Lipid Intermediates in the Biosynthesis of the Wall Teichoic Acid in *Staphylococcus lactis* I3

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1. Particulate enzyme systems have been prepared from *Staphylococcus lactis* I3 which effect the synthesis of wall teichoic acid (a polymer containing a repeating unit in which D-glycerol 1-phosphate is attached to the 4-position on *N*-acetylglucosamine 1-phosphate) from the nucleotide precursors CDP-glycerol and UDP-*N*-acetylglucosamine. By using nucleotides labelled with ^{32}P and ^{14}C it has been shown that the synthesis proceeds via lipid intermediates. 2. Two intermediates have been found. In one of these *N*-acetylglucosamine 1-phosphate is present, whereas in the other the repeating unit of the teichoic acid occurs. 3. The simultaneous formation of the teichoic acid, a poly(*N*-acetylglucosamine 1-phosphate) and an unidentified lipid, together with the poor ability of most particulate systems to synthesize polymer and the instability of the lipid intermediates themselves, have interfered with pulse-labelling experiments. Nevertheless, the biosynthetic sequence has been elucidated. It is concluded that the intermediates are derivatives of undecaprenol phosphate.

Lipid intermediates, formed by transfer of sugar, sugar 1-phosphate or polyol phosphate residues from nucleotide precursors to a lipid phosphate acceptor, have been shown to be involved in the biosynthesis of bacterial wall polymers. In some cases these lipids have been isolated and the lipid moiety identified as the C_{55} -polyisoprenol, undecaprenol. Thus polyisoprenols have been shown to participate in the biosynthesis of O-antigen (Wright *et al.*, 1967), peptidoglycan (Higashi *et al.*, 1967, 1970), mannan (Scher *et al.*, 1968), capsular polysaccharide (Troy *et al.*, 1971), and O-antigen glycosyl side chains (Nikaido & Nikaido, 1971). Related intermediates have been implicated in the biosynthesis of a poly(*N*-acetylglucosamine 1-phosphate) in the walls of *Staphylococcus lactis* N.C.T.C. 2102 (Brooks & Baddiley, 1969b), the wall teichoic acid of *Staphylococcus lactis* I3 (Douglas & Baddiley, 1968) and the two wall teichoic acids, poly(glycerol phosphate) (Anderson *et al.*, 1972) and poly(glycerol phosphate glucose) (Hancock & Baddiley, 1972) of *Bacillus licheniformis* A.T.C.C. 9945. That the lipid phosphate acceptor for teichoic acid biosynthesis is the same as that for peptidoglycan synthesis in the last two organisms has been established (Watkinson *et al.*, 1971; Anderson *et al.*, 1972). It has been concluded from this that the lipid acceptor for teichoic acid is also undecaprenol phosphate.

The wall teichoic acid (A) of *S. lactis* I3 has a repeating unit containing D-glycerol 1-phosphate attached to the 4-position of 6-O-D-alanyl-*N*-acetylglucosamine 1-phosphate (Archibald *et al.*, 1968a,

(A)

1971). Particulate enzyme preparations from the organism synthesize a polymer from the substrates UDP-*N*-acetylglucosamine and CDP-glycerol, *N*-acetylglucosamine 1-phosphate and D-glycerol 1-phosphate being transferred from the nucleotides to the polymer chain (Baddiley *et al.*, 1968). Evidence for the participation of lipid intermediates in this synthesis has been reported (Douglas & Baddiley, 1968), and the present study extends the earlier observations and establishes the course of the synthetic route.

Experimental

Materials

UDP-*N*-acetylglucosamine, UTP, ATP, CTP, glycerol kinase and calf intestinal phosphomonoesterase were purchased from Sigma Chemical Co.,

St. Louis, Mo., U.S.A. [^{14}C]Acetic anhydride, [^{14}C]glycerol, [$\gamma\text{-}^{32}\text{P}$]ATP, diphenyl [^{32}P]phosphate and carrier-free inorganic [^{32}P]phosphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. DEAE-cellulose was purchased from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Lysostaphin was kindly given by Dr. W. A. Zygmunt of the Mead Johnson Research Centre, Evansville, Ind., U.S.A. All other chemicals were of analytical grade and purchased from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Methods

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951). Radioactive material on chromatograms was located with a radiochromatogram scanner (Baird-Atomic Inc., Cambridge, Mass., U.S.A.). Low radioactivity on chromatograms was located by counting 1 cm-wide strips of the chromatogram in a Packard 4000 series Tri-Carb liquid-scintillation counter or a Beckman LS-150 liquid-scintillation counter. Quantitative measurements were made by counting radioactive areas in a scintillation counter. The liquid scintillant had the following composition: toluene, 2 litres; 2,5-diphenyloxazole, 8 g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.2 g. Radioactive samples not on chromatograms were counted in the liquid scintillant to which 1 litre of Triton X-100 (Lennig Chemicals Ltd.) had been added (Patterson & Greene, 1965).

Paper chromatography. This was carried out on the paper indicated with the following descending solvent systems: *A*, ethanol–1 M-ammonium acetate, pH 3.8 (5:2, v/v) (Paladini & Leloir, 1951) with Whatman no. 1 paper; *B*, propan-2-ol–aq. NH_3 (sp.gr. 0.88)–water (7:1:2, by vol.) (Le Cocq & Ballou, 1964) with Whatman no. 4 paper; *C*, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949) with Whatman no. 4 paper. Products were detected by the alkaline silver nitrate reagent for sugars and polyols (Trevelyan *et al.*, 1950) and molybdate– HClO_4 for phosphates (Hanes & Isherwood, 1949).

Thin-layer chromatography. Thin-layer (0.25 mm) plates of silica gel G (Merck) containing $(\text{NH}_4)_2\text{SO}_4$ (10%, w/w) were used for the separation of labelled lipid fractions (Chalvardjian *et al.*, 1962). The solvent was chloroform–methanol–water (13:6:1, by vol.) (Schlemmer, 1961). Radioactive areas were located by counting 4 mm bands from the plate in the liquid scintillant used for the paper chromatograms. When large amounts of lipid were separated thicker layers (0.5 mm) were used.

Separation of labelled lipids. Lipid extract from a reaction mixture containing 0.6 ml of the particulate enzyme suspension was applied to a thin-layer

(0.5 mm) plate and the chromatogram was developed as described. Radioactive areas were located on a narrow strip from the plate; the plate was kept at -20°C during examination and elution was started no longer than 2 h after the plate had been removed from the chromatography solvent. Elution was carried out as follows: the silica gel from an area containing labelled lipid was removed, 5 ml of chloroform–methanol (2:1, v/v) was added and the gel was washed with this solvent into a small column. The gel was then irrigated with 15 ml of chloroform–methanol (2:1, v/v) and finally with 5 ml of methanol. The effluent containing the lipids was dried *in vacuo*. Lipids obtained in this way were used for investigations without further purification.

Culture conditions. Organisms were grown essentially as described by Baddiley *et al.* (1968). Cultures (250 ml), inoculated from a nutrient agar slope, were grown overnight at 37°C with shaking in a medium of the following composition: nutrient broth no. 2 (Oxoid), 12.5 g; yeast extract (Difco), 2.5 g; K_2HPO_4 , 2.5 g; trisodium citrate, 0.5 g; glucose, 1 g; water (demineralized), 1 litre. Two of these cultures were used to inoculate a batch (10 litres) of medium in which the glucose had been increased to 10 g/l. Incubation at 37°C was continued with forced aeration for a further 3–4 h when the culture had reached the phase of late exponential growth. The cells were harvested by using a refrigerated Sharples centrifuge or a continuous-flow attachment on a Sorvall refrigerated centrifuge; they were washed once with 0.6% NaCl.

Preparation of the enzyme. The procedure was similar to that of Baddiley *et al.* (1968). The cells were resuspended in 0.05 M-tris–HCl buffer, pH 7.5, containing 0.01 M- MgCl_2 and 0.005 M-sodium thioglycollate to give a 30% (w/v) suspension. The suspension was shaken with Ballotini no. 11 beads at 4000 rev./min for 2.5 min in a Braun cell disintegrator. The beads were removed by filtration on a no. 1 sintered-glass funnel. The filtrate was centrifuged at 15000 g in a Sorvall refrigerated centrifuge to remove unbroken cells and cell walls and then at 105000 g in a Beckman model L2 ultracentrifuge for 1 h. The sedimented gel was washed with 0.15 M-tris–HCl buffer, pH 8.0. The sedimented gel was then kept at -20°C until required. In this form the enzyme suffered very little loss of activity during 3 months. Immediately before use, the gel was resuspended in 0.1 M-tris–HCl buffer, pH 8.0, to give a protein concentration of approx. 20 mg/ml. This suspension is referred to as particulate enzyme suspension in the following experiments.

Preparation of ^{32}P -labelled particulate enzyme. Medium (500 ml as above) was inoculated with a culture (50 ml), grown overnight from a nutrient-agar slope. Incubation was continued with shaking at 37°C , and cell growth was followed by measuring

turbidity. At the start of exponential growth carrier-free [^{32}P]phosphate (0.5mCi) was added and growth continued to the late exponential phase. The cells were harvested by centrifugation at 10000g in a Sorvall refrigerated centrifuge and washed once with 0.6% NaCl; eight cultures were grown. The combined cell pellets were resuspended in 0.1M-tris-HCl buffer, pH7.5 (25ml); a further quantity (75ml) of buffer containing sucrose (64g), NaCl (0.87g) and lysostaphin (2mg) was added and the mixture incubated for 2h at 30°C. Protoplasts were formed and these were sedimented by centrifugation at 30000g for 20min. The pellet was resuspended in 0.1M-tris-HCl buffer, pH7.5 (20ml), containing 0.005M-sodium thioglycollate, 0.01M-MgCl₂ and deoxyribonuclease (0.5mg). The lysed protoplasts were centrifuged at 30000g for 20min, whereupon a hard pellet of unlysed cells and an upper lighter layer of cell membrane were obtained. The membrane layer was carefully removed, resuspended in the lysis buffer and centrifuged. The sedimented gel, which contained very few intact cells, was resuspended in 0.1M-tris-HCl buffer, pH8.0, to give a protein concentration of 20mg/ml; about 0.1% of the [^{32}P]phosphate was incorporated into the membrane, and about 3% of this was in the lipids under investigation.

Preparation of substrates. CDP-glycerol was synthesized from the morpholidate of CMP (Sigma) by the general method of Roseman *et al.* (1961). CDP-[^{14}C]glycerol, UDP-*N*-[acetyl- ^{14}C]acetylglucosamine and [^{32}P]UDP-*N*-[acetyl- ^{14}C]acetylglucosamine were prepared by the methods described by Baddiley *et al.* (1968). [^{32}P]CDP-glycerol was prepared by the same method as for CDP-[^{14}C]glycerol, but [γ - ^{32}P]ATP and glycerol were used instead of ATP and [^{14}C]glycerol.

Measurement of enzymic activity of particulate suspensions. Reaction mixtures contained particulate enzyme suspension (0.15ml), MgCl₂ (4 μmol), UDP-*N*-[acetyl- ^{14}C]acetylglucosamine (0.1 μmol , 2.5×10^6 c.p.m./ μmol) or UDP-*N*-acetylglucosamine (0.2 μmol) and CDP-glycerol (0.2 μmol) or CDP-[^{14}C]glycerol (0.1 μmol , 2.5×10^6 c.p.m./ μmol) in a total volume of 0.2ml. Incubation was at 30°C for 45min. At the end of the incubation period the mixtures were cooled in ice and then applied quantitatively to Whatman no. 1 paper. The chromatograms were developed overnight in solvent A. After drying at room temperature the origin, containing synthesized polymeric material and lipid intermediates, was removed and counted for radioactivity. The enzyme activity was measured as radioactivity (c.p.m.) incorporated into material remaining at the origin of the chromatogram.

Determination of polymeric material and lipid intermediates. Butan-1-ol (0.5ml) and water (0.3ml) were added to the reaction mixtures after incubation.

The mixture was then thoroughly shaken and the aqueous and organic phases were separated by centrifugation at 10000g and 0°C. After removal of the butanol layer a second portion of butan-1-ol was added and the process was repeated. The combined butanol extracts were washed once with water and evaporated to dryness *in vacuo*, and the radioactivity of the lipid residue was measured.

To the aqueous phase of the reaction mixture was added 90% (w/w) phenol (0.5ml) and water (0.5ml). The mixture was shaken vigorously and kept with intermittent shaking for 30min at 0°C. The phases were separated by centrifugation and the aqueous phase was removed. The phenol phase was washed once with water (0.5ml) and the combined aqueous extract and washings were washed with chloroform and then evaporated to dryness *in vacuo*. The residue was applied quantitatively to Whatman no. 1 paper and the chromatogram developed in solvent A. The

Fig. 1. Separation of lipids containing *N*-acetylglucosamine

Reaction mixtures contained particulate enzyme suspension (0.15ml), MgCl₂ (4 μmol), UDP-*N*-[acetyl- ^{14}C]acetylglucosamine (0.1 μmol , 2.5×10^6 c.p.m./ μmol) and CDP-glycerol (0.2 μmol) as indicated in a total volume of 0.2ml. These were incubated at 30°C for 45min and then extracted with butan-1-ol. The lipid extracts were chromatographed on plates of silica gel G containing 10% (w/w) (NH₄)₂SO₄ in chloroform-methanol-water (13:6:1, by vol.). ●, Fraction a without CDP-glycerol; ○, fraction b with CDP-glycerol.

radioactivity at the origin indicated the amount of polymer synthesized.

Results

Formation of lipids containing N-acetylglucosamine and glycerol

When the particulate enzyme suspension from *S. lactis* I3 was incubated at 30°C with UDP-*N*-[acetyl-¹⁴C]acetylglucosamine and Mg²⁺ ions, 1.5–2.0% of the labelled precursor was incorporated into a butanol-extractable fraction (fraction a). If CDP-glycerol was also present the butanol-extractable fraction was still labelled, but this was now only 1–1.5% of the amount in the labelled precursor (fraction b). It was therefore apparent that lipid containing *N*-acetylglucosamine was formed by the particulate enzyme and that this lipid was affected by the addition of CDP-glycerol.

The separation of lipids in fractions a and b on thin-layer plates of silica gel G containing (NH₄)₂SO₄ (10%, w/w) is shown in Fig. 1. Three lipids containing *N*-acetylglucosamine were observed; all three were detected when the particulate enzyme was incubated with UDP-*N*-[acetyl-¹⁴C]acetylglucosamine and CDP-glycerol (fraction b), but only two were formed when CDP-glycerol was omitted (fraction a).

The distribution of radioactivity between the three lipids in a typical experiment is shown in Table 1. The amount of lipid III in fraction a was similar to that in fraction b. The amount of the other two lipids, however, was greatly affected by the presence of CDP-glycerol; lipid I was decreased in amount by 83% and lipid II required CDP-glycerol for its synthesis. The total radioactivity in lipids I and II in fraction a was 2258 c.p.m. and in fraction b, 1300 c.p.m.; a decrease of 44% occurred, therefore, when CDP-glycerol was present.

When the labelled precursor was CDP-[¹⁴C]-glycerol, there was no incorporation of radioactivity into lipids in the absence of UDP-*N*-acetylglucosamine. When this substrate was present, only one lipid containing [¹⁴C]glycerol (running with lipid II on t.l.c.) was formed. The radioactivity in this lipid was 924 c.p.m. (0.36% of the labelled precursor). This was in close agreement with the radioactivity in lipid II (918 c.p.m.) formed from CDP-glycerol and UDP-*N*-[acetyl-¹⁴C]acetylglucosamine at the same concentration and specific radioactivity.

Lipid I was very acid-labile, being hydrolysed completely in 5 mM-HCl at 100°C for 5 min. The amount of lipid I synthesized by the particulate enzyme was relatively constant from one preparation to another. The amount of lipid III was, however, variable, the radioactivity incorporated being between about 700 and 2500 c.p.m./0.15 ml of particulate enzyme suspension. The amount of lipid II varied with the overall activity of the enzyme for polymer synthesis.

Identity of the sugar moiety of lipids I and II

Controlled acid hydrolysis of these lipids labelled with *N*-[acetyl-¹⁴C]acetylglucosamine was carried out by heating the samples in 0.1 M-HCl for 8 min at 100°C; no labelled lipid remained after the hydrolysis. There was only one water-soluble product from lipid I, identified as *N*-acetylglucosamine by co-chromatography with authentic material in three solvent systems. Similarly, the single water-soluble product from lipid II was identified as (D-glycerol-1)(*N*-acetylglucosamine-4)phosphate (B; R = H). This was also obtained by controlled acid hydrolysis of lipid II that was labelled with [¹⁴C]glycerol. Alkaline hydrolysis of the product from lipid II that was labelled with [¹⁴C]glycerol gave D-glycerol 1-phosphate as the labelled product.

Table 1. *Distribution of N-[acetyl-¹⁴C]acetylglucosamine in lipids formed from UDP-N-[acetyl-¹⁴C]acetylglucosamine*

Incubation mixtures containing particulate enzyme preparation (0.15 ml), MgCl₂ (4 μmol), UDP-*N*-[acetyl-¹⁴C]acetylglucosamine (0.1 μmol, 2.5 × 10⁶ c.p.m./μmol) and CDP-glycerol (0.2 μmol) as indicated in a final volume of 0.2 ml were incubated at 30°C for 45 min. Lipids were extracted with butan-1-ol and the extracts chromatographed on thin-layer plates of silica gel G containing (NH₄)₂SO₄ (10%, w/w). The radioactive areas were detected and counted as described in the text.

Lipid	Radioactivity (c.p.m.)	
	Fraction a without CDP-glycerol	Fraction b with CDP-glycerol
I	2258	382
II	None	918
III	1574	1544

Incorporation into lipid I of ^{14}C and ^{32}P from $[\text{}^{32}\text{P}]\text{-UDP-N-[acetyl-}^{14}\text{C]acetylglucosamine}$

The particulate enzyme suspension (1.5ml) was incubated with the doubly labelled substrate, and the labelled lipid fraction obtained from this was fractionated on a column (2cm $\times$ 32cm) of DEAE-cellulose (Wright *et al.*, 1967) by using a linear gradient of 0–0.4M-ammonium acetate in methanol (500ml). Two labelled fractions were obtained; they were eluted at about 0.32M and 0.36M respectively. The major component (at 0.32M) contained ^{14}C and ^{32}P in the same ratio as did the precursor (5.8:1); *N*-acetylglucosamine was the only water-soluble product of its controlled acid hydrolysis. This lipid corresponds to lipid I on t.l.c. The minor component (lipid III) contained ^{14}C and ^{32}P in the ratio 4.9:1, i.e. a higher ratio of ^{32}P than in the nucleotide precursor. The explanation for this is not known, but contamination with ^{32}P from breakdown of lipid I might be possible. ^{32}P in lipid III was not investigated further. These results indicate that *N*-acetylglucosamine 1-phosphate was transferred as a unit from substrate to a lipid acceptor to form lipid I.

Incorporation into lipid II of ^{32}P and ^{14}C from $[\text{}^{32}\text{P}]\text{CDP-[}^{14}\text{C]glycerol}$

The substrate was prepared by mixing the appropriate singly labelled precursors. ^{14}C and ^{32}P from the doubly labelled substrate were incorporated into lipid II and into the teichoic acid by the particulate enzyme. Controlled acid hydrolysis of the lipid as above gave (D-glycerol-1)(*N*-acetylglucosamine-4)-phosphate (B; R = H) as the only water-soluble product. The $^{14}\text{C}/^{32}\text{P}$ ratio in this was the same as that in the nucleotide precursor, the synthesized polymer and the (D-glycerol-1)(*N*-acetylglucosamine-4)phosphate derived from it (Table 2). D-Glycerol 1-phosphate was therefore transferred as a unit from the substrate to both the lipid and the polymer.

*Lipid phosphate as the acceptor for *N*-acetylglucosamine 1-phosphate*

Fig. 2 shows the separation by t.l.c. of labelled lipids extracted from ^{32}P -labelled particulate enzyme that had been incubated with UDP-*N*-acetylglucosamine (Fig. 2a) and UDP-*N*-acetylglucosamine together with CDP-glycerol (Fig. 2b). Most of the phospholipids had R_f values greater than 0.5; some unidentified material remained near the origin. At R_f values lower than 0.5 there were three major components containing ^{32}P . With UDP-*N*-acetylglucosamine alone only two of these were present, a minor one with R_f 0.19 and a major one with R_f 0.33. The major component therefore had an R_f value closely similar to that of lipid I (R_f 0.30). That this was lipid I was suggested by the decrease in its amount when CDP-glycerol was also present (Fig. 2b). This decrease was not accompanied, however, by an increase in the lipid with R_f 0.19, but by the appearance of a third lipid with R_f 0.43, running

Table 2. *Incorporation of ^{14}C and ^{32}P from $[\text{}^{32}\text{P}]\text{CDP-[}^{14}\text{C]glycerol}$ into lipid II and teichoic acid*

The reaction mixture contained particulate enzyme suspension (0.15ml), MgCl_2 (4 μmol), UDP-*N*-acetylglucosamine (0.2 μmol) and $[\text{}^{32}\text{P}]\text{CDP-[}^{14}\text{C]glycerol}$ (0.1 μmol , ^{14}C , 2.5×10^6 c.p.m./ μmol ; ^{32}P , 2.5×10^6 c.p.m./ μmol on first day of experiment) in a total volume of 0.2ml. After incubation for 45min at 30°C, the mixture was extracted with butan-1-ol and phenol to obtain the lipid and teichoic acid fractions. Each fraction was counted for radioactivity and then subjected to controlled acid hydrolysis. The hydrolysis products were chromatographed in solvent A, eluted from the paper and counted for radioactivity. The hydrolysis product (B; R = PO_3H_2) of teichoic acid was dephosphorylated by using calf intestinal phosphomonoesterase to give (glycerol-1)(*N*-acetylglucosamine-4)phosphate which was chromatographed, eluted and counted for radioactivity (Baddiley *et al.*, 1968).

Compound	$^{14}\text{C}/^{32}\text{P}$ ratio
Lipid II	1.05
(Glycerol-1)(<i>N</i> -acetylglucosamine-4)phosphate (B; R = H) from lipid II	1.02
Teichoic acid	0.93
Phosphate (B; R = PO_3H_2) from teichoic acid	1.06
(Glycerol-1)(<i>N</i> -acetylglucosamine-4)phosphate (B; R = H) from teichoic acid	1.05
$[\text{}^{32}\text{P}]\text{CDP-[}^{14}\text{C]glycerol}$	1.00

Fig. 2. Separation of lipids containing ^{32}P and *N*-acetylglucosamine

Reaction mixtures contained ^{32}P -labelled particulate enzyme suspension (0.15 ml), MgCl_2 ($4\mu\text{mol}$), UDP-*N*-acetylglucosamine ($0.2\mu\text{mol}$) and CDP-glycerol as indicated in a total volume of 0.2 ml. These were incubated at 30°C for 45 min and then extracted with butan-1-ol. The lipid extracts were chromatographed on plates of silica gel G containing 10% (w/w) $(\text{NH}_4)_2\text{SO}_4$ in chloroform-methanol-water (13:6:1, by vol.). ●, Fraction a without CDP-glycerol; ○, fraction b with CDP-glycerol.

between lipids I and III. This new lipid was present in extracts of the particulate enzyme suspension that had not been incubated with teichoic acid precursors, and might be unsubstituted lipid phosphate acceptor. The presence of the unidentified lipid with R_F 0.19 in appreciable quantity, and running so close to lipid II (R_F 0.17), could interfere with the detection of lipid II, especially if this was only present in trace amounts.

When both UDP-*N*-[acetyl- ^{14}C]acetylglucosamine and CDP-glycerol were used as substrates with the ^{32}P -labelled enzyme suspension, lipid III with R_F 0.47 was the only major ^{14}C -labelled component, and this contained no ^{32}P . The lipids at R_F 0.19 and 0.43 were again present. We conclude that lipid III does not contain phosphorus and does not participate directly in the biosynthesis; it has the properties of a glycolipid containing *N*-acetylglucosamine.

Enzyme preparations from protoplasts were highly active, incorporating 46% of labelled substrate into polymer, compared with 20% for preparations made by mechanical disruption. Under conditions when polymer synthesis could occur, such preparations contained very little detectable lipid I or II, thereby suggesting that lipid turnover was very rapid.

Rate of formation of the lipid intermediates

Fig. 3 shows the formation of lipids I and III in the absence of CDP-glycerol. In neither case were the kinetics of formation typical of those expected for a

lipid intermediate. The synthesis of lipid III slowed with time. As the UDP-*N*-acetylglucosamine was in large excess and the ability of enzyme preparations to synthesize lipid III was unaffected by preincubation (Table 3), this falling off in the synthesis could be due to endogenous acceptor becoming depleted. Lipid I was synthesized without any marked decrease in rate during 30 min.

Fig. 4 shows the formation of the lipids in the presence of CDP-glycerol. Lipid I reached a maximum of 450 c.p.m. after 15 min, and this value was maintained for the remaining 15 min of the experiment. Lipid II was formed in a manner very similar to that for lipid I in the absence of CDP-glycerol. Lipid III was synthesized much as before, but in increased amount. The formation of lipid I in this experiment showed kinetics consistent with those expected for it being an intermediate in the formation of lipid II. The rate of synthesis of polymer was almost linear for the duration of the experiment.

Utilization of the lipids for polymer synthesis

Attempts to show that the lipids were used for polymer synthesis by pulse-labelling techniques were not very successful. Fig. 5 shows the results of one such experiment. The particulate enzyme was incubated with UDP-*N*-[acetyl- ^{14}C]acetylglucosamine for 20 min at 30°C ; the labelled substrate was removed by washing with buffer and the reconstituted enzyme was then incubated further with both UDP-*N*-acetyl-

Fig. 3. Rate of formation of lipids containing *N*-acetylglucosamine

A reaction mixture containing particulate enzyme suspension (0.90 ml), MgCl_2 ($24 \mu\text{mol}$), and UDP-*N*-[acetyl- ^{14}C]acetylglucosamine ($0.6 \mu\text{mol}$, $2.5 \times 10^6 \text{ c.p.m./}\mu\text{mol}$) in a total volume of 1.2 ml was incubated at 30°C and samples (0.2 ml) were removed at the times indicated. These were extracted with butan-1-ol and the lipid extracts separated by t.l.c. to determine the radioactivity incorporated into lipids I (■) and III (Δ).

glucosamine and CDP-glycerol. Fig. 5 shows that after incubation with unlabelled substrates for 60 min the radioactivity in the lipid fraction had decreased by 2300 c.p.m., whereas the increase in radioactivity in the polymer was only 490 c.p.m. The radioactivity lost from the lipid but unaccounted for in the synthesized polymer was found largely as water-soluble material that was identified chromatographically as (D-glycerol-1)(*N*-acetylglucosamine-4)phosphate (B; R = H) and its phosphate (B; R = PO_3H_2), containing 88 and 11.5% of the radioactivity respectively; some *N*-acetylglucosamine and *N*-acetylglucosamine 6-phosphate were also present. The products of controlled acid hydrolysis of a sample of polymer synthesized before incubation with unlabelled substrates were *N*-acetylglucosamine 6-phosphate (1297 c.p.m.) and *N*-acetylglucosamine (966 c.p.m.). These are the products expected from hydrolysis of poly(*N*-acetylglucosamine 1-phosphate) (Brooks & Baddiley, 1969a). After incubation with unlabelled substrates for 60 min the products of controlled acid hydrolysis were *N*-acetylglucosamine 6-phosphate (1435 c.p.m.), *N*-acetylglucosamine (642 c.p.m.) and the phosphate

Fig. 4. Rate of formation of lipids containing *N*-acetylglucosamine and *N*-acetylglucosamine plus glycerol

A reaction mixture containing particulate enzyme suspension (1.05 ml), MgCl_2 ($28 \mu\text{mol}$), UDP-*N*-[acetyl- ^{14}C]acetylglucosamine ($0.7 \mu\text{mol}$, $2.5 \times 10^6 \text{ c.p.m./}\mu\text{mol}$) and CDP-glycerol ($1.4 \mu\text{mol}$) in a total volume of 1.4 ml was incubated at 30°C . Samples (0.2 ml) were removed at the times indicated. These were extracted with butan-1-ol and the lipid extracts separated on t.l.c. to determine the radioactivity incorporated into lipids I (■), II (□) and III (Δ), and also the radioactivity in the teichoic acid (●).

(B; R = PO_3H_2) (580 c.p.m.). The lipid fraction after incubation with unlabelled substrates contained lipids I, II and III. These results indicate that during incubation with unlabelled substrates lipid II was formed from lipid I, and *N*-[acetyl- ^{14}C]acetylglucosamine was transferred from the lipid fraction to the polymer fraction. Part of this *N*-acetylglucosamine was used for the synthesis of poly(*N*-acetylglucosamine 1-phosphate), the average chain length from the ratio *N*-acetylglucosamine 6-phosphate/*N*-acetylglucosamine (Hussey *et al.*, 1969) being increased from 1.3 to 2.2 units. Part was incorporated into the wall teichoic acid (A) as shown by the hydrolysis products.

Fig. 5. Pulse labelling of lipid intermediates and polymer

A reaction mixture containing particulate enzyme suspension (1.8ml), MgCl_2 (48 μmol), and UDP-*N*-[acetyl- ^{14}C]acetylglucosamine (1.2 μmol , 2.5×10^6 c.p.m./ μmol) in a final volume of 2.4ml was incubated at 30°C for 20min. 0.1 M-tris-HCl buffer, pH8.0, containing 0.005 M-sodium thioglycollate (5ml) was added and the mixture centrifuged at 30000g for 15min. The sedimented gel was washed twice with 5ml portions of the buffer and then resuspended in 0.1 M-tris-HCl buffer, pH8.0, to give a volume of 1.8ml. To this was added MgCl_2 (48 μmol), UDP-*N*-acetylglucosamine (2.4 μmol) and CDP-glycerol (2.4 μmol) to make a final volume of 2.4ml. This mixture was incubated at 30°C and samples (0.2ml) were taken at the times indicated. The samples were washed once with water (1.0ml) and then the radioactivity in the lipid fraction, teichoic acid and in the washings for each sample was determined. ●, Teichoic acid; □, lipid; Δ, teichoic acid plus washings.

It is not known whether the origin of the soluble products was lipid intermediates or polymers.

Fig. 6(a) shows the decrease in radioactivity of the lipid fraction which occurred when CDP-glycerol was added to reaction mixtures that had incorporated UDP-*N*-[acetyl- ^{14}C]acetylglucosamine during 40min. The decrease continued for 20min, after which the radioactivity began to rise. This indicated that some lipid was utilized for polymer synthesis immediately the CDP-glycerol was added. There was a corresponding sharp rise in the amount of label in the polymers as the synthesis of teichoic acid (A) started.

Fig. 6(b) shows the results of a similar experiment in which the starting substrates were both CDP-[^{14}C]glycerol and UDP-*N*-acetylglucosamine. Unlabelled CDP-glycerol was added after 20min in sufficient quantity to lower the specific radioactivity of the CDP-glycerol by a factor of 3. The addition of the CDP-glycerol resulted in a decrease in the radioactivity of the lipid fraction, consistent with the

formation and utilization of a lipid intermediate containing labelled glycerol phosphate. More radioactivity was incorporated into the polymer in the first 10min after the addition of CDP-glycerol than would have been expected if only CDP-[^{14}C]glycerol were being used for polymer synthesis; this presumably came from lipid I.

Stability of the enzyme during incubation

When the particulate enzyme was incubated with MgCl_2 and 0.1 M-tris-HCl buffer, pH8.0, at 30°C for 30min, followed by the addition of UDP-*N*-[acetyl- ^{14}C]acetylglucosamine and incubation for a further 45min at 30°C, there was a decreased ability to synthesize lipid I (Table 3, tube 2). If the particulate enzyme was washed after the preincubation, the ability to synthesize lipid I was even more greatly decreased (Table 3, tubes 3-5). The particulate enzyme was unstable, therefore, during incubation

Fig. 6. Utilization of lipid intermediates for teichoic acid synthesis

(a) A reaction mixture containing particulate enzyme suspension (2.1 ml), MgCl_2 ($56 \mu\text{mol}$) and UDP-*N*-[acetyl- ^{14}C]acetylglucosamine ($1.4 \mu\text{mol}$, 2.5×10^6 c.p.m./ μmol) in a final volume of 2.8 ml was incubated for 40 min at 30°C . Samples (0.2 ml) were taken at the times indicated. After 40 min the reaction mixture was divided into two parts (1 ml each). CDP-glycerol ($0.5 \mu\text{mol}$ in 0.05 ml of water) was added to one part and water (0.05 ml) to the other. Incubation was continued and samples again taken as indicated. Lipid and polymer were determined in each sample. (b) A reaction mixture containing particulate enzyme suspension (1.8 ml), MgCl_2 ($48 \mu\text{mol}$), UDP-*N*-acetylglucosamine ($2.4 \mu\text{mol}$) and CDP-[^{14}C]glycerol ($1.2 \mu\text{mol}$, 2.5×10^6 c.p.m./ μmol) in a final volume of 2.4 ml was incubated at 30°C . Samples (0.2 ml) were taken at the times indicated. After 20 min the reaction mixture was divided into two parts (0.8 ml). To one was added CDP-glycerol ($0.8 \mu\text{mol}$ in 0.04 ml) and to the other water (0.04 ml). Incubation was continued and the remaining samples were taken. Lipid and teichoic acid were determined in each sample. ■, Lipid without CDP-glycerol; □, lipid with CDP-glycerol; ●, teichoic acid without CDP-glycerol; ○, teichoic acid with CDP-glycerol.

at 30°C . The ability to synthesize lipid III was substantially unaffected by preincubation or washing.

Solubilization of the enzyme system

The particulate enzyme suspension (0.15 ml) which had been preincubated with 0.01 M-tris-HCl buffer, pH 8.0 (0.05 ml) and MgCl_2 ($4 \mu\text{mol}$) at 30°C for 30 min was centrifuged at $30000g$ for 20 min. The supernatant (0.1 ml) was removed and to this was added UDP-*N*-[acetyl- ^{14}C]acetylglucosamine ($0.1 \mu\text{mol}$, 2.5×10^6 c.p.m./ μmol) and CDP-glycerol ($0.2 \mu\text{mol}$). This mixture was incubated at 30°C for

45 min and then transferred quantitatively to Whatman no. 1 paper and chromatographed overnight in solvent A. Labelled material occurred at the origin of the chromatogram, indicating polymer formation. When this material was hydrolysed in 0.1 M-HCl for 8 min at 100°C the products were *N*-acetylglucosamine 6-phosphate (860 c.p.m.), *N*-acetylglucosamine (2720 c.p.m.) and the phosphate (B; $\text{R} = \text{PO}_3\text{H}_2$) (820 c.p.m.). These were the products expected from the hydrolysis of poly(*N*-acetylglucosamine 1-phosphate) and the wall teichoic acid (A). It appeared, therefore, that preincubation of the particulate enzyme suspension caused solubilization of some of

Table 3. *Effect of preincubation and washing on the ability of the particulate enzyme to synthesize lipid I*

A reaction mixture containing particulate enzyme suspension (1.5 ml) and MgCl_2 ($40\mu\text{mol}$) in a total volume of 1.6 ml was incubated at 30°C for 30 min. The mixture was cooled in ice and duplicate samples (0.16 ml) were removed. The remaining suspension was centrifuged at $30000g$ for 20 min at 0°C ; the supernatant was decanted and the residue washed twice with cold 0.1 M-tris-HCl buffer, pH 8.0. The washed residue was resuspended in buffer to give a volume of 1.30 ml. Duplicate samples (0.16 ml) were taken. The remaining suspension was then centrifuged, washed, resuspended and samples were taken for a third and fourth time. To all of the samples was added UDP-*N*-[acetyl- ^{14}C]acetylglucosamine ($0.1\mu\text{mol}$, 2.5×10^6 c.p.m./ μmol) and 0.1 M-tris-HCl buffer, pH 8.0 (0.04 ml). MgCl_2 ($4\mu\text{mol}$) was added to washed samples. The samples were then incubated for 45 min at 30°C . Butanol extraction was carried out; one extract from each pair of samples was counted for radioactivity to determine total lipid, the other chromatographed to determine lipid I and lipid III. The control pair containing particulate enzyme suspension (0.15 ml) and MgCl_2 ($4\mu\text{mol}$) were preincubated at 0°C and then treated as described above.

Tube no.	Treatment	Radioactivity (c.p.m.)		
		Lipid I	Lipid III	Total lipid extract
1	Control	4000	940	4760
2	Preincubated	2960	1000	3900
3	Preincubated and washed twice	—	—	1930
4	Preincubated and washed three times	—	—	1940
5	Preincubated and washed four times	400	1170	1840

the polymer-synthesizing enzyme system. The *N*-acetylglucosamine content of the hydrolysate was high and could be the product of hydrolysis of lipid I. The soluble preparation had only about 10% of the enzymic activity of the particulate enzyme that had not been preincubated; the enzymic activity of the residual particulate material was decreased to 39% of the activity of the untreated material.

Discussion

The particulate enzyme preparation from *S. lactis* I3 synthesizes a teichoic acid with a repeating structure (A). The origin of the repeating structure has been shown to be CDP-glycerol and UDP-*N*-acetylglucosamine, which transfer respectively glycerol phosphate and *N*-acetylglucosamine 1-phosphate (Baddiley *et al.*, 1968).

The particulate enzyme also synthesizes lipids containing *N*-acetylglucosamine 1-phosphate and glycerol phosphate from endogenous lipid acceptors and the appropriate nucleotides (Fig. 1). Three lipids are produced when both nucleotide precursors are present, and it has been shown that two of these are closely interdependent. Lipid I contains the *N*-acetylglucosamine 1-phosphate residue and accumulates in the reaction mixtures only in the absence of CDP-glycerol. When this nucleotide is present lipid I is converted into lipid II containing the repeating unit of the polymer, and the total amount of labelled lipid is decreased (Fig. 1, Table 1). Both lipid I and lipid II have been shown to 'turn over' to some extent in pulse-labelling experiments, the sugar

phosphate and glycerol phosphate-sugar phosphate moieties appearing in the teichoic acid (Figs. 5, 6a and 6b). These lipids therefore are intermediates in the biosynthesis of the teichoic acid, and Scheme 1 shows the probable route for this synthesis. Controlled acid hydrolysis of lipids I and II gave in each case a single radioactive product, *N*-acetylglucosamine and the phosphate (B; R = H) respectively. The nature of lipid III is not known; the acceptor for its synthesis in the membrane does not contain phosphate. As all lipid intermediates so far examined contain an isoprenol phosphate residue it is, therefore, unlikely that lipid III is a lipid intermediate. Its synthesis was unaffected by the addition of CDP-glycerol and the kinetics of formation were not those of a lipid intermediate (Figs. 4 and 5). The amount synthesized varied from one preparation to another and was unrelated to the polymer-synthesizing ability of the enzyme preparations. Amounts of glycolipids, glucosyldiglyceride and diglucosyldiglyceride, synthesized by enzyme preparations from *B. licheniformis* A.T.C.C. 9945 showed similar wide variation (I. C. Hancock & J. Baddiley, unpublished work). It is possible, therefore, that lipid III is a glycolipid containing *N*-acetylglucosamine. As none of the intermediates described in this paper yield disaccharides containing glucosamine and muramic acid, it is concluded that they are not related to the synthesis of peptidoglycan.

It is noteworthy that the particulate preparation was able to synthesize a poly(*N*-acetylglucosamine 1-phosphate). This is apparently the one already known as a wall polymer in *S. lactis* N.C.T.C. 2102

Scheme 1. Lipid intermediates in the synthesis of wall teichoic acid in *S. lactis* I3

(Archibald *et al.*, 1968*b*; Brooks & Baddiley, 1969*a*) in which the phosphodiester linkage is at the 6-position on the *N*-acetylglucosamine; it had not been observed hitherto in wall preparations from *S. lactis* I3.

The nature of the lipid phosphate acceptor has been studied by direct chemical and physical examination. That it contains phosphate has been demonstrated by using ^{32}P -labelled enzyme preparations (Fig. 2), and it has also been shown that the lipid intermediates are very acid-labile. Both these observations are consistent with it being a polyisoprenol phosphate. It is known that the lipid acceptor for peptidoglycan synthesis in *Micrococcus lysodeikticus* and in *Staphylococcus aureus* is undecaprenol phosphate (Higashi *et al.*, 1967, 1970). It is also known that the lipid phosphate acceptor for teichoic acid synthesis in *S. lactis* I3 is the same as that for peptidoglycan synthesis in this organism, and that lipid phosphate molecules are shared by the two biosynthetic routes (Watkinson *et al.*, 1971). If it is assumed that the acceptor for peptidoglycan synthesis in all micro-organisms is undecaprenol phosphate then we can conclude that the acceptor for teichoic acid synthesis in *S. lactis* I3 is also undecaprenol phosphate.

The complete 'turnover' of labelled lipids has not been demonstrated. The enzyme preparation, during incubation at 30°C, lost some of its ability to synthesize lipid I (Table 3), and there was further

deterioration when the preparation was washed with buffer. Incubation followed by washing are requirements for pulse-labelling experiments of the type shown in Fig. 5; thus, if the ability to synthesize lipid I was greatly decreased in the early stages of the experiment then this would explain the rather poor ability of preparations to transfer label from lipid I into polymer. The results in Figs. 6(*a*) and 6(*b*), however, also indicate only partial turnover of the lipids. It is concluded that not all of the lipid synthesized can participate in the biosynthesis of the teichoic acid. As lipids I and II give reasonably sharp bands on t.l.c., and as each gives rise to only one water-soluble product on acid hydrolysis, it is probable that they are single lipids and not mixtures. The ability of the particulate enzyme to use only a part of the lipid for polymer synthesis may be the result of partial disruption during cell breakage of an orderly arrangement of enzymes on the membrane. The kinetics of formation of the labelled lipids are not those typical of lipid intermediates, and this is also consistent with this interpretation. It is significant that these particulate enzyme preparations produce lipid intermediate(s) for peptidoglycan synthesis; 15% of UDP-*N*-acetylmuramyl- ^{3}H pentapeptide was incorporated by them into water-insoluble products, but of this only 20–40% represented polymer, the remainder occurring in lipid intermediates (Watkinson *et al.*, 1971). Similar observations have been made with *B. licheniformis*

A.T.C.C. 9945 (R. Anderson, H. Hussey & J. Baddiley, unpublished work) and with *S. aureus* (Matsuhashi *et al.*, 1965). Particulate enzyme preparations from cells of both of these organisms that had been broken by the glass-bead method incorporated UDP-*N*-acetylmuramyl-pentapeptide and UDP-*N*-acetylglucosamine only into lipid intermediates. It is likely that this method of breaking cells results in considerable damage to the membrane, possibly fragmenting it in such a manner as to separate enzymes transferring residues from nucleotides to lipid acceptors from the enzymes utilizing the lipid intermediates for polymer synthesis. This would lead to production of intermediates that could not be utilized for polymer synthesis, and possibly to conversion of all the lipid phosphate acceptor in the membrane into lipid intermediates, thus giving rise to the kinetics of formation of the type observed (Figs. 3 and 4).

When ³²P-labelled particulate enzyme obtained from protoplasts was incubated with UDP-*N*-acetylglucosamine in the absence of CDP-glycerol, a lipid intermediate (lipid I) accumulated. If CDP-glycerol was also present there was no accumulation of lipid II, but a compound tentatively identified as the acceptor lipid phosphate was regenerated (Fig. 2). The enzyme preparations from protoplasts were more active in polymer synthesis than were those prepared mechanically, and appeared to utilize lipid intermediates more efficiently. The integrity of the membrane may well have been better preserved in these preparations than in those from cells that had been broken with glass beads. Partial solubilization of the enzyme has been achieved but this is associated with considerable loss of activity, possibly because here also the organization of the membrane has been disturbed. It is highly probable that a precise arrangement of enzymes and lipid phosphate acceptor is required in the membrane if nucleotides and lipid intermediates are to be efficiently used for polymer synthesis (Anderson *et al.*, 1972). An orderly arrangement of the enzymes would be required not only for efficient synthesis but also for the vectorial nature of the process of formation of polymer lying outside of the membrane from nucleotide precursors within the cell.

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