane teichoic acid, the enzyme systems are insensitive to added Mg^{2+}. The membrane appears to interact preferentially with the constant concentration of Mg^{2+} that is bound to the phosphate groups of teichoic acid in the wall and on the membrane. When the wall is removed by the action of lysozyme the enzymes again become dependent on an external supply of Mg^{2+}. 4. A membrane preparation that retained its membrane teichoic acid was still dependent on Mg^{2+} in solution, but the dependence was damped so that the enzymes exhibited near-maximal activity over a much greater range of concentrations of added Mg^{2+}; this preparation contained Mg^{2+} bound to the membrane teichoic acid. The behaviour of this preparation could be reproduced by binding membrane teichoic acid to membranes in the presence of Mg^{2+}. Addition of membrane teichoic acid to reaction mixtures also had a damping effect on the Mg^{2+} requirement of the enzymes, since the added polymer interacted rapidly with the membrane. 5. Other phosphate polymers behaved in a qualitatively similar way to membrane teichoic acid on addition to reaction mixtures. 6. It is concluded that in whole cells the ordered array of anionic wall and membrane teichoic acids provides a constant reservoir of bound bivalent cations with which the membrane preferentially interacts. The membrane teichoic acid is the component of the system which mediates the interaction of bound cations with the membrane. The anionic polymers in the wall scavenge cations from the medium and maintain a constant environment for the membrane teichoic acid. Thus a function of wall and membrane teichoic acids is to maintain the correct ionic environment for cation-dependent membrane systems.

Teichoic acids and structurally related sugar 1-phosphate polymers occur covalently attached to the peptidoglycan in the walls of most Gram-positive bacteria (Archibald & Baddiley, 1966; Baddiley, 1972). Other teichoic acids, exclusively derivatives of poly(glycerol phosphate), are located in or on the surface of the cytoplasmic membrane of probably all Gram-positive bacteria (Hay *et al.*, 1963); these have been called membrane teichoic acids and are covalently attached to glycolipids or phosphatidylglycolipids in the membrane (Wicken & Knox, 1970; Toon *et al.*, 1972; Coley *et al.*, 1972). When *Bacillus subtilis* was grown in a chemostat under conditions of phosphate limitation, the wall teichoic acid was no longer present, but the cell walls possessed another anionic polymer containing uronic acid residues (Tempest *et al.*, 1968). Even under these conditions of phosphate limitation, however, membrane teichoic acid was present and under all other conditions of growth wall teichoic acid was also found (Ellwood & Tempest, 1968). These observations, together with the widespread occurrence of membrane teichoic acid, suggest that Gram-positive bacteria require membrane teichoic acid for their normal cellular activity, and they also require an anionic polymer in their walls; the wall polymer should preferably be a teichoic acid but, at least in some bacteria, other anionic polymers may suffice (Heptinstall *et al.*, 1970).

The presence of membrane teichoic acid between the cell wall and the surface of the cytoplasmic membrane could provide an integrated cation-exchange system between the exterior of the cell, through the wall, to the membrane itself. There, high concentrations of Mg^{2+} are required for membrane stability (Weibull, 1967; Reaveley & Rogers, 1969) and for the activity of a variety of enzymic reactions including, in bacilli, phospholipid synthesis (Patterson & Lennarz, 1971), teichoic acid synthesis (Burger & Glaser, 1964) and peptidoglycan synthesis

(Anderson *et al.*, 1972). The optimum concentrations vary from 8mM (for phospholipid) to 60mM (for peptidoglycan), most requirements falling in the range 10–30mM. We have reported preliminary results of experiments with a broken-cell preparation containing both wall and membrane (Hughes *et al.*, 1971) from which direct evidence was obtained for the function of teichoic acids as cation controllers. It was found that a membrane-bound enzyme system that required Mg^{2+} for optimum activity was fully activated by the Mg^{2+} bound to the endogenous teichoic acid and was insensitive to externally added Mg^{2+}. This contrasted with the behaviour of the same enzyme system in isolated cytoplasmic membranes lacking teichoic acid.

In the present paper we report more detailed studies and in particular experiments in which the effect of membrane teichoic acid on the Mg^{2+} requirement of membranes is compared with the behaviour of the wall-membrane system. The enzymes chosen for this work are believed to be a part of a multi-enzyme system responsible for complete wall synthesis, in which different polymer syntheses share a common undecaprenol phosphate acceptor (Anderson *et al.*, 1972; Hancock & Baddiley, 1972). Thus synthesis of each polymer is a multi-step process and it is not known which individual enzymes require bivalent cations, although it is likely that all of the reactions directly involving sugar nucleotides are cation-dependent. It is found that, not only does the teichoic acid system of wall and membrane create a suitable cationic environment for membrane enzymes independent of the external conditions, but the enzymes utilize preferentially those cations that are associated with teichoic acid rather than others provided in the suspending medium. A partial reconstruction of the teichoic acid–membrane system has been achieved by adding membrane lipoteichoic acid to a membrane preparation that is itself free from teichoic acid.

Experimental

Materials

UDP-glucose (sodium salt), lysozyme, RNAase* A from bovine pancreas, DNAase from bovine pancreas and cytidine phosphoromorpholidate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. CDP-glycerol (lithium salt) was prepared from cytidine phosphoromorpholidate by the general procedure described by Roseman *et al.* (1961).

[1-^{14}C]Glycerol and α-D-[G-^{14}C]glucose 1-phosphate were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. CDP-[^{14}C]glycerol was synthesized enzymically as described by Baddiley *et al.* (1968) from [1-^{14}C]glycerol. UDP-[^{14}C]glucose was prepared enzymically from α-D-[G-^{14}C]glucose 1-phosphate as described by Hancock & Baddiley (1972).

* Abbreviations: RNAase, ribonuclease; DNAase; deoxyribonuclease.

Methods

Growth of bacteria and preparation of enzymes. *Bacillus licheniformis* A.T.C.C. 9945 was grown in the complex medium previously described (Hancock & Baddiley, 1972), by using a 2% (v/v) inoculum of overnight culture in the same medium, under forced aeration for 3.5h at 37°C. The cells were harvested by centrifugation, and washed once in 0.6% NaCl solution.

For enzyme preparation A the washed cells were treated with lysozyme by a modification of the procedure of Burger & Glaser (1966). Cells (40g wet wt.) were suspended in 400ml of 0.05M-Tris–HCl buffer, pH7.5, containing 1mM-EDTA and lysozyme (1mg/ml), RNAase (5μg/ml) and DNAase (5μg/ml). The suspension was incubated at 37°C until cell lysis was complete as judged by microscopic examination; this was normally within 30min. The crude membrane fraction was collected by centrifugation at 25000*g* for 20min at 0°C, then washed once with the above Tris–HCl buffer, twice with aq. 0.15M-sodium pyrophosphate buffer, pH7.5, and finally with 0.05M-Tris–HCl buffer, pH7.5. The washed membranes were resuspended at the concentration shown in Table 1 in 0.05M-Tris–HCl buffer, pH7.5, containing 5mM-sodium thioglycollate.

Preparation B was obtained in the same way, except that $MgCl_2$ to a final concentration of 30mM was included in the buffer at each stage, and the pyrophosphate washes were replaced by washes in 0.05M-Tris–HCl buffer, pH7.5. The membranes were finally resuspended at the concentration given in Table 1 in 0.05M-Tris–HCl buffer, pH7.5, containing 5mM-sodium thioglycollate.

Preparation C was obtained by rupture of the cells in a French pressure cell. Cells (40g wet wt.) were suspended in 25ml of 0.05M-Tris–HCl buffer, pH7.5, containing 10mM-$MgCl_2$ and 1mM-EDTA. The slurry was passed through the cell at 4°C at the minimum pressure required for breakage (1.76×10^7 N/m^2) and the resulting suspension was incubated at 4°C for 15min with RNAase and DNAase, each at a concentration of 5μg/ml. Unbroken cells were removed by centrifugation at 5000*g* for 15min and the wall-membrane fraction was collected from the supernatant by centrifugation at 25000*g* for 30min at 0°C. The pellet was washed as described for preparation A and finally suspended at the concentration given in Table 1 in 5mM-sodium thioglycollate. All the preparations were kept frozen at −20°C in 2ml batches.

Some samples of preparations A and C were preincubated with $MgCl_2$ before assay. The membranes

were incubated with 25mM-$MgCl_2$ at pH6 for 1h at 4°C, and unbound Mg^{2+} was removed by washing four times at 0°C with water adjusted to pH6.8 with dilute aq. NH_3. Preparations treated in this way were kept frozen in water at pH6.8.

Determination of phosphate, Mg^{2+}, hexoses and protein. The phosphorus content of teichoic acids, of extracted lipids and of enzyme preparations, before and after extraction of phospholipids by the method of Bligh & Dyer (1959), and of aqueous phenol extracts was determined by the method of Chen *et al.* (1956).

Mg^{2+} was determined with an atomic absorption spectrometer (Unicam SP.20 series 2) after extraction of samples containing 0–10μg of Mg^{2+} with 5ml of 1.0M-$HClO_4$ at room temperature for 30min and removal of protein by centrifugation. Standard solutions of $MgCl_2$ containing 0–2μg of Mg^{2+}/ml were freshly prepared in 1M-$HClO_4$. Hexose was determined by the sulphuric acid–phenol method of Dubois *et al.* (1956).

Protein in enzyme preparations was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurement of the nucleic acid content of preparations. Samples (1.0ml) of the enzyme preparations were mixed with an equal volume of aq. 90% (w/v) phenol at 4°C for 30min. The phases were separated by centrifugation and the phenol phases and protein pellets were washed once for 30min at 4°C with 1.0ml of water. The combined water layers were washed repeatedly with batches (1ml) of chloroform until the aqueous phases were clear and gave a constant E_{260} reading. An approximate conversion factor of 3μg of phosphorus/ml per E_{260} unit for degraded nucleic acid was applied. A 1cm light-path was used.

Preparation and purification of teichoic acids. Cell walls of *B. licheniformis* were obtained by breaking cells (50% wet wt./vol.) with an equal volume of no. 11 Ballotini beads in a Braun disintegrator for 1min. Walls were recovered by centrifugation at 17000*g* for 30min and were cleaned by exhaustive washing in aq. 0.6% NaCl, followed by six washes with water.

Wall teichoic acids were extracted from lysozyme-treated walls as follows. Cell walls (500mg) were suspended in 50ml of 0.05M-Tris–HCl buffer, pH7.5, and incubated with lysozyme (1mg/ml) for 30min at 37°C. The suspension was mixed with an equal volume of aq. 80% (w/v) phenol at 4°C and stirred at that temperature for 4h. The aqueous phase was dialysed against water for 24h and purified by gel filtration on Sephadex G-200 as described by Burger & Glaser (1966). The molar proportions of phosphate/hexose/glycerol were 1.00:0.91:1.10, indicating that poly(glycerol phosphate hexose) comprised 90% of the material and poly(glycerol phosphate) comprised 10%. Wall teichoic acids prepared by this method are covalently linked through one end of the polymer chain to fragments of peptidoglycan (Burger & Glaser, 1966).

Membrane teichoic acid was isolated from preparation B by phenol extraction as described above, and partially purified by gel filtration on Sephadex G-200. Nucleic acids, estimated from the E_{260} of a solution, comprised less than 0.6% of the total phosphate in the preparation, but analytical ultracentrifugation showed that the preparation still contained a small amount of contaminating material; this sedimented with $s = 2.2$S as a diffuse peak and might have been degraded nucleic acid or wall teichoic acid, although the latter could not be detected chemically. The main component sedimented as a very sharp peak with $s = 6.0$S. Alkali hydrolysis of 10mg of the freeze-dried preparation yielded glycerol, glycerol phosphates and glycerol diphosphate(s) as the only observed products. However, treatment of the membrane teichoic acid (30mg) with 60% (w/v) HF at 0°C for 24h as described by Toon *et al.* (1972), followed by extraction with chloroform, yielded chloroform-soluble material, which was examined by t.l.c. on silica gel G in chloroform–methanol–water (65:25:4, by vol.) and diethyl ether–light petroleum (b.p. 40–60°C)–acetic acid (30:70:3, by vol.). The chromatograms were stained by the periodate–Schiff reagents and the major component developed colour slowly, turning blue-brown overnight. It had R_F 0.6 and 0.05 in the two solvent systems and co-chromatographed with authentic diglucosyldiglyceride from *B. licheniformis*. Smaller amounts of components, with R_F 0.2 and 0.72 in chloroform–methanol–water, were also observed by t.l.c.

The chromatographic properties of the membrane teichoic acid on gel filtration and ultracentrifugation, and the release of lipid from it by HF treatment, indicated that the polymer was a poly(glycerol phosphate) lipoteichoic acid of the type described by Wicken & Knox (1970) and Toon *et al.* (1972); it was not characterized further.

Enzymic activity and Mg^{2+}-dependence studies. The membrane and wall-membrane preparations were obtained as described above and were kept at −20°C. Biosynthesis of poly(glycerol phosphate glucose) was measured by the incorporation into the polymer of ^{14}C from UDP-[^{14}C]glucose in the presence of unlabelled CDP-glycerol. The standard reaction mixtures were as follows: for the synthesis of poly(glycerol phosphate glucose), mixtures contained 0.05ml of enzyme suspension, 0.02ml of UDP-[^{14}C]glucose (10mM, 3.5μCi/ml), 0.01ml of 20mM-CDP-glycerol, $MgCl_2$ at the appropriate concentration, and any other additions as described in the Results and Discussion section, in a total volume of 0.1ml; for the synthesis of poly(glycerol phosphate), mixtures contained 0.05ml of enzyme suspension, 0.01ml of CDP-[^{14}C]glycerol (5mM, 5μCi/ml) and 0.01ml of 20mM-CDP-glycerol (unlabelled), 0.01ml of

Tris–HCl buffer (pH 7.5) containing 1 mM-EDTA, the appropriate concentration of $MgCl_2$, and additions as described in the Results and Discussion section, in a total volume of 0.1 ml. The reaction mixtures were incubated at 37°C for 1 h, and reactions were terminated by heating in a boiling-water bath for 2 min. All assays were carried out in duplicate. Incorporation of ^{14}C into products was measured by chromatography as bands (2.5 cm) on Whatman no. 4 paper, in solvent *A* for the glucose-labelled polymer and in solvent *B* for the glycerol-labelled polymer (see below); chromatograms were run for at least 18 h. Base-line areas were cut out and radioactivity was measured in 10 ml of toluene scintillant (see below). In each of the systems saturating substrate concentrations were used so that the amount of enzyme was rate-limiting.

Hydrolysis. Acid hydrolysis of teichoic acids was carried out in 1 M-HCl at 100°C for 3 h. The product was evaporated to dryness *in vacuo* at 30°C. Alkali hydrolysis was carried out in 1 M-NaOH at 100°C for 3 h. The solution was passed through a column (10 cm × 1 cm) of Dowex 50 (NH_4^+ form) and evaporated to dryness *in vacuo*. The products were examined by paper chromatography as described below.

Chromatography. Paper chromatography of the hydrolysis products of teichoic acid was carried out on Whatman no. 1 or no. 4 paper. The solvents were: *A*, ethanol–0.1 M-ammonium acetate, pH 7.5 (5:2, v/v) (Paladini & Leloir, 1952); *B*, ethanol–0.1 M-ammonium acetate, pH 3.8 (5:2, v/v) (Paladini & Leloir, 1952); *C*, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.).

T.l.c. of lipids was carried out on layers (0.4 mm) of silica gel G (E. Merck, Darmstadt, Germany) on plates (5 cm × 20 cm) that were activated by heating at 110°C for 30 min before use.

Radioactivity measurements. Bands of chromatography paper were counted for radioactivity in 10 ml of scintillant that contained 2,5-diphenyloxazole (8 g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.2 g) in 2 litres of toluene. The efficiency of counting was about 45% for ^{14}C on paper. Aqueous samples were adjusted to 1.5 ml in water for counting in 10 ml of scintillant made by mixing the toluene scintillant with Triton X-100 (2:1, v/v); the efficiency of counting for these samples was about 70% for ^{14}C. The instrument was a Beckman LS 150 liquid-scintillation spectrometer. Counts were accumulated to a counting error of ±2% or less.

Results and Discussion

Enzyme preparations

Three different particulate preparations from *B. licheniformis* A.T.C.C. 9945, all with enzymic activity for the biosynthesis of the several teichoic acids of the organism, were used. Preparation A was obtained by treatment of exponentially growing cells with lysozyme in Tris–EDTA solution in the absence of added Mg^{2+}. Preparation B was similar to preparation A except that all the preparative steps were carried out in the presence of 30 mM-Mg^{2+}. For preparation C the cells were broken in a French pressure cell, and subsequent steps were the same as those used for preparation A after cell lysis. Table 1 gives some of the properties of the three systems and shows the ability of preparation C to bind Mg^{2+} when incubated with 25 mM-$MgCl_2$ (see under 'Methods'). The specific activity of each preparation for the biosynthesis of poly(glycerol phosphate glucose) in 15 mM-Mg^{2+} under standard conditions is given. Unless otherwise stated, preparation C was used after being preincubated with 25 mM-Mg^{2+}.

The presence of teichoic acids was investigated by chromatographic examination of the products of acid hydrolysis of preparations from which lipids had been removed by the procedure of Bligh & Dyer (1959). Preparations B and C yielded glycerol, glycerol monophosphates and glycerol diphosphate, the characteristic products of acid hydrolysis of poly(glycerol phosphate). These products were also detected in the hydrolysates of aqueous phenol extracts of preparations B and C. The nature of the material extracted by aqueous phenol from preparation B has been described above: it appeared to be a lipoteichoic acid. Similar material has been extracted from preparation C, and thus both preparations B and C contained membrane teichoic acid. Preparation C also contained wall teichoic acid and peptidoglycan as indicated by the presence of hexosylglycerol phosphates and isosaccharinic acids in alkali hydrolysates of the lipid-free material. Hydrolysates of preparation A after lipid extraction, and of phenol extracts of preparation A, revealed no products characteristic of teichoic acids or peptidoglycan. Also, as described by Hughes *et al.* (1971), preparation A derived from cells that had been grown in the presence of [G-^{14}C]-glucose contained no radioactive material that could be extracted by aqueous phenol, indicating the absence of teichoic acid. The absence of teichoic acids from preparation A is also reflected in its extremely low non-lipid phosphate content. Nucleic acids, as measured by E_{260}, comprised less than 6% of the total phosphate of aqueous phenol extracts of all the preparations, and hence the amounts of non-lipid phosphate given in Table 1 can be regarded as an approximate measure of the total teichoic acid contents of the preparations.

The results in Table 1 show that isolation of the enzyme preparation in the presence of Mg^{2+} caused the retention of the membrane teichoic acid of the cell, poly(glycerol phosphate), on the membrane in preparation B; this is in agreement with a similar finding with *Streptococcus faecalis* (Hay *et al.*, 1963),

Table 1. *Properties of the enzyme preparations*

Methods of preparation and analysis are given in the Experimental section. The dry weights were obtained by freeze-drying 1 ml samples of the preparations to constant weight. Specific activities are for the synthesis of poly(glycerol phosphate glucose) at 15 mM-Mg^{2+} in the standard procedure described under 'Methods'. Non-lipid phosphate was measured in two ways: by phosphate estimation of samples before and after removal of lipid by the procedure of Bligh & Dyer (1959), and also by phosphate estimation of aqueous phenol extracts of the preparations. The results of the two methods were in very close agreement. The values for nucleic acid content are based on the E_{260} of aqueous phenol extracts which had been exhaustively washed with chloroform (see under 'Methods').

Preparation	Dry wt. (mg/ml)	Protein (mg/ml)	Specific activity (c.p.m./mg of protein)	Teichoic acid	Membrane teichoic acid (μmol of P/mg dry wt.)	Non-lipid P (μmol/mg dry wt.)	Nucleic acid P (μmol/mg dry wt.)	Mg^{2+} (μequiv./mg)	
								Not incubated with Mg^{2+}	Incubated with Mg^{2+}
A	31.0	16.4	7800	Not detectable	0.05	0.10	0.005	0.056	0.096
B	26.0	9.4	7600	Poly(glycerol phosphate)	0.43	0.47	0.025	—	0.380
C	28.5	7.4	3800	Poly(glycerol phosphate) Poly(glycerol phosphate hexose)	0.47	1.30	0.020	0.08	1.42

and was reflected in the greater amounts of phosphate and Mg^{2+} in preparation B than in preparation A. The presence of the bound teichoic acid in preparation B caused an increase of 0.28 μequiv. of Mg^{2+} for an increase of 0.37 μmol of phosphate, compared with preparation A. On the other hand, incubation of preparation A with Mg^{2+} did not raise its content of Mg^{2+} to a significant value. About 75% of the phosphate groups of the bound teichoic acid in preparation B were associated with Mg^{2+}.

Preparation C contained wall teichoic acids; these included poly(glycerol phosphate hexose), in which the hexoses were glucose and galactose, in addition to poly(glycerol phosphate). Electron microscopy (Plate 1) showed that almost intact walls enclosed the membrane vesicles, and analysis showed that the wall components comprised about 30% of the total dry weight of the preparation. Roughly one-third of the weight of the wall material was teichoic acid, consisting of poly(glycerol phosphate) (10%) and poly(glycerol phosphate hexose) (90%). Membrane teichoic acid comprised 36% of the total teichoic acid, and the preparation thus had a similar content of membrane teichoic acid to preparation B. The only other component detected was peptidoglycan. Thus in preparation C wall-membrane contacts are almost exclusively between the outer surface of the membrane, carrying membrane teichoic acid, and the inner surface of the cell wall. This contrasts with an artificial mixture of walls and membranes in which the membrane is exposed mainly to the outside of the wall. The properties of these two systems will be compared below. Other preparations in which the membrane is retained substantially within the wall have been described for other organisms and were obtained by similar methods (Hunt *et al.*, 1959; Francis *et al.*, 1963; Cole & Hughes, 1965). The presence of the wall teichoic acid explains the very high Mg^{2+}-binding capacity of this preparation; the ratio of 1.4 μequiv. of Mg^{2+} for 1.3 μmol of non-lipid phosphate shows that virtually every phosphate group in the wall was associated with a Mg^{2+} ion; the wall therefore provided a large reservoir of bound Mg^{2+}. The specific enzymic activity of preparation C was appreciably lower than those of preparations A and B; however, the activity of a given amount of preparation C for the synthesis of poly(glycerol phosphate glucose) at the optimum Mg^{2+} concentration did not change when the wall was removed by using lysozyme. Thus the lower specific activity was not caused by interaction of wall material with the membrane, but possibly was a consequence of the method employed for preparation of the material.

For convenience all Mg^{2+}-requirement curves have been plotted with a vertical axis representing the percentage of the maximum activity displayed at each concentration of Mg^{2+}; the amount of enzyme was always rate-limiting under the conditions used.

Requirement of the enzyme preparations for Mg^{2+}

The requirements for added Mg^{2+} of the enzymes catalysing the synthesis of poly(glycerol phosphate glucose) in the three preparations are shown in Fig. 1(*a*). As was found previously (Hughes *et al.*, 1971) preparation C had no requirement for Mg^{2+} in the suspending medium when it had been pre-incubated with Mg^{2+} and then washed. After treatment with lysozyme it behaved exactly like preparation A. The effect of the bound membrane teichoic acid in preparation B was especially noteworthy; although it did not abolish the requirement of the enzyme system for Mg^{2+}, it considerably damped the response to increasing concentrations of Mg^{2+}, so that the system exhibited near-maximum activity over a greater range of concentrations of the cation. The same effects were observed for the enzyme system synthesizing poly(glycerol phosphate) (Fig. 1*b*). Since the retention of membrane teichoic acid during preparation of the enzyme was dependent on the presence of Mg^{2+}, the possibility of removing the bound teichoic acid from preparation B by treatment with EDTA was investigated. Table 2 shows the effects of various washing procedures on the amounts of bound teichoic acid and Mg^{2+} in preparation B. Washing in water in the absence of Mg^{2+} did not by itself remove bound Mg^{2+} or teichoic acid. The presence of a chelating agent in large excess was necessary for the extraction of bound Mg^{2+} (24μmol of EDTA or 400μmol of Tris–HCl buffer to remove 0.22μequiv. and 0.13μequiv. of Mg^{2+} respectively). It is particularly interesting that teichoic acid was not released at an equivalent rate to Mg^{2+} on treatment with EDTA, and this phenomenon requires close examination. The membrane obtained after washing twice with EDTA gave Mg^{2+}-dependence curves for the synthesis of teichoic acids identical with those for preparation A shown in Fig. 1, confirming that the difference in shape between the curves for preparations A and B was probably due to their different contents of bound Mg^{2+} and teichoic acid.

Effects of teichoic acids on cation requirement

The results described above showed that only in preparation C, where the membrane is surrounded by the cell wall, was the Mg^{2+}-dependence of the membrane-bound enzymes almost eliminated. Experiments were therefore carried out to investigate the contributions of the individual components of the system to this phenomenon.

Fig. 2 shows the effect of adding Mg^{2+}-saturated cell walls that were free of both membrane and membrane teichoic acid to preparations A and B. Even with preparation B, which already contained membrane teichoic acid, addition of walls did not reproduce the cation-independence observed with preparation C. There was some damping of the cation-dependence in both preparations, however, and this is discussed below. Apparently, for the almost complete independence observed with preparation C the membranes must be enclosed by the cell walls and therefore able to make contact with the walls' inner surfaces.

The possibility of reconstituting preparation B from preparation A by the addition of membrane teichoic acid was investigated. Since the detachment of membrane teichoic acid from a protoplast during removal

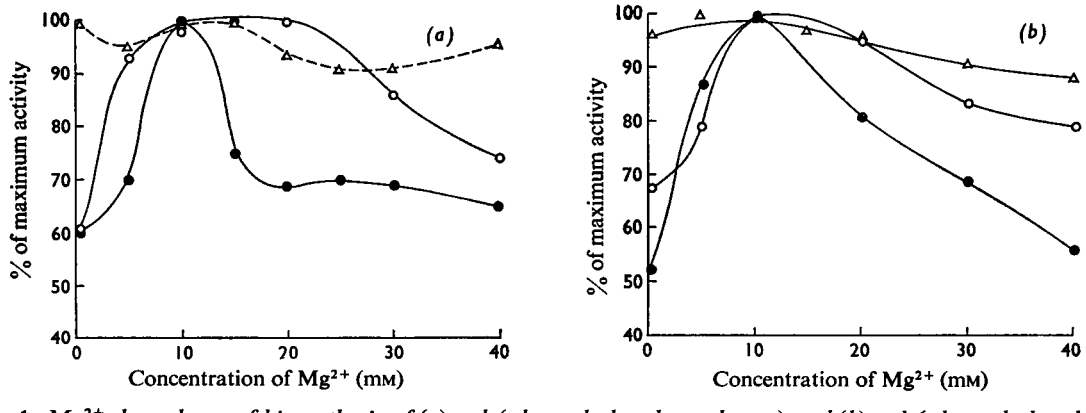
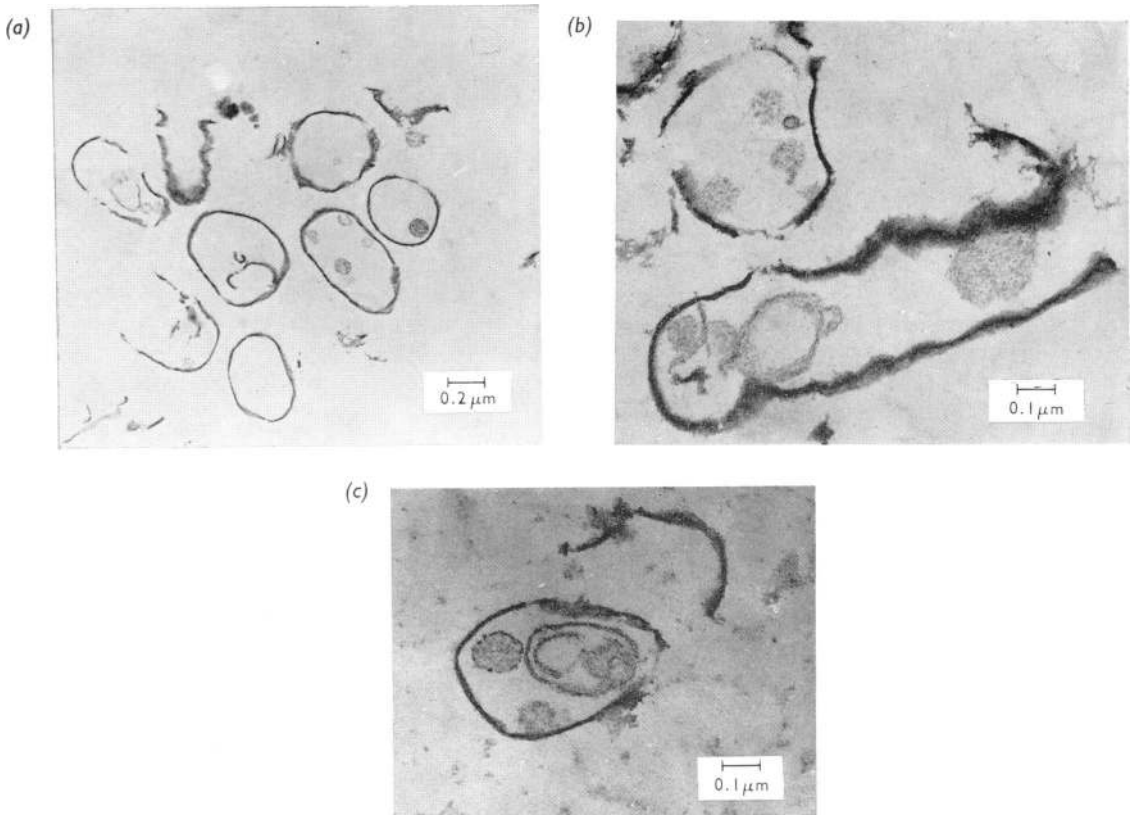


Fig. 1. *Mg^{2+}-dependence of biosynthesis of (a) poly(glycerol phosphate glucose) and (b) poly(glycerol phosphate) in preparations A, B and C*

The preparations were at the concentrations given in Table 1 and the standard assay procedure described under 'Methods' was used. Individual points on each curve were reproducible to within ±2% of maximum activity in four experiments. ●, Preparation A; ○, preparation B; △, preparation C.



EXPLANATION OF PLATE 1

Electron micrographs of thin sections of wall-membrane preparation C

The specimens were fixed with osmium tetroxide, stained with uranyl acetate, sectioned and post-stained with lead citrate (Highton, 1969).

Table 2. *Removal of teichoic acid and Mg^{2+} from preparation B*

Washings were all carried out at a concentration of 1 mg dry wt. of preparation B/ml of washing solution. The suspensions were left at 0°C for 30 min before recovery of the membrane by centrifugation at 25000*g* for 20 min. The Tris buffer was 0.05 M-Tris–HCl, pH 7.5, and the EDTA was 3 mM-EDTA (disodium salt), pH 7.5; 4.0 ml samples were used. After the membrane had been recovered by centrifugation it was resuspended in 1.5 ml of water for analysis as described for Table 1.

Procedure	Non-lipid P (μmol/mg dry wt.)	Bound Mg^{2+} (μequiv./mg dry wt.)
Original preparation	0.47	0.38
Washed twice in Tris buffer	0.37	0.25
Washed with Tris buffer as above then once with water	0.36	0.24
Washed with Tris buffer as above then twice with water	0.36	0.24
Washed with Tris buffer as above then once with EDTA	0.24	0.044
Washed with Tris buffer as above then twice with EDTA	0.15	0.026

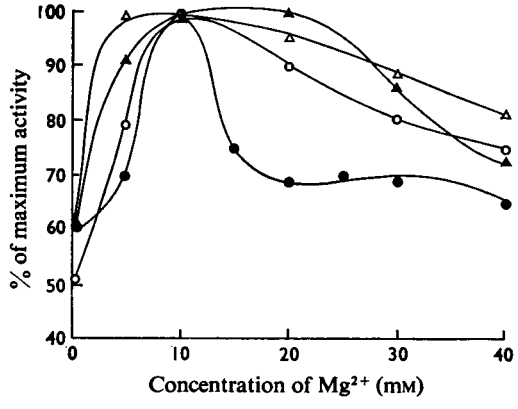


Fig. 2. *Effect of wall material containing bound Mg^{2+} on the Mg^{2+}-dependence of poly(glycerol phosphate glucose) biosynthesis in preparations A and B*

Walls of *B. licheniformis* were prepared and loaded with bound Mg^{2+} as described under 'Methods'. Wall was added to the standard assay system to a final concentration of 15 mg dry wt./ml. Each incubation thus contained 5.1 μmol of added phosphate. ●, Preparation A; ▲, preparation B; ○, preparation A+wall; △, preparation B+wall.

of the cell wall depends on the concentration of Mg^{2+} in the medium, we examined the effect of added Mg^{2+} on adsorption of radioactive poly(glycerol phosphate) to the membrane in preparation A. The labelled teichoic acid was prepared biosynthetically and was probably a mixture of wall and membrane poly(glycerol phosphate); the results are shown in Fig. 3. Adsorption of the teichoic acid was complete within 30 min at 2°C and the amount adsorbed was not increased at higher concentrations of added teichoic acid; binding showed an optimum at 10 mM-Mg^{2+}. The slight decrease in adsorption at higher Mg^{2+} concentrations was reproducible and is not understood. The binding of labelled poly(glycerol phosphate glucose) showed an identical cation-dependence profile, but a lower maximum concentration of bound phosphate was obtained (Table 3).

For a study of the effects of adsorbed polymers on enzymic activity, wall and membrane teichoic acids were purified and characterized. The membrane polymer was a lipoteichoic acid, but the lipid component has not been identified. The amounts of phosphate and Mg^{2+} in preparations containing adsorbed teichoic acids are given in Table 3. Preparation A to which membrane teichoic acid had been bound closely resembled preparation B in its content of phosphate and Mg^{2+}, although there was no indication of whether the teichoic acid interacted with the membrane in the same way in both preparations; it is possible, however, that lipophilic interactions with the membrane lipids participate in the binding as well as cation-bridging between phosphate groups in the polymer and the membrane.

Fig. 4(*a*) shows the Mg^{2+}-response of the enzyme system for synthesis of poly(glycerol phosphate) in preparation A before and after adsorption of teichoic acids. A similar study for synthesis of poly(glycerol phosphate glucose) is shown in Fig. 4(*b*). Preparation A to which membrane teichoic acid had been added beforehand closely resembled preparation B, so apparently the nature of the binding of the polymer to the membrane was sufficiently similar in both cases that they behaved in an almost identical manner; it thus seems likely that the relationship between teichoic acid, cation and membrane was similar to that in intact cells. The effect of bound wall teichoic acid was less pronounced, and this perhaps reflected the smaller amounts of phosphate and Mg^{2+} bound in this system, or the wider spacing of phosphate groups

in the poly(glycerol phosphate glucose). For these and subsequent experiments a new preparation of system A was used. It had a slightly less sharp optimum for Mg^{2+} than did the previous preparation, and this was probably due to the presence in it of traces of membrane teichoic acid. In agreement with this interpretation it contained appreciably more non-lipid phosphate and Mg^{2+} than did the original preparation, possibly arising from the presence of Mg^{2+} in the growth medium of the new cells.

Addition of teichoic acid to reaction mixtures

Since the binding of teichoic acid to the membrane preparation in the presence of Mg^{2+} was rapid and essentially complete at cation concentrations of 10mM and higher (Fig. 3), it seemed likely that the addition of teichoic acid to the reaction mixture would reproduce the effect of pre-binding the teichoic acid to the membrane. The effect of added membrane teichoic acid on the Mg^{2+}-requirement for the synthesis of poly(glycerol phosphate glucose) with preparation A is shown in Fig. 5. The same concentration of wall teichoic acid had no detectable effect on the shape of the curve, but 2mg of wall teichoic acid/ml, which has approximately the same phosphate content as 1mg of poly(glycerol phosphate)/ml, did alter the shape slightly. Membrane teichoic acid added to 0.2mg/ml did not affect the Mg^{2+}-requirement of preparation A.

Added teichoic acid thus had a qualitatively similar effect to that of membrane teichoic acid that had been bound beforehand to the membrane, but quantitative comparison would be difficult because of the dependence of binding on the concentration of Mg^{2+} in the solution and the dependence of the amount of bound Mg^{2+} on the quantity of adsorbed phosphate. In these experiments the effect of cation-complexing by the bound teichoic acid on the concentration of Mg^{2+} in aqueous solution would be small; thus if all of the added teichoic acid were bound to the membrane, and each phosphate group

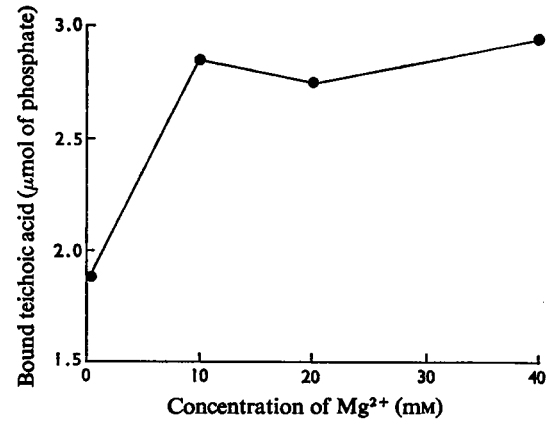


Fig. 3. *Effect of Mg^{2+} on binding of poly(glycerol phosphate) to membrane in preparation A*

^{14}C-labelled poly(glycerol phosphate) was prepared biosynthetically from a 20-fold enlargement of the standard assay system for poly(glycerol phosphate) synthesis. Water-soluble products were extracted from the reaction mixture after 1h at 37°C by mixing with an equal volume of aq. 90% (w/v) phenol for 30min at room temperature. Polymer was isolated and purified from the aqueous phase by dialysis and chromatography on Sephadex G-200 as described for the preparation of unlabelled membrane teichoic acid (see under 'Methods'). It was diluted with unlabelled membrane teichoic acid to give a final concentration of 90μmol of phosphate/ml (150000c.p.m./ml). For measurement of binding 0.25ml of preparation A, 0.2ml of labelled teichoic acid and 0.05ml of $MgCl_2$ of the appropriate concentration were mixed for 30min at 4°C. The membrane was recovered by centrifugation at 30000*g* for 15min and was then washed twice in 0.01M-Tris–HCl buffer, pH7.5, containing the same concentration of Mg^{2+} as was used in the incubation mixture. The membrane pellet was suspended in Triton–toluene scintillant for measurement of radioactivity.

Table 3. *Mg^{2+} and non-lipid phosphate content of enzyme preparations with and without bound teichoic acids*

Measurements were made as described for Table 1 and under 'Methods'. Membrane teichoic acid was bound to preparation A as follows: 10mg of purified membrane teichoic acid, 2.0ml of preparation A and 10mM-$MgCl_2$ were incubated in a total volume of 4.0ml at 4°C for 30min. The membranes were then collected by centrifugation at 25000*g* for 20min and washed once with the same buffer without Mg^{2+}. The membranes were finally resuspended in 2.0ml of the latter buffer. A similar procedure was used for binding wall teichoic acid.

Preparation	Non-lipid phosphate (μmol/mg dry wt.)	Bound Mg^{2+} (μequiv./mg dry wt.)
A	0.10	0.056
A+bound membrane teichoic acid	0.42	0.380
B	0.47	0.380
A+bound wall teichoic acid	0.30	0.130

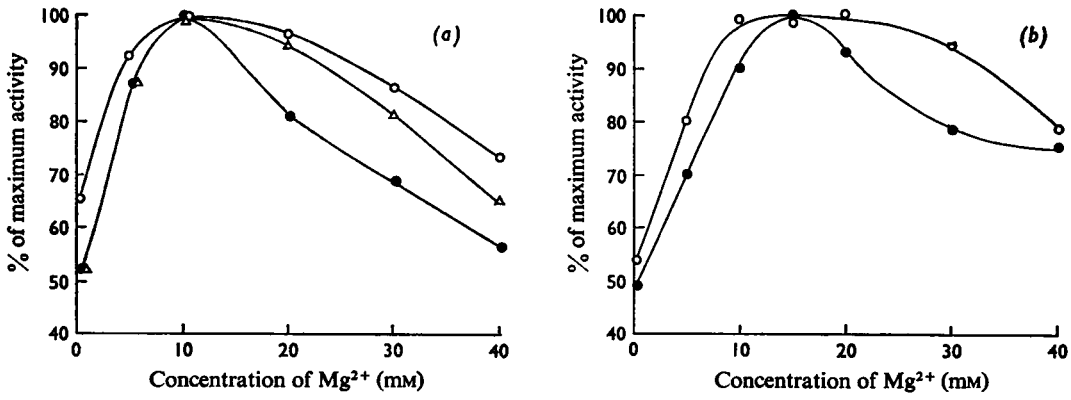


Fig. 4. *Mg^{2+}-dependence of biosynthesis of (a) poly(glycerol phosphate) and (b) poly(glycerol phosphate glucose) in preparation A to which teichoic acid had been bound*

Preparation A (2ml) was incubated with 10mg of the appropriate teichoic acid at 10mM-$MgCl_2$ in a total volume of 4.0ml for 30min at 4°C. The membrane was isolated by centrifugation at 30000*g* for 10min and washed twice with 0.01M-Tris–HCl buffer, pH7.5, containing 10mM-$MgCl_2$. The membrane was finally resuspended in 2.0ml of 0.1M-Tris–HCl buffer, pH7.5, and the suspension measured for enzymic activity by the standard procedure. All points on the curves were reproducible to within ±2% of maximum activity in three experiments. ●, Preparation A; ○, preparation A+membrane teichoic acid; △, preparation A+wall teichoic acid.

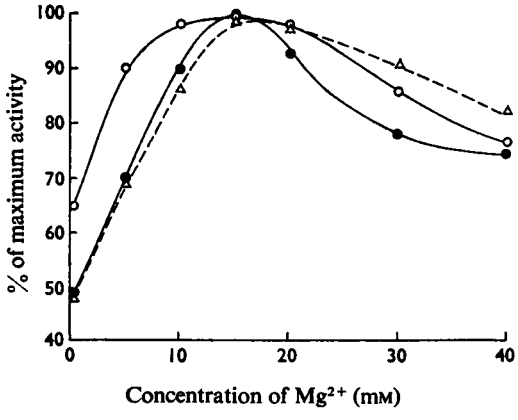


Fig. 5. *Effect of teichoic acids added to the reaction mixture on the Mg^{2+}-dependence of biosynthesis of poly(glycerol phosphate glucose) by preparation A*

The standard assay procedure was used. Membrane teichoic acid prepared as described under 'Methods' was added in aqueous solution to a final concentration of 1mg/ml, representing 0.6μmol of phosphate in each incubation mixture. Wall teichoic acid prepared as described under 'Methods' was added to a final concentration of 2mg/ml, also representing approx. 0.6μmol of phosphate in each incubation mixture. ●, Preparation A; ○, preparation A+membrane teichoic acid; △, preparation A+wall teichoic acid.

in this polymer were to combine with 1 equiv. of Mg^{2+}, then the concentration of Mg^{2+} in solution would be decreased only by about 1.5mM and there would thus be a negligible shift of the position of the dependence curve along the [Mg^{2+}] axis. The observed effects were therefore not caused simply by complexing of the added Mg^{2+} with teichoic acid, but by the interaction between teichoic acid, Mg^{2+} and the membrane.

This combined interaction provided a method for examining the effects of other polymers on the system, and it was used to investigate whether RNA could reproduce the effect of membrane teichoic acid (Fig. 6). In fact RNA led to a change in Mg^{2+}-dependence very similar to that caused by the addition of wall teichoic acid at a similar concentration of polymer phosphate. This was not unexpected, as wall teichoic acids vary considerably in their chemical structure and include polymers of glycerol phosphate or ribitol phosphate with and without glycosyl substituents, mixed polymers of glycerol phosphate and sugar 1-phosphates, as well as the closely related polymers of hexose 1-phosphate or oligosaccharide 1-phosphate (Archibald & Baddiley, 1966; Baddiley, 1972). Moreover, the substitution in some organisms of polysaccharides containing uronic acid residues for wall teichoic acids under conditions of phosphate limitation of growth indicates that acidic polymers in general are able to fulfil the function of cation binding in walls. As shown in Table 1, all the enzyme preparations contained small amounts of nucleic acid, but

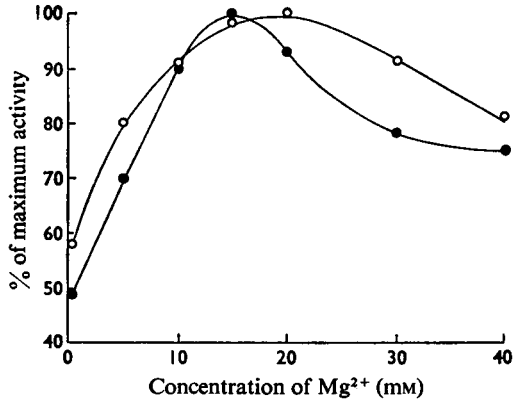


Fig. 6. *Effect of added RNA on Mg^{2+}-dependence of the biosynthesis of poly(glycerol phosphate glucose) by preparation A*

The experiments were carried out as for Fig. 5. RNA was added to a concentration of 0.65 μmol of phosphate to each incubation. ●, Preparation A; ○, preparation A+RNA.

the contents were only about 5% of those of membrane teichoic acid, and were too low to affect the Mg^{2+}-requirement curves.

The effects of addition of a bacterial-wall preparation to membrane preparations A and B (Fig. 2) have been discussed briefly above. The curves resemble those observed on the addition of purified teichoic acids to preparation A, and it is likely that the effect arises from the interaction of wall teichoic acid exposed on the surface of the wall with the membrane preparation in a similar manner to the corresponding interaction with isolated wall teichoic acid.

The most straightforward interpretation of our results is that the Mg^{2+}-dependent components of the enzyme systems that we have studied interact preferentially with Mg^{2+} ions provided by the wall-membrane teichoic acid system, rather than with those in aqueous solution. When only a relatively small amount of membrane teichoic acid is present, as in preparation B, cations must be supplied from the suspending solution to maintain the equilibrium concentration of bound Mg^{2+}; hence the system is dependent on added cations but shows little response to higher concentrations of Mg^{2+} in solution within a limited range. When wall is also present, as in preparation C, it provides a very large reservoir of bound cations, which can maintain the equilibrium concentration of Mg^{2+} in the membrane teichoic acid, the component that interacts directly with the membrane. In this case the system is almost completely insensitive to the amounts of Mg^{2+} in aqueous solution. The response of preparation C, when it had not been preincubated with Mg^{2+}, to added Mg^{2+} was previously described (Hughes *et al.*, 1971). When the teichoic acid in this preparation had not been loaded with Mg^{2+} the enzyme system synthesizing poly(glycerol phosphate glucose) showed a sharp Mg^{2+}-dependence with maximal activity at 30mM-Mg^{2+}. It was suggested (Hughes *et al.*, 1971) that this reflected a slow rate of equilibration of bound Mg^{2+} relative to the rapid initial rate of polymer synthesis, but this could not explain the inhibition by very high concentrations of Mg^{2+}. It is possible that the absence of bound Mg^{2+} from the cell wall alters the interaction between the membrane teichoic acid and the wall, and that on addition of Mg^{2+} a slow reorganization takes place.

Our results should help to emphasize the fact that membranes prepared from Gram-positive bacteria in the presence of Mg^{2+} usually contain large amounts of bound membrane teichoic acid, and hence of bound Mg^{2+}. The presence of this bound Mg^{2+}–teichoic acid is a factor that has not generally been appreciated in studies by others on membrane-bound enzyme systems. Lastras & Munoz (1972) reported differences in the behaviour of membrane-bound adenosine triphosphatase from *Micrococcus lysodeikticus* in membranes prepared in the presence and in the absence of Mg^{2+}. They found that membrane adenosine triphosphatase prepared in the presence of Mg^{2+} became more sensitive to Mg^{2+} in the assay mixture after the membranes had been washed with Tris–EDTA solution, a procedure which we have found removes bound membrane teichoic acid (Table 2). Membranes that had been prepared in the absence of Mg^{2+} showed a much less dramatic change of Mg^{2+}-dependence after washing. Lastras & Munoz (1972) concluded that the change in cation-response might be due to the loss of components that regulate the action of bivalent cations on the membrane-bound enzyme. Our work would suggest that one of these components is membrane teichoic acid.

The interaction of bivalent cations with phosphates and other ligands has been examined in detail by Hammes and his co-workers. The binding of Mg^{2+} to phosphate occurs in two steps (Hammes & Levison, 1964); the first is the formation of an ion-pair, and the second is the loss of one or more molecules of water from the first hydration shell of the cation to produce the complex. The second step is rate-limiting, and thus the rate of formation of magnesium complexes of this type is independent of the nature of the ligand. For example, Co^{2+} and Ni^{2+} complex at similar rates with pyrophosphate, tripolyphosphate, glycine, imidazole and glycylglycine (Hammes & Morell, 1964; Hammes & Steinfeld, 1962). Since the dehydration process is rate-limiting in all of these systems it is possible that direct transfer of bivalent cations from ligand to ligand, without intermediate solvation of the cation, would be energetically and kinetically more favourable than would be binding of a fully hydrated ion from aqueous solution. Thus

Table 4. *Transfer of Mg^{2+} between wall and membrane teichoic acid*

Clean cell walls, some of which had been equilibrated with Mg^{2+} and washed as described under 'Methods', were suspended at 50mg/ml in water, pH7. Membrane teichoic acid containing Mg^{2+} was prepared by phenol extraction as described under 'Methods' in the presence of 25mM-Mg^{2+}. To free this preparation of Mg^{2+} a solution was dialysed against 3mM-EDTA at 4°C for 48h, and then dialysed against water for a further 18h. Both teichoic acid preparations were made up to 10mg/ml. Reaction mixtures for the experiments contained 0.1ml of donor and 0.1ml of acceptor. They were incubated at 37°C, and wall was removed by centrifugation at 12000*g* for 15min. Mg^{2+} in pellet and supernatant was measured as described under 'Methods': 5mg of preincubated wall contained 7μmol of Mg^{2+}.

Donor	Acceptor	Incubation time (min)	Mg^{2+} transferred to acceptor (% of donor-Mg^{2+})
Wall-Mg^{2+}	Water	60	5.0
Wall-Mg^{2+}	Membrane teichoic acid	5	45.6
Wall-Mg^{2+}	Membrane teichoic acid	60	50.0
Membrane teichoic acid-Mg^{2+}	Wall	60	53.5

direct transfer of bound Mg^{2+} from wall to membrane teichoic acid, and hence to ligand groups in the membrane, could provide a satisfactory explanation of the role of teichoic acids in the bacterial-cell envelope. In this connexion we have examined the transfer of Mg^{2+} from a suspension of cell walls to membrane teichoic acid in water (Table 4). Complete equilibration of bound Mg^{2+} occurred within 5min at 37°C between wall and soluble teichoic acid, but predictably there was negligible release of Mg^{2+} from walls into water in the absence of teichoic acid acceptor and other anions. It should be noted that in the experiments in which soluble teichoic acid was added the polymer was the only anion present in the water and it therefore seems likely that the transfer of Mg^{2+} from the wall to the soluble teichoic acid was direct. Detailed kinetic studies will be required to elucidate the nature of the cation-transfer processes.

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