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Synthesis of Peptidoglycan and Teichoic Acid in *Bacillus subtilis*: Role of the Electrochemical Proton Gradient

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The effects of several ionophores and uncouplers on glycerol and *N*-acetylglucosamine incorporation by *Bacillus subtilis* 61360, a glycerol auxotroph, were tested at different pH values. In particular, the effect of valinomycin on the synthesis of teichoic acid and peptidoglycan was examined in more detail in both growing cells and in vitro biosynthetic systems. Valinomycin inhibited synthesis of wall teichoic acid and peptidoglycan in whole cells but not in the comparable in vitro systems. It did not inhibit formation of free lipid or lipoteichoic acid. The results were consistent with a role for the electrochemical proton gradient in maintaining full activity of cell wall synthetic enzymes in intact cells. Such an energy source would be required for a model in which rotation or reorientation of synthetic enzyme complexes is envisaged for the translocation of wall precursor molecules across the cytoplasmic membrane (Harrington and Baddiley, J. Bacteriol. 155:776–792, 1983).

The cell walls of *Bacillus subtilis* contain peptidoglycan and either teichoic acid or teichuronic acid, which are synthesized from appropriate nucleotide precursors formed in the cytoplasm. The molecular mechanism by which the substrates reach their destination in the wall is not understood. The problem of translocation has been investigated recently (4, 22) with in vitro systems in which nucleotide precursors are supplied externally to an intact plasma membrane. In a study with protoplasts of *B. subtilis* W23, Bertram et al. (4) proposed that the teichoic acid biosynthetic enzyme complex spans the membrane and either rotates or reorients in such a way that the polymer dissociates at the outer surface, and the sites for nucleotide interaction become temporarily exposed to the external environment. We previously studied (22) the synthesis of peptidoglycan by intact cells undergoing partial wall autolysis and reached similar conclusions, although it was still possible that the wall-synthesizing complexes had been displaced from the membrane in the in vitro systems. Whereas displacement of the complexes does not occur in vivo, rotation of transmembrane complexes remains a plausible explanation for translocation in vivo; energy for such rotation or reorientation might arise from the electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) as previously suggested (22).

The electron transport chain and the ATPase complex in bacteria generate the electrogenic transport of protons out of the cell. Several molecular devices exist whereby protons can return to the cytoplasm while performing work, e.g., ATP synthesis, transhydrogenation of pyridine nucleotides, motility, and the transport of many metabolites (20). Proton movement has two consequences: (i) it produces an asymmetric distribution of protons (pH gradient, ΔpH) in which the cytoplasm is alkaline with respect to the outside, and (ii) it creates an electrical voltage gradient (membrane potential, $\Delta\psi$, interior negative). Together, ΔpH and $\Delta\psi$ constitute $\Delta\tilde{\mu}_{H^+}$ according to the equation:

$$\Delta\tilde{\mu}_{H^+} = \Delta\psi - \frac{2.3\,RT}{F}\Delta\text{pH}$$

where R, T, and F are the gas constant, absolute temperature, and Faraday constant, respectively. Selective ionophores (16, 38) can be used to manipulate ΔpH and $\Delta\psi$ qualitatively in media of different pH values and ionic compositions without direct measurement of the individual parameters. For example, this has been done in the study of active transport in vesicles from *Escherichia coli* (27). At low external pH values, the major component of $\Delta\tilde{\mu}_{H^+}$ is ΔpH, which is sensitive to carboxylic ionophores such as nigericin or monensin, which exchange cations for protons. At higher external pH values, the ΔpH decreases, and the remaining $\Delta\psi$ is sensitive to electrogenic ionophores such as valinomycin, which selectively conducts K^+ ions, or to the lipophilic cation methyltriphenyl phosphonium. On the other hand, lipophilic weak acids such as 2.4-dinitrophenol (DNP) or carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) increase the conductance of protons through membranes and act as classical uncouplers by uncoupling ATP synthesis from the $\Delta\tilde{\mu}_{H^+}$ and dissipating both the $\Delta\psi$ and ΔpH.

In a preliminary report (23) we showed that peptidoglycan formation in *B. subtilis* W23 is inhibited by the uncouplers DNP and CCCP; this occurs with an accumulation of the nucleotide precursors involved in peptidoglycan synthesis. However, since these uncouplers also affect the transport of *N*-acetylglucosamine (GlcNAc) (23) and have been reported to initiate autolytic activity (26), the study was extended to the synthesis of teichoic acid in vivo. A glycerol auxotroph (34) was used to allow maximum incorporation of glycerol into teichoic acid. In addition to containing peptidoglycan, the cell walls of the parent strain (*B. subtilis* 168) contain a poly(glycerol phosphate) substituted with α-D-glucopyranosyl and D-alanyl residues (5, 14, 48) and two wall polymers containing *N*-acetylgalactosamine (10, 42). Recently, the existence of covalently linked proteins or polypeptides in the walls of *B. subtilis* 168 has been reported (35). In addition, a poly(glycerol phosphate)-containing membrane teichoic acid (lipoteichoic acid, LTA) is present (8). Although valinomycin causes little or no inhibition of glycerol incorporation into the cell or into the nucleotide precursor cytidine 5′-diphosphate (CDP) glycerol within the cell under appropriate conditions, we observed a marked inhibition of wall teichoic acid but not LTA formation, implicating the electrochemical proton gradient in translocation during wall formation. Studies on peptidoglycan synthesis also showed inhibition by valinomycin.

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MATERIALS AND METHODS

Chemicals. The following radioactive compounds were obtained from Amersham International (Amersham, England) p.l.c.: UDP-*N*-acetyl-D-[*U*-^{14}C]glucosamine (282 Ci/mol), *N*-acetyl-D-[1-^{14}C]glucosamine (54 Ci/mol), D-[*U*-^{14}C]glucose (292 Ci/mol), [2-^{3}H]glycerol (1 Ci/mmol), [*U*-^{14}C]glycerol (170 Ci/mol), L-[*U*-^{14}C]arginine · HCl (10.7 Ci/mol), and L-[*ring*-2-^{14}C]histidine (33 Ci/mol). Hexokinase, inorganic pyrophosphatase, glycerol kinase, DL-α-glycerol-1-phosphate, α-glucose-1,6-diphosphate, chloramphenicol, nonradioactive nucleotides, ionophores, and uncouplers were obtained from Sigma Chemical Co. Ltd. (London, England). Polyethylene glycol 6000 was purchased from Hopkin and Williams (Chadwell Heath, England), Amberlite IR-120 was purchased from BDH Laboratories (Poole, England), and other chemicals and solvents were of analytical reagent grade or purchased as before (22).

Analytical procedures. Protein and radioactivity determinations and preparation of tunicamycin solutions were as described previously (22). Descending paper chromatography on Whatman 3MM or no. 1 paper (acid-washed before preparative or analytical separations) was done at room temperature in one of the following solvent systems: A, 96% ethanol–0.5 M ammonium acetate (pH 3.8) (5:2, vol/vol); B, isobutyric acid–0.5 M aqueous ammonia (5:3, vol/vol); C, propan-1-ol–ammonia (specific gravity of 0.88)–water (6:3:1 vol/vol/vol). Standards were visualized on chromatograms by alkaline silver nitrate (45) for reducing sugars, UV light for nucleotides, and a dip for phosphoric esters (19). Free lipids were separated by thin-layer chromatography on Polygram SilG plates (0.25 mm thick; Camlab, Cambridge, England), developed in chloroform-methanol-water (65:25:4 vol/vol/vol), and visualized by autoradiography.

Organisms and cultivation media. *B. subtilis* 168 (*trp*) from laboratory stock was maintained and cultivated as described previously (22). *B. subtilis* 61360 (*trp his glyC glpD*), originally supplied by E. Freese (37), was a gift from I. C. Hancock (University of Newcastle upon Tyne, England). It was isolated from *B. subtilis* 168 by Mindich (34) and is a double mutant that lacks the NAD-dependent *sn*-glycerol-3-phosphate dehydrogenase and requires glycerol for growth. It was maintained on agar plates containing tryptone (1%; Difco Laboratories, East Molesey, England), yeast extract (0.5%; Difco), and glycerol (100 μg/ml). *B. subtilis* 61360 was harvested from overnight cultures in antibiotic medium no. 3 (Difco) containing glycerol (100 μg/ml). Cells were inoculated (5%, vol/vol) into a minimal salts medium [0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% trisodium citrate dihydrate, 0.02% $MgSO_4 \cdot 7H_2O$] (43) supplemented with 0.5% glucose and a 20-μg/ml concentration each of tryptophan, histidine, and glycerol. This medium had a pH of 7.0, and the mean generation time in it was 90 min. Cells were also grown in the same medium with 2% glucose and with the pH adjusted to 5.8 or 7.5 by using HCl or NH_3 solution, respectively. The mean generation time was the same at both pH values (80 min). *Saccharomyces cerevisiae* S288-C, obtained from R. O. Nicholas, was grown in yeast extract (1%), Bacto-Peptone (2%; Difco), and glucose (2%) at 30°C with aeration.

Preparation of nucleotides. UDP-*N*-acetylmuramyl pentapeptide was prepared as described previously (22). UDP-[^{14}C]glucose (292 Ci/mol) was prepared from [*U*-^{14}C]glucose by using a 40 to 60% ammonium sulfate fraction obtained from autolyzed *S. cerevisiae* S288-C (47) as a source of UDP-glucose pyrophosphorylase. The reaction mixture contained [*U*-^{14}C]glucose (100 μCi, 340 nmol), UTP (1.5 μmol), Tris-hydrochloride (6.75 μmol, pH 7.8), inorganic pyrophosphatase (1 U), hexokinase (0.8 U), phosphoglucomutase (3.7 U), and UDP-glucose pyrophosphorylase (0.5 mg of protein) in a total volume of 0.25 ml. The mixture was incubated for 2.5 h at 30°C, stopped by immersion in a boiling water bath for 2 min, applied to Whatman 3MM paper, and developed in solvent A for 18 h. UDP-glucose was eluted with water, treated with alkaline phosphatase to remove traces of glucose phosphates, and separated again by paper chromatography.

CDP-glycerol was prepared from the morpholidate of CMP by the general procedure described by Roseman et al. (40). CDP-[2-^{3}H]glycerol was prepared enzymically. [2-^{3}H]glycerol was phosphorylated by using ATP and glycerokinase at 37°C in glycine-sodium hydroxide (50 mM, pH 9.8) containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. CDP-glycerol pyrophosphorylase was isolated from *B. subtilis* 168, fractionated as described by Shaw (41), and used to convert L-α-[2-^{3}H]glycerol phosphate to CDP-[2-^{3}H]glycerol (1 Ci/mmol) as described by Baddiley et al. (3).

The concentration of nucleotides was based on a molar extinction coefficient at 262 nm (ε_{262}) for uridine of 10,100 and an ε_{280} for cytidine of 12,800, both at pH 2.

Preparation of protoplasts, partly autolyzed cells, and membranes. Protoplasts and partly autolyzed cells were prepared as described previously (22). However, protoplast formation was complete in 30 min at 37°C with *B. subtilis* 168.

Membranes were prepared by sonication of protoplasts suspended in either Tris-hydrochloride (50 mM, pH 7.5) containing 10 mM $MgCl_2$ (buffer A) or the same buffer containing 20% (wt/vol) polyethylene glycol 6000 (buffer B). The former membranes were harvested at 100,000 × *g* for 60 min at 4°C, and the latter were harvested at 12,000 × *g* for 10 min and washed once with buffer B. Both membrane fractions were suspended in buffer A at a concentration of ca. 0.5 mg of membrane protein ml^{-1}.

Enzyme determinations. Peptidoglycan synthesis was measured as described previously (22) by using partly autolyzed cells. Teichoic acid synthesis was measured by monitoring the incorporation of [2-^{3}H]glycerol from CDP-[2-^{3}H]glycerol into material that remained immobile at the origins of the paper chromatograms. Standard incubation mixtures (125 μl) containing CDP-[2-^{3}H]glycerol (1.07 mM, 0.75 Ci/mol), 10 mM $MgCl_2$, 40 mM Tris-hydrochloride (pH 7.5), 0.5 M sucrose, and either a cell or protoplast suspension (100 μl) were incubated at 37°C, and synthesis was stopped by immersion in a boiling water bath for 2 min. The reaction mixture was applied to Whatman 3MM paper, developed in solvent A for 18 h, and processed as described before for peptidoglycan synthesis (22). For the separation of linkage unit lipid intermediates, chromatograms were developed in solvent B for 20 h as described previously (33).

Glucosylation of teichoic acid was determined similarly. The standard incubation mixture (125 μl) contained the following nucleotides: CDP-glycerol (1.07 mM), UDP-GlcNAc (0.4 mM), and UDP-[^{14}C]glucose (0.4 mM, 1 Ci/mol). Endogenous lipids and galactosamine-containing polymer (10, 42) may also accept glucose from UDP-glucose in this assay. Similar results were obtained by a filtration assay in which the polymer was precipitated by 0.15 M perchloric acid (6).

GlcNAc incorporation. Routinely, cultures (100 ml) were grown in minimal salts medium (pH 7.5) with glucose (0.5%) to mid-exponential phase. At ca. 0.13 mg (dry weight of cells) ml^{-1}, [^{14}C]GlcNAc (2 μCi, 58 Ci/mol) was added, and

incubation was continued at 37°C. Cultures were harvested at 4°C, washed twice with water, and finally suspended in water (5 ml). The cell pellet (obtained by centrifugation at 40,000 × *g* for 10 min) was then treated as described by Mobley et al. (36) to give five fractions; four were soluble in 5% (wt/vol) trichloroacetic acid (TCA; 0°C, 16 h), DNase with RNase (37°C, 4 h, pH 7.0), trypsin (37°C, 4 h, pH 7.3), and lysozyme (37°C, 16 h, pH 6.5) respectively, and there was an insoluble residue. Radioactivity in all the fractions was estimated with Triton-toluene scintillation cocktail (3 ml). Most of the radioactivity was found in the TCA (soluble precursors) and the lysozyme (peptidoglycan) extracts.

Glycerol incorporation. *B. subtilis* 61360 was grown in minimal salts medium, which was supplemented with amino acids, glycerol, and glucose, to mid-exponential phase (ca. 0.13 mg [dry weight] ml^{-1}). To 100 ml of culture, [2-^{3}H]glycerol (100 μCi, 1 Ci/mmol) or [*U*-^{14}C]glycerol (17 μCi, 170 Ci/mol) was added, and incubation was continued at 37°C. Cells were collected by centrifugation or on Whatman GF/C filters (21 mm in diameter) and washed with distilled water. Free lipids were extracted with chloroform-methanol (2:1, vol/vol) as described previously (34) and backwashed with 0.88% KCl as described by Folch et al. (12). Lipids were concentrated to dryness under nitrogen and radioactivity was counted in toluene scintillation cocktail (10 ml).

Material not extracted by the chloroform-methanol mixture was routinely estimated for radioactivity remaining on the filters, which was referred to as the total teichoic acid fraction. For detailed analyses, LTA and wall teichoic acids were separated. Defatted pellets were suspended with sodium acetate (50 mM, pH 4.7), and LTA was extracted by mixing the suspension with an equal volume of 80% (wt/vol) aqueous phenol at 4°C for 40 min (8). The upper phase (LTA) was retained, and the phenol layer and interface were filtered through Whatman GF/C filters (21 mm in diameter), which were rinsed with distilled water. The wall teichoic acid was solubilized from the filters by dilute alkali (0.1 M sodium hydroxide at 35°C for 16 h) under nitrogen (25). Sodium ions were removed on a small column of Amberlite IR-120 (H^+ form). Less than 5% of the radioactivity remained insoluble after this treatment.

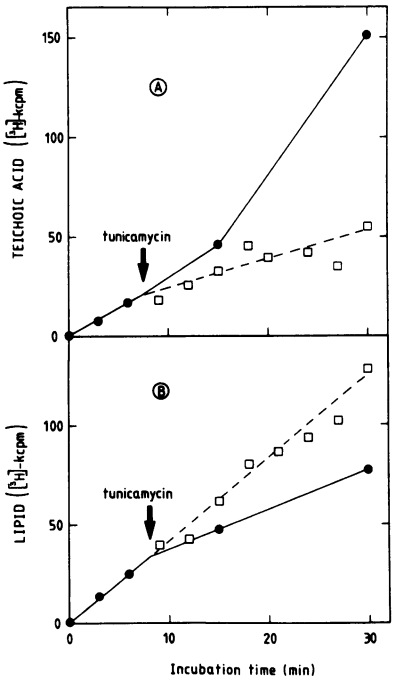

FIG. 1. Effect of tunicamycin on glycerol incorporation in *B. subtilis* 61360. Cells grown at pH 7.0 were incubated with [2-^{3}H]glycerol, and samples (10 ml) were separated into total teichoic acid (A) and free lipid (B) fractions, as described in the text. Tunicamycin (5 μg/ml; □) or alkali (control; ●) was added to cultures after 8 min (arrows).

Analysis of teichoic acids. Teichoic acids were hydrolyzed with 1 M sodium hydroxide at 100°C for 3 h (29), and sodium ions were removed on columns of Amberlite IR-120 (H^+ form). Acid hydrolysis was done with 2 M hydrochloric acid at 100°C for 2 h. The hydrolysate was frozen, and HCl was removed in vacuo over KOH. Hydrolysates were either treated with alkaline phosphatase (50 U) in 50 mM ammonium carbonate (pH 9.3, 37°C, 30 min) and separated by paper chromatography on Whatman no. 1 paper developed in solvent C for 16 h or separated directly in the same solvent system (29).

Analysis of TCA extracts. TCA was removed from extracts by washing with 3 equal volumes of ether. Neutralized extracts were separated on Whatman no. 1 paper developed in solvent A (peptidoglycan) or solvents A and C (teichoic acid) for the appropriate soluble cell wall precursors.

Amino acid uptake. *B. subtilis* 61360 was grown to mid-exponential phase as described above. Cultures (50 ml) were used to measure the uptake of [^{14}C]arginine (0.5 μCi, 1.75 Ci/mol) or [^{14}C]histidine (2 μCi, 0.3 Ci/mol) directly at 37°C. Samples, in duplicate, were filtered rapidly through Whatman GF/C filters (21 mm in diameter) and washed with either 5 ml of minimal salts solution (room temperature) for total pool size or 5 ml of ice-cold 10% (wt/vol) TCA for incorporation into protein. Filters were dried and counted in toluene scintillation cocktail (3 ml).

Preparation of ionophores and uncouplers. Stock solutions of ionophores and uncouplers were dissolved in acetone (valinomycin, 5 mg/ml) or ethanol (nigericin, 100 μM; methyltriphenyl phosphonium bromide, 100 mM; DNP, 10 mM; CCCP, 1 mM; monensin, 1 mg/ml).

RESULTS

Glycerol incorporation by *B. subtilis* 61360. Exponential cultures starved of glycerol continued to grow as fast as did cultures supplemented with glycerol (20 μg/ml) for at least 2 h. However, since glycerol incorporation and the rate of lipid formation were not affected significantly by brief starvation (30 min), cells were not washed free of glycerol before the addition of radioactive glycerol. Incorporation was identical with [^{3}H]- or [^{14}C]glycerol and amounted to ca. 0.4 nmol of glycerol incorporated per min per ml of culture. This represented 5.6% uptake after 30 min of incubation, and the incorporation was linear with time for at least 40 min. The incorporation of glycerol into free lipid and total teichoic acid fractions is shown in Fig. 1, and see Fig. 2. Tunicamycin (5 μg/ml) inhibited teichoic acid synthesis (Fig. 1A), whereas there was an increase in the amount of free lipid formed (Fig. 1B), indicating that the lipid fraction did not include significant amounts of the lipid intermediates involved in wall teichoic acid synthesis. The remaining tunicamycin-resistant teichoic acid formation (Fig. 1A) probably reflected the synthesis of LTA (see below). The lipid fraction consisted of phospholipids and neutral lipids based upon their separation by thin-layer chromatography (data not shown), although their exact nature was not characterized.

Teichoic acids were analyzed from cultures pulsed with [^{14}C]glycerol. LTA, which was extracted with aqueous

phenol, made up only 7 to 8% of the total teichoic acid fraction. Less than 5% of the LTA and wall teichoic acid fractions migrated from the origins of chromatograms developed in solvent C for 16 h. Alkaline hydrolysis of both fractions gave results that were consistent with the existence of polymers of glycerol phosphate substituted to various extents with α-D-glucopyranosyl residues (14): substituted polymer was resistant to alkali but was hydrolyzed to a mixture of glycerol phosphates and glycerol by hydrochloric acid, whereas nonsubstituted polymer was completely hydrolyzed to a mixture of glycerol phosphates and glycerol with both alkali and acid; glycerol phosphates were converted to glycerol by alkaline phosphatase. Although glucosyl glycerol was not isolated by hydrofluoric acid degradation of the polymers (14), the results of alkaline hydrolysis indicated that the extents of glucose substitution were 30 and then 15% for LTA and 11 and 35% for wall teichoic acid (the two values correspond to samples taken 15 and 30 min, respectively, after the addition of [^{14}C]glycerol). D-Alanyl residues that substituted for wall polymers would have been lost during their alkaline isolation.

Effect of ionophores and uncouplers on glycerol incorporation by *B. subtilis* 61360. Valinomycin (10 μg/ml) did not affect the incorporation of glycerol at pH 7.0 (data not shown) but did inhibit total teichoic acid synthesis (70% inhibition); this was accompanied by an increase in lipid formation (Fig. 2). There was no drastic change in the mixture of free lipids separated by thin-layer chromatography for cells treated with valinomycin and for those not treated (data not shown). To manipulate $\Delta\psi$ and ΔpH, we tested several ionophores and uncouplers on glycerol incorporation by cells growing in medium at two pH values; the results are shown as dose-response curves (see Fig. 3 to 5). The growth curves were identical for cells growing at pH 5.8 and 7.5, and the pH values of the culture supernatants by the end of the experiments were 5.8 and 7.3, respectively.

Valinomycin, with K^+ in the medium, inhibited teichoic acid synthesis while causing an increase in the formation of free lipids at pH 7.5. There was no inhibition at pH 5.8 (Fig. 3). Nigericin, at a concentration as low as 0.05 μM, was a potent inhibitor of teichoic acid synthesis and caused a breakdown of lipid at pH 5.8 (Fig. 3). On the other hand, at pH 7.5 teichoic acid synthesis was not inhibited at low concentrations of nigericin, although higher concentrations (1 μM) inhibited the formation of both fractions. The effects of methyltriphenyl phosphonium bromide and monensin were essentially similar to those of valinomycin and nigericin (Fig. 4).

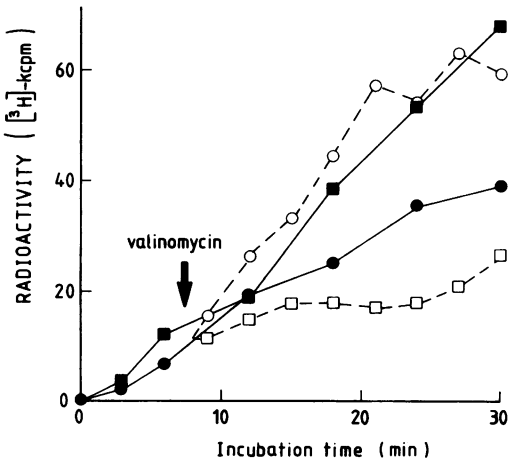

FIG. 2. Effect of valinomycin on glycerol incorporation in *B. subtilis* 61360. Cells grown at pH 7.0 were incubated with [2-^{3}H]glycerol, and samples (5 ml) were separated into total teichoic acid (■, □) and free-lipid (●, ○) fractions as described in the text. Valinomycin (10 μg/ml; □, ○) or acetone (control; ■, ●) was added after 7.5 min (arrow).

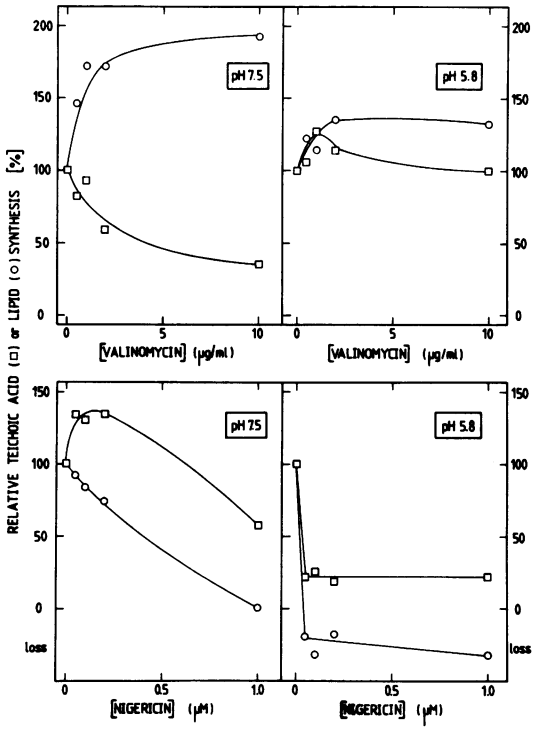

FIG. 3. Effect of valinomycin and nigericin on glycerol incorporation in *B. subtilis* 61360. Cultures (50 ml) grown at pH 5.8 or 7.5 were incubated with [2-^{3}H]glycerol. After 16 min, 5 ml of the culture was treated with ionophore, and samples were fractionated as described in the text. Results are expressed as the relative amounts of total teichoic acid (□) or free lipid (○) formed compared with 100% for untreated cells over a 15-min period. Values below 0% indicate the percent loss of lipids formed during the first 16 min of incubation before the addition of ionophore (plotted on the same scale).

The uncouplers DNP and CCCP inhibited formation of both lipid and teichoic acid fractions (Fig. 5). CCCP had no effect at pH 7.5, presumably as a result of the pK of the proton conductor itself (6.09; see, e.g., reference 27), since DNP inhibited the formation of both products at pH 5.8 (data not shown) and pH 7.5 (Fig. 5). Dithiothreitol was included to negate possible sulfhydryl effects of CCCP (28), although such effects appear to be minimal at the highest concentration of uncoupler used (Fig. 5).

The effect of valinomycin on the formation of the wall teichoic acid precursor CDP-glycerol was analyzed (Table 1 and Fig. 6). In the TCA extract from untreated cells, there was a small amount of material which comigrated with CDP-glycerol, whereas most of the radioactive material remained at the origin as a result of TCA extraction of teichoic acid (2). Formation of teichoic acid extractable by TCA (Fig. 6) was inhibited to the same extent as that of the teichoic acid subsequently solubilized with alkali (Table 1). Nevertheless, valinomycin-treated cells accumulated both glycerol phosphate and CDP-glycerol (Fig. 6A). It should be noted that longer treatments with valinomycin (30 min [Table 1]) result-

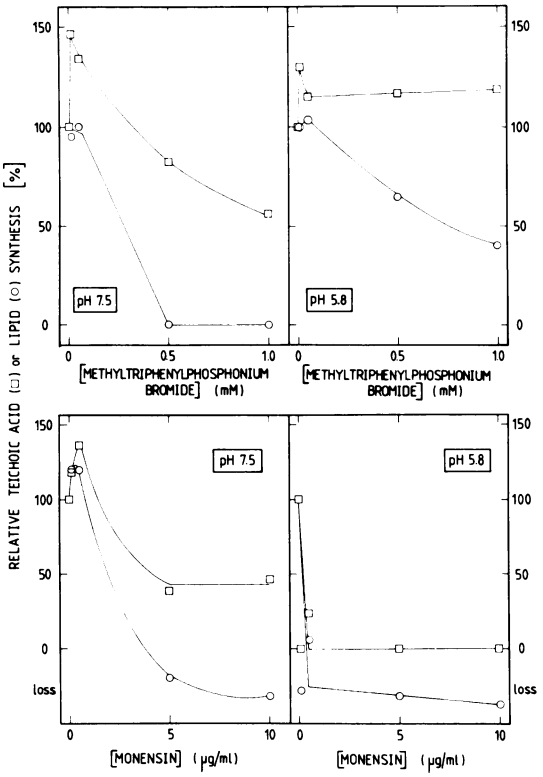

FIG. 4. Effect of methyltriphenyl phosphonium bromide and monensin on glycerol incorporation in *B. subtilis* 61360. Experimental conditions were as described in the legend to Fig. 3.

ed in decreased lipid formation, possibly as a result of weakened cell walls. With 5 mM DNP, CDP-glycerol and glycerol phosphate accumulated in cells grown at pH 7.5. At this pH value there was some accumulation of glycerol phosphate, but not of CDP-glycerol, under conditions (0.02 μM nigericin) which did not affect teichoic acid synthesis (Fig. 3). It is possible that a feedback control mechanism might prevent the accumulation of excess CDP-glycerol during the inhibition of teichoic acid synthesis. Uptake of

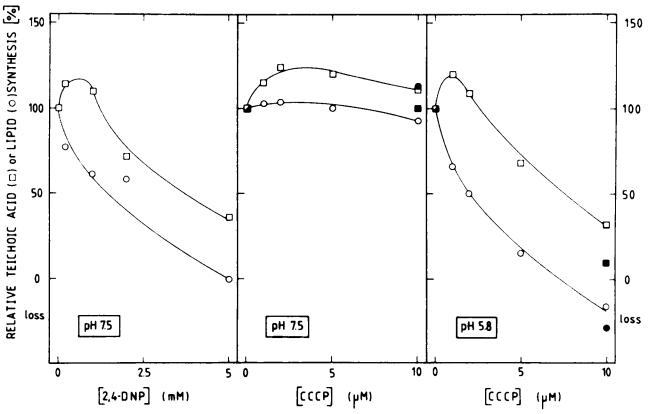

FIG. 5. Effect of DNP and CCCP on glycerol incorporation in *B. subtilis* 61360. Experimental conditions were as described in the legend to Fig. 3. Cells were treated with CCCP in the presence (□, ○) or absence (■, ●) of 1 mM dithiothreitol added before [2-^{3}H]glycerol.

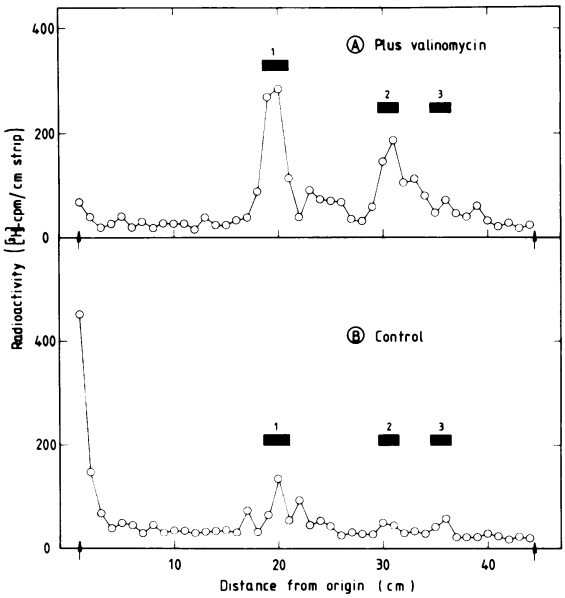

FIG. 6. Effect of valinomycin on CDP-glycerol formation. TCA extracts (Table 1) were separated on Whatman no. 1 paper developed in solvent A for 18 h. The extracts were those from cells treated with (A) or without (B) valinomycin (10 μg/ml) for 5 min and then pulsed with [2-^{3}H]glycerol for 10 min. The standards, indicated by the hatched areas, were: 1, CDP-glycerol; 2, glycerol phosphate; and 3, glycerol.

glycerol appears to be inhibited by 5 mM DNP and 0.02 μM nigericin at pH 5.8 since there was no accumulation of glycerol, glycerol phosphate, or CDP-glycerol.

Teichoic acids synthesized from [^{14}C]glycerol in the presence of valinomycin at pH 7.0 were examined. The small amount of LTA synthesized was increased by 22% with valinomycin, whereas the formation of wall teichoic acid was decreased by 88% during a 15-min incubation. Chemical and enzymic degradations of the teichoic acids indicated that valinomycin did not alter the extent of LTA glucosylation (13% of the polymer substituted with glucose). However, the wall teichoic acid synthesized before the addition of valinomycin (15-min pulse) had become further glucosylated in the presence of the ionophore and, as a result, was 64% substituted after 30 min (35% without valinomycin).

Effect of valinomycin on GlcNAc incorporation by *B. subtilis* 61360. Formation of peptidoglycan from [^{14}C]GlcNAc in *B. subtilis* 61360 was also inhibited by valinomycin, and there was a simultaneous increase in the amount of radioactive material extractable by TCA (Table 2). Analysis of the TCA extract showed that UDP-*N*-acetylmuramyl pentapeptide accumulated after a 10-min pulse with [^{14}C]GlcNAc (Fig. 7), and after a 30-min pulse (data not shown) more UDP-*N*-acetylmuramyl pentapeptide was formed at the expense of UDP-GlcNAc. Polymeric material was also extracted with TCA (Fig. 7), although the small amount of such material from valinomycin-treated cells (less than 5% of the extract) did not increase between the 10- and 30-min pulses.

Effect of valinomycin on synthesis of wall polymers in vitro. Valinomycin was tested on cell wall synthetic enzymes in a K^+-free medium to examine whether its action was due to dissipation of $\Delta\psi$ or whether it was merely acting as an inhibitor of such enzymes.

B. subtilis 168 was used as the parent strain of *B. subtilis*

TABLE 1. Effect of valinomycin on [2-^{3}H]glycerol incorporation in *B. subtilis* 61360[a]

Incubation time (min)	Addition	Radioactivity (^{3}Hkcpm)[b]					Recovery (%)[c]
		Total uptake	TCA extract	Lipid extract	Teichoic acid extract	Residue	
10	None	215	20	53	108	4	86
	Valinomycin	216	47	113	54	0	99
30	None	1,165	40	337	632	14	88
	Valinomycin	262	45	120	65	4	89

[a] Exponential cultures (40 ml, pH 7.0, 0.5% glucose) were incubated for 5 min with or without valinomycin (10 μg/ml); [2-^{3}H]glycerol (40 μCi, 1 Ci/mmol) was then added, and the cells were incubated for a further 10 or 30 min. Cells were harvested and washed twice with water.

[b] Radioactivity in washed cells was estimated for total uptake, and then the cells were extracted with 5 ml of 5% TCA (wt/vol) at 0°C for 16 h. The pellet remaining after centrifugation was washed with water and extracted first with chloroform-methanol (lipid extract) and then with mild alkali (total teichoic acid extract) as described in the text. Finally, the radioactivity remaining in the residue was estimated.

[c] The radioactivity measured in the three extracts and the residue was expressed as a percentage of the total radioactivity incorporated into the cells.

61360, and the assay system developed for ribitol teichoic acid synthesis in *B. subtilis* W23 protoplasts (4) was adapted for this glycerol teichoic acid-containing strain. Protoplasts of *B. subtilis* 168 were highly efficient at synthesizing polymer from externally supplied CDP-[2-^{3}H]glycerol. With various concentrations of the nucleotide (1.4 μM to 1.6 mM), the K_m for CDP-glycerol was 0.31 mM, and the apparent V_{max} was 561 nmol per mg of membrane protein per h. Routinely, 1.07 mM CDP-glycerol was used, and the synthesis of teichoic acid increased almost linearly with time for 60 min at 37°C. Partly autolyzed cells were much less effective at synthesizing the polymer. Polymer synthesis by protoplasts was not affected significantly by the presence of UDP-GlcNAc (0.4 mM) or by the addition of tunicamycin (10 μg/ml). At very low concentrations of CDP-glycerol (0.4 μM), polymer synthesis was slightly stimulated by UDP-GlcNAc (0.4 mM; 15% stimulation); that this indicated the synthesis of teichoic acid linkage unit lipids was confirmed by the observation that such stimulation was completely inhibited by tunicamycin (10 μg/ml). Therefore, in contrast to *B. subtilis* W23 protoplasts (4), protoplasts of *B. subtilis* 168 synthesized teichoic acid largely independent of linkage unit precursors. On the other hand, such precursors were detected when isolated membrane fractions were used. Membranes prepared either in the presence or the absence of polyethylene glycol 6000 were equally active in synthesis of teichoic acid. The latter preparations were unaffected by the presence of GTP in the assay system, whereas the membranes prepared in the presence of polyethylene glycol 6000 were slightly stimulated (18%) by 0.2 mM GTP. This stimulation was not as marked as that caused by GTP in the synthesis of cellulose by *Acetobacter xylinum* membranes prepared in polyethylene glycol (1). The membrane fraction incorporated radioactivity from CDP-[2-^{3}H]glycerol into two products which had R_f values, in solvent B, of 0.62 and 0.80, respectively, and whose synthesis was entirely dependent on the addition of UDP-GlcNAc (0.4 mM). These were characteristics of two of the linkage unit lipid intermediates (lipids II and III) synthesized by membranes of *Staphylococcus aureus*, *Micrococcus varians* (33), and *B. subtilis* W23 (18).

Valinomycin (1 to 20 μg/ml) caused no more than 8% inhibition of teichoic acid synthesis by protoplasts (1.07 mM CDP-glycerol in assay). At low concentrations of CDP-glycerol (0.4 μM), with 0.4 mM UDP-GlcNAc, there was actually 8% stimulation of polymer synthesis by valinomycin (20 μg/ml), indicating that neither polymer synthesis nor linkage unit synthesis was inhibited by valinomycin in vitro.

Under the standard assay conditions, incorporation of glucose from UDP-glucose into polymer by protoplasts of *B. subtilis* 168 increased linearly for 10 min at 37°C. It was unaffected by 0.4 mM UDP-GlcNAc but stimulated by 1.07 mM CDP-glycerol (121% of the control value) and by a combination of 1.07 mM CDP-glycerol and 0.4 mM UDP-GlcNAc (131% of the control value; values are the mean of 10 separate determinations, standard error of the mean = 1.6%). By varying the UDP-glucose concentration (1.6 μM to 1.6 mM) and using 10-min incubations, we found that the K_m for UDP-glucose was 24 μM, with an apparent V_{max} of 435 nmol (mg of membrane protein)$^{-1}$ h^{-1}. Valinomycin (20 μg/ml) had no significant effect on glucosylation from UDP-glucose whether present singly, with CDP-glycerol, or with a combination of CDP-glycerol and UDP-GlcNAc. Similarly tunicamycin (10 μg/ml) had no effect on glucosylation as expected.

Peptidoglycan synthesis by partly autolysed cells of *B. subtilis* 168 (assay developed for *B. subtilis* W23 [22]) was only 8% inhibited by valinomycin (10 μg/ml).

Amino acid uptake. Uptake of arginine and histidine (an auxotrophic requirement) by *B. subtilis* 61360 increased linearly for 10 and 20 min, respectively. Protein synthesis in

TABLE 2. Effect of valinomycin on [^{14}C]GlcNAc incorporation in *B. subtilis* 61360[a]

Incubation time (min)	Addition	[^{14}C]GlcNAc uptake (fraction)[b]	Radioactivity solubilized (%) by:				Insoluble residue (%)	Recovery (%)[c]
			TCA	RNase + DNase	Trypsin	Lysozyme		
10	None	0.40	15.6	5.9	7.1	65.0	3.1	96.7
	Valinomycin	0.29	36.9	6.0	5.3	40.8	3.6	92.6
30	None	0.48	7.5	6.1	8.4	72.5	2.7	97.2
	Valinomycin	0.29	39.9	6.7	6.5	30.1	3.1	86.3

[a] Exponential cultures (80 ml, pH 7.0, 0.5% glucose) were incubated with or without valinomycin (10 μg/ml) for 5 min; [^{14}C]GlcNAc (2 μCi, 58 Ci/mol) was then added, and the cells were incubated for a further 10 or 30 min. The cells were harvested, washed twice with water, and fractionated as described in the text.

[b] [^{14}C]GlcNAc in the washed cell pellet was expressed as a fraction of the total radioactivity added.

[c] Radioactivity measured in the four extracts and the residue was expressed as a percentage of the total radioactivity incorporated into the cells.

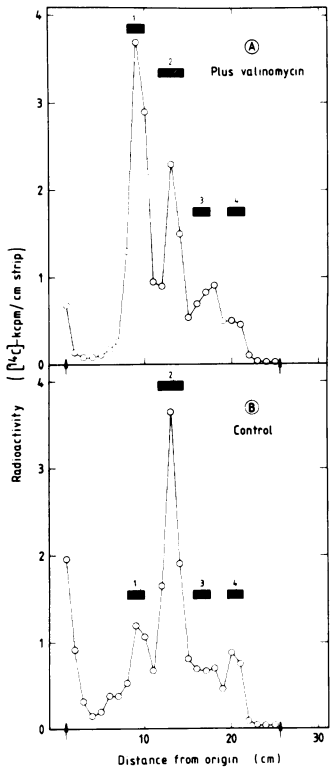

FIG. 7. Effect of valinomycin on the formation of peptidoglycan precursors. TCA extracts, from the experiment described in Table 2, were separated on Whatman no. 1 paper developed in solvent A for 18 h. The extracts were those from cells treated with (A) or without (B) valinomycin (10 μg/ml) for 5 min and then pulsed with [^{14}C]GlcNAc for 10 min. The standards, indicated by the hatched areas, were: 1, UDP-*N*-acetylmuramyl pentapeptide; 2, UDP-GlcNAc; 3, GlcNAc-1-phosphate; and 4, GlcNAc.

both cases was completely inhibited by chloramphenicol (70 μg/ml), although uptake continued briefly. However, valinomycin (10 μg/ml) stopped the uptake of both amino acids within 1 min. Other uncouplers were not tested on amino acid uptake, although it is known that active transport of amino acids by membrane vesicles of *B. subtilis* is inhibited by azide, DNP, and valinomycin (31).

DISCUSSION

Several ionophores and uncouplers were found to be inhibitory to the synthesis of teichoic acid by the glycerol auxotroph, *B. subtilis* 61360. Only valinomycin, in a high K^+-containing medium, was inhibitory to peptidoglycan and teichoic acid synthesis but did not inhibit LTA synthesis, lipid formation, or the synthesis of wall precursor nucleotides. The observation that valinomycin inhibits wall teichoic acid but not LTA formation is indicative of the different mechanisms involved in their synthesis. Wall teichoic acid is synthesized from CDP-glycerol and UDP-glucose (14), whereas the glycerol phosphate residues of LTA in *B. subtilis* are probably derived from L-α-phosphatidylglycerol as they are in several other gram-positive bacteria (11, 13, 15, 30, 44). Glycosylation of wall teichoic acid does not appear to be as sensitive to valinomycin as is the formation of the glycerol phosphate chain.

Alterations in $\Delta\tilde{\mu}_{H^+}$ are liable to exert many indirect effects on cell wall formation. Transport of substrates may be affected. Although glycerol and GlcNAc transport might have been inhibited in cells treated with carboxylic ionophores or uncouplers, valinomycin did not inhibit transport of these precursors. Active transport of amino acids in *B. subtilis* is also inhibited by valinomycin and uncouplers (31). However, since wall synthesis is unaffected by chloramphenicol or amino acid starvation (39), deprivation of amino acids would not have affected wall polymer formation during the 15-min treatments used in this study. However, a decrease in the ATP content of the cell might affect the synthesis of wall precursor nucleotides. Although carboxylic ionophores and the uncouplers reduce the ATP content of *B. subtilis* (26), valinomycin does not (26), and the accumulation of CDP-glycerol supports this. Finally, disruption of $\Delta\tilde{\mu}_{H^+}$ could activate either wall autolysins that hydrolyze peptidoglycan (26) or enzymes that degrade membrane lipids, as indicated in this study. Although a teichoicase acting on the glucosylated glycerophosphate polymer formed by *B. subtilis* 168 has been characterized recently (32), the presence of teichoic acid-degrading enzymes has been detected only during sporulation or phosphate starvation of *B. subtilis* (17, 46), and their activity in vegetative cells treated with ionophores or uncouplers is not known. Nevertheless, the absence of significant quantities of degradation products of teichoic acid or peptidoglycan, and the accumulation of soluble precursor nucleotides, suggest that polymer breakdown was not the major cause of inhibition of polymer formation by valinomycin.

The results obtained with valinomycin suggest that it is the $\Delta\tilde{\mu}_{H^+}$, rather than either of its individual components, which is important in cell wall synthesis. At pH 7.5, valinomycin inhibited peptidoglycan and teichoic acid synthesis by dissipating the $\Delta\psi$. On the other hand, the ΔpH in valinomycin-treated cells at pH 5.8 must have been sufficient to compensate for a decrease in the $\Delta\psi$ to allow teichoic acid synthesis to remain unaffected. Although the other ionophores and uncouplers inhibited teichoic acid synthesis, their affect was nonspecific, and incorporation and lipid formation were also affected. At high concentrations of nigericin (1 μM) the ionophore can also affect the $\Delta\psi$ in *B. subtilis* (26), acting like an uncoupler (16). Since ionophores were only tested in a medium with high K^+ and low Na^+ contents, it was not possible to detect differences between the actions of, say, nigericin and monensin since both still have an affinity for K^+, although monensin shows a slight preference for Na^+ over K^+ ions (16, 38).

Valinomycin did not affect several cell wall synthetic enzymes that were expressed on the external surface of protoplasts or of cells undergoing partial wall autolysis: glycerol teichoic acid synthesis and its glucosylation, formation of teichoic acid-peptidoglycan linkage unit precursor lipids, and peptidoglycan synthesis. In these systems, K^+ was not present in the stabilizing buffer and so any $\Delta\psi$ should not have been affected by valinomycin. These results rule out the possibility that the ionophore acted directly on the enzymes.

The results obtained with valinomycin suggest that the synthetic enzymes involved in cell wall synthesis in the intact living cell do require the maintenance of the $\Delta\tilde{\mu}_{H^+}$ for optimal activity and indicate that the energy liberated during the formation of chemical bonds is not itself sufficient for cell wall synthesis. Such conclusions are consistent with our earlier hypothesis (22) that an energized membrane is required to enable rotation or reorientation, by conformational changes, of synthetic enzyme complexes. At present it is not possible to decide how directly the $\Delta\tilde{\mu}_{H^+}$ is involved in cell wall synthesis.

It should be possible to compensate for the loss of either $\Delta\psi$ or ΔpH, provided that either the other parameter or the capacity for substrate level phosphorylation is of sufficient magnitude to sustain adequate energy metabolism. This concept is illustrated in the study by Harold and van Brunt (21), who reported that the fermentative *Streptococcus faecalis*, in a specially devised medium, grows normally under conditions in which both the ΔpH and $\Delta\psi$ are dissipated. These results suggest that proton circulation is not obligatory for the synthesis of macromolecules, such as those of the cell wall, for DNA replication, or for cell division. Nevertheless, the regulation by $\Delta\tilde{\mu}_{H^+}$ of several processes involved in bacterial cell wall assembly has since been implicated for a variety of respiratory or facultative organisms: (i) regulation of autolysins in *B. subtilis* 168 by $\Delta\tilde{\mu}_{H^+}$ (26); (ii) maintenance of cellulose synthesis in *A. xylinum* by $\Delta\psi$ (9); and (iii) insertion of *E. coli* outer membrane proteins promoted by $\Delta\tilde{\mu}_{H^+}$ (49). Our results suggest a further role for $\Delta\tilde{\mu}_{H^+}$ which is consistent with the concept of the mobile enzyme complex discussed previously (4, 22). Direct proof of this and speculation (22) whether such a system operates during the glycosylation of eukaryotic proteins must await the availability of purified synthetic enzymes inserted into artificial liposomes. Such an approach has been used recently by Haselbeck and Tanner (24) to study the enzymology of yeast mannosyltransferases, which involves a dolichyl phosphate intermediate. Translocation of sugar residues, involved in the synthesis of microbial cell wall polymers, which does not proceed via prenyl intermediates may provoke additional hypotheses, and the possibility of pore structures being formed by chitin synthase in yeasts has been suggested recently by Cabib et al. (7) to account for translocation of GlcNAc. In this case, no source of energy other than the free energy of hydrolysis of UDP-GlcNAc was required for synthesis and extrusion of chitin through isolated plasma membranes.

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