outer surface, similar protrusion inwards might be expected with isolated walls, whereas a difference in the gel structure of the wall could explain the morphological difference, and also the finding (Burger, 1966) that isolated walls bind little more antibody than do whole bacteria.

The inaccessibility of new teichoic acid to concanavalin A at the bacterial surface shows that the teichoic acid chains do not extend through the thickness of the wall; it also shows that concanavalin A molecules cannot penetrate the gel structure of the interior region of the wall (Lang & Archibald, 1983). This is consistent with previous work showing that walls are of low porosity (Scherrer et al., 1977). It follows that large proteins exported from the wall are unlikely to be freely diffusible through the wall both because of pore size and because of the possibilities of ionic and specific binding interactions between proteins and wall polymers. In a preliminary study (with Dr. C. R. Harwood) we have found that there is a delay between synthesis and export of proteins in B. subtilis (see Fig. 2). This delay is of the same order as the time taken for inside to outside movement of the cell wall, suggesting that a dynamic interaction between wall assembly and macromolecular export is possible. Whether export can be added to the list of functionally important interactions between proteins and teichoic acids remains to be demonstrated. However, the importance of teichoic acids in microbial physiology and in medical and industrial microbiology ensures that the next 25 years, like the last, will see increasing attention paid to the properties and functions of these most interesting polymers.

trans-membrane synthesis of cell wall polymers

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I greatly value the honour conferred upon me by the Biochemical Society and the Society for General Microbiology in arranging this Symposium. The fact that work on bacterial cell walls is being carried out today at least as vigorously as it was 30 or more years ago, when my colleagues and I discovered the nucleotides CDP-glycerol and CDP-ribitol, illustrates the steadily increasing realization of the importance of the cell wall in microbial biochemistry. This includes aspects of immunology, antibiotic action, cell biology and biotechnology.

We now understand much about the structure and biosynthesis of wall polymers in Gram-positive bacteria, although some of the details are still unsettled. My contribution to this Symposium concerns the less well under-
stood matter of translocation of material across the membrane during wall synthesis. The walls of many Gram-positive bacteria comprise a single mixed layer of peptidoglycan and teichoic acid covalently attached to each other. These polymers are synthesized from nucleotide precursors which are themselves synthesized from UTP and CTP together with the appropriate sugar phosphates and polyl phosphates. The synthesis of the nucleotide precursors is considered to be an intracellular event, although there is some evidence that the pyrophosphorylases concerned are loosely associated with the membrane. In any event, the enzymes responsible for the synthesis of the polymers from these precursors are located in the membrane and studies leading to our understanding of wall polymer synthesis have made use of membrane fragments as the enzyme source.

The steps in peptidoglycan synthesis have been established for a number of organisms, and involve the successive transfer of residues from nucleotide precursors to membrane-associated undecaprenyl phosphate. Teichoic acid synthesis occurs in an analogous manner; a linkage unit is first formed by transfer to undecaprenyl phosphate of N-acetylglucosamine phosphate, then, in at least some cases, N-acetylmuramic acid, followed by trehalose 6-phosphate residues. The presence of N-acetylmannosamine in linkage units has been established recently (Kaya et al., 1984) for a number of bacteria and this has been confirmed by C. R. Harrington (unpublished work) for Staphylococcus aureus H in a biosynthesis study where UDP-N-acetylmannosamine is required for linkage unit synthesis. However, no such requirement was found for Micrococcus varians ATCC 29750 and it is not yet known how widespread the presence of N-acetylmannosamine in linkage units.

The main chain of teichoic acid can be built up by transfer of polyol phosphate from the appropriate nucleotide to the terminal glycerol in the linkage unit, prenyl pyrophosphate. After the acquisition of D-alanyl ester residues (Neuhaus, 1985), the completed teichoic acid is transferred from its prenyl phosphate carrier, by a mechanism not yet demonstrated in vitro, to a muramic acid residue in peptidoglycan.

In an attempt to establish whether any of the steps in teichoic acid biosynthesis occur at the outer surface of the membrane, we examined synthesis using intact protoplasts of Bacillus subtilis W23 and found, surprisingly, that both main chain and linkage unit synthesis were synthesized with great efficiency from the appropriate nucleotides added to the suspending buffer (Bertram et al., 1981). Thus the complete synthesis appeared to occur at the outer surface. Moreover, synthesis was inhibited by trypsin or p-chloromercuribenzenesulphonate in the suspending buffer, neither of which is able to penetrate the membrane. However, if the enzymes are normally located on the outer surface of the membrane, then the nucleotide precursors and their products UMP and CMP would have to pass through the membrane during synthesis in whole cells, since the precursors in whole cells are intracellular and, as there is no loss of CMP or UMP during synthesis, these nucleotide products would have to return to the cell. It was shown that in our experiments with protoplasts there was no loss of cellular nucleotides to the medium during synthesis, nor did CMP or UMP penetrate the membrane in either direction.

If we next consider the alternative model in which the enzymes are located on the inner surface of the membrane, similar difficulties are encountered. In this case the normal situation is one in which both nucleotide precursors and the products CMP and UMP remain within the cell and it is necessary to postulate a pore or similar device through the membrane to allow passage to the outside of synthesized polymer. However, the results of the experiments with protoplasts would not be explained readily. As well as the difficulty in explaining the experiments with inhibitors, it would be necessary to postulate transport of both nucleotide precursors and the nucleotide products across the membrane, in conflict with our findings.

The possible participation of membrane-bound carriers was also considered. Under normal conditions in the living cell this would imply a reaction at the inner surface of the membrane between the nucleotide precursors and the carrier(s) yielding nucleotide products within the cell and carrier complex(es) in which the sugar phosphate or polymer phosphate residue(s) are covalently attached to the presumably protein carrier. After re-arrangement or rotation the residues, now on the outer surface, are transferred to the growing polymer. In the experiments with protoplasts, nucleotide precursors in the suspending medium would react with acceptor sites on those carriers which happen to be directed towards the outside. If membrane-bound carriers are involved, however, it should be possible to demonstrate with protoplasts an exchange reaction between the carrier(s) and CDP-ribitol when these nucleotides are present in the suspending buffer. No such interchange was observed, either with protoplasts or with membrane fragments, and we conclude that translocation does not occur in this manner.

The studies on teichoic acid synthesis have been followed by an examination of the ability of protoplasts to synthesize peptidoglycan from externally supplied nucleotide precursors (Harrington & Baddiley, 1983). In contrast to the vigorous synthesis observed for teichoic acid, there was barely detectable synthesis of peptidoglycan from the appropriate labelled nucleotide precursors. This was understandable, as the protoplasts were prepared by the removal of the walls of the bacteria by incubation with lysozyme, some of which invariably adheres to the surface of the protoplasts. Efforts to remove this were unsuccessful but the synthesis cycle must have occurred as labelled lipid intermediates were detected and their formation was inhibited by tunicamycin and by bacitracin. We then resorted to the use of the autolysins present naturally in the bacillus. Whole bacteria are considered to be impermeable to nucleotides, and this was supported by the observation that suspensions of fresh cells did not synthesize either teichoic acid or peptidoglycan from nucleotides in the suspending medium. Partly autolysed, osmotically stabilized cells were obtained by incubation for about 20 min in a buffer, at pH 8.5, containing sucrose. This preparation, in which the cell membranes were intact, readily synthesized peptidoglycan from the two nucleotide precursors, UDPGlcNAc and UDP-MurNAc-pentapeptide, in the suspending buffer. Synthesis was inhibited by trypsin and by p-chloromercuribenzenesulphonate and in these experiments also no passage of nucleotides occurred in either direction between the cells and the medium. No transphosphorylation reaction was observed with UDPGlcNAc and the partly autolysed cells.

It appears then that with protoplasts or partly autolysed, stabilized cells both of the cell wall polymers are readily synthesized from externally supplied nucleotides, the enzymic activity can be detected on the outer surface of the membrane and there is neither transphosphorylation via carriers nor transfer of nucleotides across the membrane during synthesis.

It was shown that the teichoic acid-synthesizing enzymes in M. varians could be solubilized with Triton X-100 and density gradient centrifugation indicated that they com-
prise a tight complex or small number of complexes containing only a few polypeptide chains to which the undecaprenyl phosphate is firmly associated (Leaver et al., 1981). Taking this into account, a simple interpretation of the experiments with protoplasts and autolysed cells would be that the complex, together with its bound prenyl phosphate, occupies a trans-membrane location and either rotates or re-orients itself such that active sites are first exposed on the inner surface where successive interactions with nucleotide precursors occur, thereby transferring residues to the prenyl phosphate. As loading of the lipid occurs the complex either rotates or re-arranges so that growing polymer attached to its lipid emerges from the outer surface. At this point active sites on the enzymes become temporarily exposed on the outer surface and it is this that enables synthesis to be demonstrated on the outer surface or protoplasts (Fig. 1). In the illustration the model comprises a single rotating complex; two or more adjacent complexes could be envisaged, however, and re-arrangement within the complex, rather than rotation, is a possibility.

A major question posed by such a model is the amount and source of energy required to bring about translocation. The nature of the environment within the membrane in the immediate vicinity of the enzyme complex is not known and so it is not possible to assess the forces exerted between complex and membrane. Nevertheless, it seems unlikely that the bond energy changes during synthesis of the polymers from precursors would be able to provide the necessary driving force. We have therefore examined the possibility that the electrochemical proton gradient of the membrane might provide the energy (Harrington & Baddiley, 1984).

In the glycerol auxotroph, Bacillus subtilis 61360, peptidoglycan synthesis was inhibited by the uncouplers dinitrophenol and carbonylcyanide m-chlorophenylhydrazone, with the accumulation of nucleotide precursors. These uncouplers also affect the transport of N-acetylgalactosamine and have been reported to initiate autolytic activity, so the study was extended to teichoic acid synthesis in vitro and the effect of the ionophore valinomycin. It was found that whereas valinomycin caused little or no inhibition of glycerol incorporation into the cell, or into the nucleotide precursor CDP-glycerol within the cell, under certain conditions marked inhibition of wall teichoic acid synthesis was observed. Similarly, inhibition of peptidoglycan synthesis was noted, accompanied by the accumulation of UDP-MurNAc-peptides. In contrast, valinomycin did not inhibit the synthesis of wall teichoic acid by membrane preparations, nor did it inhibit lipoteichoic acid synthesis in vitro. From the action of valinomycin at different pH values and ionic concentrations, it was concluded that the electrochemical proton gradient, rather than either one of its components (Δψ and ΔpH), is essential for wall synthesis in the living cell. This dependence of wall synthesis on the integrity of the electrochemical proton gradient is consistent with our model.

I should like to close by acknowledgement of my appreciation of the skills, ingenuity and devoted efforts of my many co-workers over the years. They are too numerous to name individually but I have had the greatest pleasure in our association and friendship.

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The cell walls of Bacillus subtilis and Bacillus licheniformis consist mainly of peptidoglycan, teichoic acids and teichuronic acid. In the work described here we have used mainly B. subtilis W23, in which the teichoic acid is poly(β-glucosyl ribitolphosphate) attached to a muramic acid residue of peptidoglycan by a 'linkage unit' consisting of three glycerol phosphate residues, and an N-acetyl glucosamine 1-phosphate residue that is phosphodiester-linked to the muramic acid (Coley et al., 1978). Recent work by Ito's group indicates that an additional N-acetylmannosamine residue may intervene between N-acetylgalactosamine and the first glycerol phosphate linkage unit (Kojima et al., 1985) although it is not clear whether this is essential for biosynthesis in vitro. The linkage unit is synthesized from UDP-N-acetylgalactosamine and CDP-glycerol and is assembled on a polysoprenylphosphate-carrier lipid. Subsequently the main poly(ribitolphosphate) chain is assembled on the lipid-bound linkage unit (lipid-LU) by sequential additions of ribitolphosphate units from CDP-ribitol. All these reactions are catalysed by membrane-bound enzymes (Hancock, 1981). Teichuronic acid, which only appears in the walls of B. subtilis at low phosphate concentrations but is synthesized constitutively by B. licheniformis, is a polymer of equimolar amounts of glucuronic acid and N-acetylgalactosamine, probably with a disaccharide repeating unit structure. Ward & Curtis (1982)

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The regulation of synthesis of wall polymers and of wall assembly in Bacillus

B. subtilis is an auxotroph for glycerol, the precursor for teichoic acid synthesis. The cell walls of B. subtilis and B. licheniformis consist mainly of peptidoglycan, teichoic acids and teichuronic acid. In the work described here we have used mainly B. subtilis W23, in which the teichoic acid is poly(β-glucosyl ribitolphosphate) attached to a muramic acid residue of peptidoglycan by a 'linkage unit' consisting of three glycerol phosphate residues, and an N-acetyl glucosamine 1-phosphate residue that is phosphodiester-linked to the muramic acid (Coley et al., 1978). Recent work by Ito's group indicates that an additional N-acetylmannosamine residue may intervene between N-acetylgalactosamine and the first glycerol phosphate linkage unit (Kojima et al., 1985) although it is not clear whether this is essential for biosynthesis in vitro. The linkage unit is synthesized from UDP-N-acetylgalactosamine and CDP-glycerol and is assembled on a polysoprenylphosphate-carrier lipid. Subsequently the main poly(ribitolphosphate) chain is assembled on the lipid-bound linkage unit (lipid-LU) by sequential additions of ribitolphosphate units from CDP-ribitol. All these reactions are catalysed by membrane-bound enzymes (Hancock, 1981). Teichuronic acid, which only appears in the walls of B. subtilis at low phosphate concentrations but is synthesized constitutively by B. licheniformis, is a polymer of equimolar amounts of glucuronic acid and N-acetylgalactosamine, probably with a disaccharide repeating unit structure. Ward & Curtis (1982)