LIPID INTERMEDIATES IN THE BIOSYNTHESIS OF THE LINKAGE UNIT BETWEEN TEICHOIC ACIDS AND PEPTIDOGLYCAN

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1. Introduction

Chemical studies have shown that the wall teichoic acid in *Staphylococcus aureus* H and the wall poly(*N*-acetylglucosamine 1-phosphate) in *Micrococcus* sp. 2102 are covalently attached to muramic acid residues in the peptidoglycan by a linkage unit containing three glycerol phosphate residues [1–3]. The synthesis of this unit from CDP-glycerol in the presence of UDP-*N*-acetylglucosamine (UDP-GlcNAc) has been demonstrated using membrane preparations from both species [4–6]. In the case of *S. aureus* H it was demonstrated that the linkage unit must be assembled as a membrane-bound lipid before attachment of the teichoic acid, poly(ribitol phosphate), and it was suggested that the lipid to which the glycerol phosphate units became attached was either the prenyl phosphate disaccharide intermediate of peptidoglycan synthesis or *N*-acetylglucosamine linked to prenyl phosphate [4,6]. Extracts from reaction mixtures containing radioactively labelled CDP-glycerol and UDP-GlcNAc with organic solvents gave radioactive lipids that were not identified [4]; similar lipids have been observed using wall + membrane preparations from *B. subtilis* W23 [7]. The synthesis of such lipid extracts in a membrane preparation from a mutant of *S. aureus* H was examined [8]. Lipids extracted with 70% aq. ethanol, which contained radioactivity from both nucleotide substrates, were found to be converted to a water-soluble polymer by incubation with CDP-ribitol in the presence of membranes. The product behaved like poly(ribitol phosphate) synthesised by the same membrane preparation, when examined by polyacrylamide gel electrophoresis.

The antibiotic tunicamycin inhibited the synthesis of this lipid and also inhibited the synthesis of poly(ribitol phosphate) attached to linkage unit [6,8]. This antibiotic is known to inhibit the transfer of *N*-acetylglucosamine 1-phosphate and *N*-acetylmuramyl pentapeptide 1-phosphate residues from the corresponding uridine nucleotides to prenyl monophosphate [9–11]. Since *N*-acetylglucosamine, but not *N*-acetylmuramyl pentapeptide, could be detected in the lipid [8] it appeared that the lipid intermediate contained glycerol phosphate residues attached to a prenylpyrophosphoryl-*N*-acetylglucosamine. Following the demonstration of a requirement for UDP-GlcNAc in the synthesis of linkage [4], and the finding that the phosphate attached to muramic acid in the wall of *B. licheniformis* is derived from UDP-GlcNAc [12], chemical studies have shown that the tri(glycerol phosphate) moiety of the linkage unit is attached by a single *N*-acetylglucosamine 1-phosphate residue to the 6-hydroxyl of a muramic acid residue (fig.1) in the cell wall of *S. aureus* H [13] and *Micrococcus* sp. 2102 [2]. A similar linkage unit also occurs in the walls of *Bacillus subtilis* W23 (E. Tarelli, unpublished). We present here evidence that at least three lipid intermediates participate in the synthesis of linkage units.

2. Methods

CDP-ribitol and CDP-glycerol were prepared from cytidine phosphoromorpholidate as in [4]. CDP-[2-^{3}H glycerol and [β-^{32}P]CDP-glycerol were prepared enzymically from [2-^{3}H]glycerol and [γ-^{32}P]ATP (Radiochemical Centre, Amersham, England) [14].

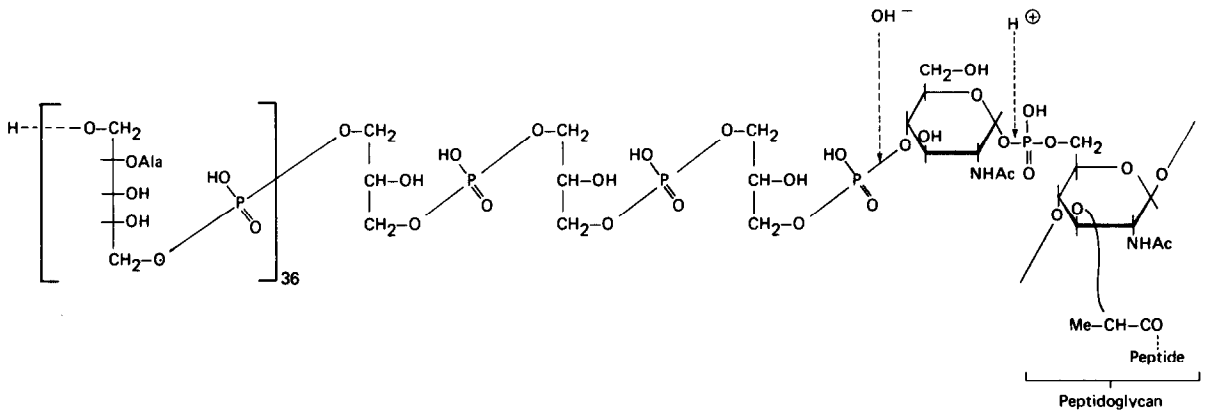

Fig.1. Scheme for the linkage of ribitol teichoic acid to peptidoglycan in a mutant of *S. aureus* H, showing the bonds that are labile in dilute acid and alkali.

The specific activity of CDP-[^{3}H]glycerol was 580 μCi/μmol and that of [^{32}P]CDP-glycerol was 250 μCi/μmol on the day of preparation. UDP-*N*-acetyl[U-^{14}C]glucosamine was purchased from the Radiochemical Centre, Amersham (300 μCi/μmol).

Micrococcus sp. 2102, grown as in [15] and *S. aureus* H [5] were harvested at mid-exponential phase. Membranes were isolated from the bacteria after disruption with glass beads as in [4]. Membranes were suspended in 0.05 M Tris–HCl, pH 8.0, containing 5 mM dithiothreitol and were kept at −15°C in 1 ml samples (20 mg protein/ml). Each sample was used once only.

Small-scale reaction mixtures contained 0.1 ml membrane suspension, 20 mM magnesium acetate and, in the case of *S. aureus* H, 1.7 mM ATP. Additions were made where required to the following concentrations: CDP-[^{3}H]glycerol, 0.025 mM (10^6 dpm); [^{32}P]CDP-glycerol, 0.067 mM (4×10^5 dpm); UDP-[^{14}C]GlcNAc, 0.33 mM (2.8×10^5 dpm); CDP-glycerol, CDP-ribitol, UDP-GlcNAc, 0.67 mM; tunicamycin 10 μg/ml. Reaction mixture total vol. was 0.15 ml. Reaction was terminated by immersion in boiling water for 2 min or by addition of ethanol, to 20%, by vol. The products were separated by application of the complete reaction mixture to a 1 × 2.5 cm band on Whatman 3MM paper and chromatography for 20 h in solvent (A), isobutyric acid–1 M ammonia (5:3, v/v). The dried papers were cut into 1 cm bands for radioactivity measurement as in [4]. In double isotope experiments the relative efficiencies of counting and correction for overlapping energy spectra were calculated using internal standards of single isotopes (on paper or in solution as required.) Radioactivity in aqueous solution was measured (samples up to 1 ml) in 3 ml of a scintillant containing: diphenyloxazole (0.4% w/v) in toluene–Triton X-100 (1:1, v/v) counted in 5 ml polyethylene vials. Tunicamycin was a generous gift from Professor G. Tamura (Lot T-12-06) and Glaxo Ltd (Lot BAH 6/5). They had identical potency.

Hydrolysis of the lipids was carried out on the paper cut from the appropriate regions of the chromatogram. The paper was cut into small pieces and macerated in 5 ml 5 mM HCl or 0.5 M sodium hydroxide. For acid hydrolysis the suspension of paper was then heated in a boiling water bath for 10 min. This process was repeated once. For alkali hydrolysis the suspension was kept at room temperature for 2 h. At the end of the hydrolysis the suspension was filtered through a glass-fibre disc (Whatman grade GF/D) and the paper sediment washed with a further 10 ml distilled water. The filtrates were combined. Acid extracts were neutralised with aq. ammonia and evaporated to dryness in a rotary evaporator at 30°C. Alkali extracts were passed through a Dowex 50 (X 8) column (1 × 10 cm) in the ammonium form, then ammonia and water were removed by evaporation in vacuo at 30°C. Remaining ammonia was removed by a further evaporation of water at 30°C Ion-exchange chromatography was carried out on a DEAE-cellulose column (1.6 × 40 cm) (Whatman DE52) acetate form and eluted with a linear gradient

of 400 ml pyridinium acetate, pH 5.5 (0–1.0 M acetate) at 60 ml/h, collecting 4 ml fractions. Paper chromatography in solvent B (propanol/aq. NH_3 0.88 sp.gr/water, 6:3:1, by vol.) was carried out on Whatman No. 4 paper that had been washed with 2 M acetic acid, dried, then washed with distilled water, and on Whatman No. 1 paper in solvent C (pyridine/ethyl acetate/acetic acid/water, 5:5:1:3, by vol.) High-voltage paper electrophoresis was carried out on Whatman No. 1 paper in Shandon-Southern L24 equipment, at 35 V/cm in pyridine/acetic acid/water (25:1:225, by vol.) at pH 6.5, for 1 h.

Authentic samples of *N*-acetylglucosamine and DL-glycerol-1-phosphate were purchased from Sigma (St Louis, MO). Glycerylphosphorylglycerol was prepared chemically from DL-glycerol-1-phosphate and 2,3-epoxypropanol as in [16]. 4-Glycerylphosphoryl-*N*-acetylglucosamine was prepared from the wall teichoic acid of *Staphylococcus lactis* I3 as in [17].

Alkaline phosphatase treatment was carried out using 50 units calf intestinal alkaline phosphatase (Boehringer Corp. Mannheim, FRG) in 1 ml of 0.1 M ammonium carbonate, pH 10, at 20°C overnight.

3. Results

When membranes from *S. aureus* H were incubated with radioactive CDP-glycerol at 30°C for 20 min, radioactive lipids were formed that could be extracted into 70% ethanol as observed in [8]. Incorporation into lipids was stimulated 15-fold by the addition of UDP-GlcNAc and was inhibited 90% by tunicamycin. Addition of CDP-ribitol, the substrate for poly(ribitol phosphate) synthesis, caused a 75% decrease of radioactive lipid synthesised in the presence of UDP-GlcNAc.

Lipids were synthesised by *S. aureus* membranes during a 10 min pulse from CDP-[^{3}H]glycerol and UDP-GlcNAc; tunicamycin was then added to prevent further lipid synthesis, followed by the addition of CDP-ribitol to allow poly(ribitol phosphate) synthesis to proceed. Radioactivity present in 70% ethanol-extractable lipids decreased, and label appeared in water-soluble polymeric material that remained at the origin of a chromatograph run in solvent (A). Similar results have been reported [8]. Over a period of 30 min 9300 dpm (per ml membrane suspension) were lost from lipid and 9100 dpm/ml appeared in polymer. No radioactive polymer was synthesised if tunicamycin was added at the start of the 10 min pulse of CDP-[^{3}H]glycerol. Incorporation into lipid in the presence of tunicamycin continued throughout the experiment; this represented 30% of the radioactive lipid at the end of the 10 min pulse and accounted for all the radioactivity in lipid after a further 30 min.

Thin-layer chromatography of the ethanol-extracted lipids from both bacteria on silica gel G in chloroform/methanol/water (65:25:4, by vol.) failed to separate the products, but chromatography on paper in solvent (A), the solvent used to resolve the lipid intermediates of peptidoglycan biosynthesis [18], revealed two distinct labelled components. Therefore in subsequent experiments the complete reaction mixture was applied to the origin of Whatman 3MM paper and developed overnight in solvent (A). The lipids (II and III) had R_F values of 0.77 and 0.66 (R_F of glycerol, 0.5). These were formed by membranes of both *S. aureus* H and *Micrococcus* sp. 2102. The synthesis of both lipids was dependent upon the presence of UDP-GlcNAc and was inhibited by tunicamycin at 1 μg/ml.

The lipid whose synthesis was not inhibited by tunicamycin chromatographed in about the same place as lipid III. Experiments with UDP-[^{14}C]GlcNAc (vide infra) showed that it did not contain *N*-acetylglucosamine and in the case of *S. aureus* its synthesis was unaffected by the presence of CDP-ribitol. It is phosphatidylglycerol synthesised from glycerol phosphate formed by the enzymic breakdown of CDP-glycerol (unpublished, F.M.R.).

When membranes were incubated with UDP-[^{14}C]-GlcNAc in the presence of CDP-glycerol, lipids II and III again became labelled; incorporation of radioactivity into both these lipids was dependent upon the presence of CDP-glycerol. Another lipid (lipid I) running just ahead of lipid II and only partially resolved from it was also present; however the synthesis of this lipid, also occurred in the absence of CDP-glycerol. Synthesis of all three lipids was inhibited by tunicamycin.

Whilst membranes of *S. aureus* H and *Micrococcus* sp. 2102 gave qualitatively the same results, much larger quantities of lipid could be obtained from the *Micrococcus* preparation incubated at 30°C. This was used in subsequent experiments. Figure 2 shows the

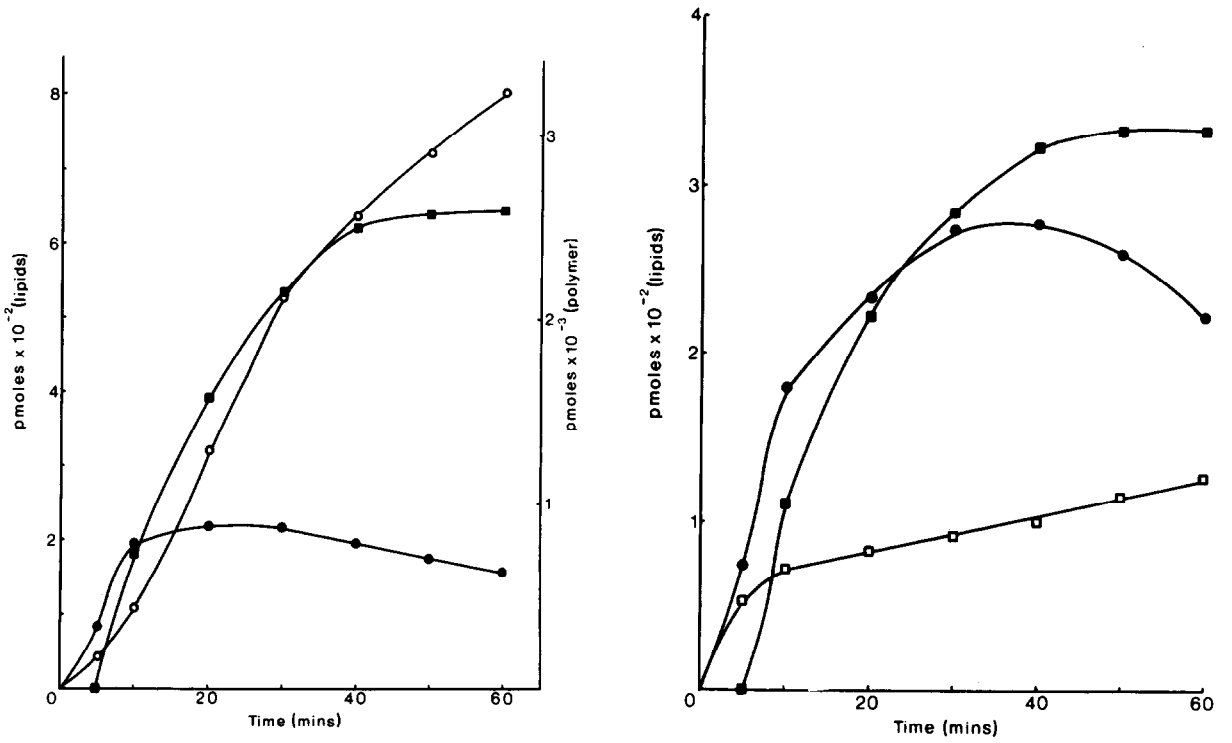

Fig.2. Reaction mixtures containing CDP-[^{3}H]glycerol and UDP-[^{14}C]GlcNAc were incubated at 30°C for the appropriate time. The reaction was then terminated by boiling and the products were separated by chromatography in solvent (A). (2a) shows the incorporation of glycerol from CDP-[^{3}H]glycerol into lipid II (•), lipid III (▪) and linkage unit attached to polymer (○). (2b) shows the incorporation of *N*-acetylglucosamine from UDP-[^{14}C]GlcNAc into lipids I (□), II (•), and III (▪). After 60 min 75% of the CDP-[^{3}H]glycerol and 40% of the UDP-[^{14}C]GlcNAc had been metabolised.

rates of incorporation into lipids I, II and III of ^{3}H from CDP-glycerol (2a) and of ^{14}C from UDP-GlcNAc (2b) in reaction mixtures containing both radioactive substrates. Polymer labelled from CDP-glycerol (fig.2) is linkage unit attached to poly(*N*-acetylglucosamine 1-phosphate) [6]. Lipid I was synthesised rapidly only during the first 5 min incubation and contained only ^{14}C from UDP-GlcNAc (fig.2). Lipid II continued to be synthesised rapidly for a longer period than I. Synthesis of lipid III was not detected after 5 min but this lipid subsequently accumulated to a high level; this was accompanied by a delay in the synthesis of polymer attached to the linkage unit before the maximum rate was reached. Lipids II and III contained both ^{3}H and ^{14}C in the approx. molar ratios 1:1 and 2:1, respectively. This behaviour is consistent with the view that lipids I, II and III are successive intermediates in a pathway in which glycerol phosphate units are transferred to lipid I from CDP-glycerol and that lipid III is a precursor of the linkage-unit teichoic acid polymer.

3.1. *Structure of the intermediates*

Treatment of the radioactive lipid areas of the chromatogram with 5 mM HCl extracted nearly all of the radioactivity, all of the ^{14}C incorporated from UDP-GlcNAc was extracted, but about 20% total radioactivity incorporated from CDP-glycerol remained on the paper from the lipid III region and was believed to be phosphatidylglycerol. Little radioactivity was extracted into water at pH 7.0. Since lipids I and II were not completely resolved in double-label experiments lipid II contained a small variable amount of lipid I. For subsequent analysis lipid I was obtained from that part of the chromatogram that did not contain lipid II.

Dilute acid hydrolysates of the lipid synthesised from [β-^{32}P]CDP-glycerol and UDP-*N*-acetyl-[U-^{14}C]-glucosamine in membranes of *S. aureus* H were neutralised and applied in 10 ml water to a DEAE-cellulose column; elution was carried out in a linear gradient of pyridinium acetate (0–1.0 M acetic acid). Lipid I yielded only *N*-acetylglucosamine on treatment with dilute acid. Lipid II (contaminated with lipid I) yielded two components. One containing ^{14}C eluted rapidly from the column, emerging in 0.02 M acetate; the other, which contained both ^{32}P and ^{14}C in a constant ratio through the peak, emerged in 0.25 M acetate. The ^{14}C-labelled product chromatographed as a single compound on paper in solvents B and C, and corresponded to *N*-acetylglucosamine arising from contaminating lipid I. The doubly-labelled product chromatographed as a single compound on paper in solvent B with $R_{\text{glycerol phosphate}}$ (R_{GP}) 1.6, and had the same mobility as authentic glycerylphosphoryl-*N*-acetylglucosamine. The proportions of radioactivity in the two isotopes in this compound are shown in table 1. They indicate that it contained 1 glycerol phosphate residue/residue of *N*-acetylglucosamine.

Lipid III was treated with dilute acid and the single radioactive product was eluted from the DEAE-cellulose column at 0.3 M acetic acid. This product contained both ^{14}C and ^{32}P in a contstant ratio throughout the peak and it chromatographed as a single compound on paper in solvent B (R_{GP} 1.3). It contained glycerol phosphate and *N*-acetylglucosamine in the ratio 2:1 (see table 1).

The acid hydrolysis products of lipids II and III had mobilities on paper in solvent B greater than that of glycerol phosphate, and so it was unlikely that they contained phosphomonoester groups. It seemed then that they contained glycerol phosphate residues attached by phosphodiester linkage to *N*-acetylglucosamine in the molar ratios 1:1 and 2:1, respectively. In the intact linkage unit in the bacterial wall, the linkage between glycerol phosphate and *N*-acetylglucosamine is labile towards alkali [13]. The acid hydrolysis products of lipids II and III were therefore treated with 0.5 M NaOH for 2 h at room temperature and the products were examined by chromatography on DEAE-cellulose. Both sets of products yielded an unadsorbed compound containing only ^{14}C which was identified as *N*-acetylglucosamine by paper chromatography in solvent C. Lipid II yielded one other product, labelled only with ^{32}P, which emerged from the column in 0.4 M acetate and chromatographed on paper with glycerol phosphate. The products from lipid III also included only one

Table 1
The products of dilute acid hydrolysis of the lipids

	Lipid I	Lipid II	Lipid III
$R_{\alpha\ GP}$ solvent A	2.6	1.6[a]	1.3
$R_{\alpha\ GP}$, pH 6.5, electrophoresis	0.25	0.5[a]	0.7
Micrococcus sp. 2102			
dpm in ^{14}C (22 μCi/μmol)	24 440	49 080	59 060
dpm in ^{3}H (45 μCi/μmol)	0	101 290	23 430
molar ratio ^{3}H/^{14}C	–	0.99	1.93
(in two experiments)		0.98	1.91
Staphylococcus aureus H			
dpm in ^{14}C (2.5 μCi/μmol)	350	1440	9320
dpm in ^{32}P (10.9 μCi/μmol)	0	5160	77 600
molar ratio ^{32}P/^{14}C		0.82	1.91
(in two experiments)		0.85	1.96

[a] The hydrolysate also contained a trace of the product obtained from lipid I

^{32}P-labelled compound, which eluted from the DEAE cellulose in 0.45 M acetate and chromatographed on paper in solvent B with R_{GP} 1.18 (in electrophoresis R_{GP} 1.2). Identical experiments, with the same results, were carried out with lipids labelled from CDP-[^{3}H]glycerol and UDP-[^{14}C]GlcNAc in membranes of *Micrococcus* sp. 2102 (table 1). Thus the phosphodiester bond to *N*-acetylglucosamine in the acid hydrolysis products from the lipids was labile towards alkali in the same way as the similar linkage in the walls [3]. Since all of the *N*-acetylglucosamine in these lipids appeared as free *N*-acetylglucosamine in the above degradations the lipids must have contained glycerol phosphate residues attached to a single *N*-acetylglucosamine residue. Lipid II must therefore have contained one glycerol phosphate residue and one *N*-acetylglucosamine, while lipid III contained two glycerol phosphate residues and one *N*-acetylglucosamine.

The phosphate-containing products from the alkaline degradations were treated with alkaline phosphatase, chromatographed on paper in solvent B and examined by paper electrophoresis. The alkaline hydrolysis product from lipid II, which appeared to be glycerol phosphate, gave rise on treatment with phosphatase to [^{32}P]P_i (from ^{32}P-labelled lipid) or [^{3}H]glycerol (from ^{3}H-labelled lipid), confirming its structure. On treatment with phosphatase the alkali product from lipid III, labelled with ^{32}P, yielded [^{32}P]P_i and ^{32}P-labelled glycerylphosphorylglycerol (R_{GP} 1.9). Figure 3 shows the complete sequence of degradation of the lipids.

The structures of the acid hydrolysis products from lipids I, II and III were therefore *N*-acetylglucosamine, glycerylphosphoryl-*N*-acetylglucosamine (GPGlcNAc) and bis(glycerylphosphoryl)-*N*-acetylglucosamine (GPGPGlcNAc). Since these compounds were formed from the lipids by hot dilute acid we conclude that they were attached to the apolar part of the lipid by a *N*-acetylglucosamine-1-phosphate linkage. The nature of the apolar moiety has not been established unequivocally but as the synthesis of the lipids was inhibited by tunicamycin this strongly suggests that it is a polyisoprenol to which the *N*-acetylglucosamine residue is attached by a pyrophosphate bond [19,20]. The synthesis of a lipid that resembles lipid I, by membranes of *Bacillus subtilis* W23 was stimulated 2-fold by the addition of C_{55}-polyisoprenyl phosphate (G. Wiseman, personal communication). The phosphodiester and pyrophosphodiester linkages between polyprenols and *N*-acetylglucosamine are known to be stable towards dilute alkali [21], whereas glyceride lipids are deacylated. Thus, when lipid III was treated on the chromatography paper with dilute alkali GPGP was released, but all of the *N*-[^{14}C]acetylglucosamine remained on the paper but could be extracted from the washed paper with hot dilute acid. The provided further confirmation of the nature of the lipid.

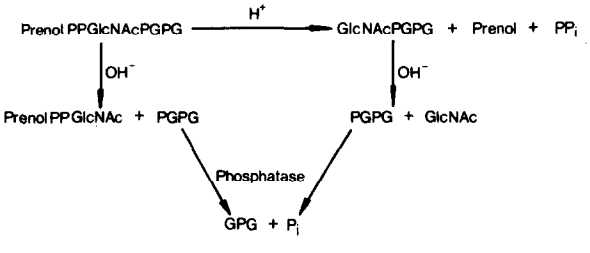

Fig.3. The chemical degradation of lipid III by acid (H^+), alkali (OH^-) and alkaline phosphatase.

4. Discussion

The wall teichoic acid of *S. aureus* H has a chain of poly(*N*-acetyl-glucosaminylribitol phosphate) and the anionic wall polymer of *Micrococcus* sp. 2102 is poly(*N*-acetylglucosamine 1-phosphate). When membranes of *S. aureus* and *Micrococcus* sp. 2102 are incubated with CDP-glycerol, UDP-GlcNAc and the substrate for main chain synthesis (CDP-ribitol and UDP-GlcNAc, respectively), polymer attached to a linkage unit containing glycerol phosphate residues and *N*-acetylglucosamine is formed in addition to polymer that does not carry the linkage unit [4,6]. Lipids are also synthesised from CDP-glycerol and UDP-GlcNAc [4] and turnover has been demonstrated from a crude lipid extract into polymer in *S. aureus* [8].

The present work shows that the lipid fraction that is a precursor of linkage unit attached to main chain contains at least three lipids, I, II and III, that are successive intermediates in the synthesis. The structures of the lipid intermediates are prenyl-PP-GlcNAc, prenyl-PP-GlcNAcPG and prenyl-PP-GlcNAcPGPG. In

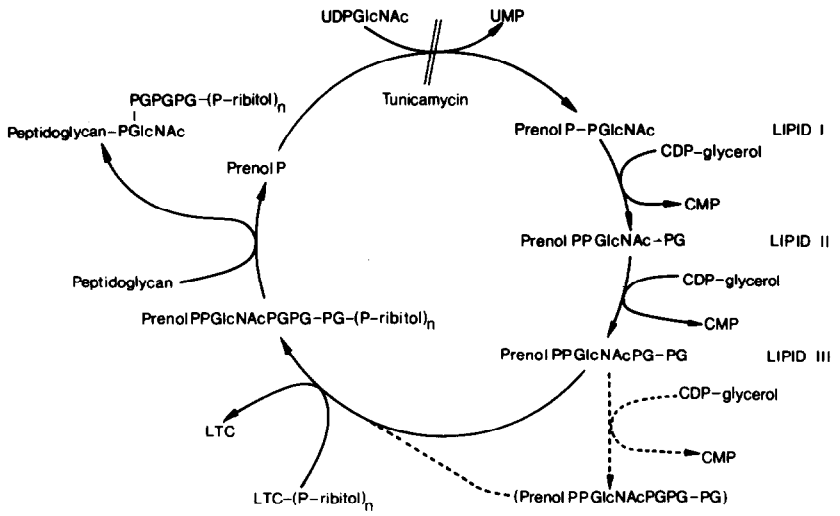

Fig.4. Scheme for the assembly of ribitol teichoic acid-linkage unit in *S. aureus*. PG represents a glycerophosphoryl residue; GlcNAc, *N*-acetylglucosamine.

the intact wall the linkage unit contains three glycerol phosphate residues and one *N*-acetylglucosamine, but we have been unable to detect a fourth lipid intermediate bearing all three glycerol phosphate residues. If the addition of a third glycerol phosphate residue to lipid III from CDP-glycerol is the rate-limiting step in the synthesis and attachment of the linkage unit, then the fourth lipid intermediate might not appear in detectable quantity in our experiments, particularly as such a lipid would be expected to chromatograph in a similar position to the nucleotide substrates required for its synthesis. On the other hand, we cannot rule out the possibility that the third glycerol phosphate residue is transferred with the pre-assembled teichoic acid chain to lipid III. If this were the case, then the glycerol phosphate residue to which the teichoic acid is attached might arise from phosphatidylglycerol via lipoteichoic acid carrier (LTC), rather than from CDP-glycerol. The attachment of poly(ribitol phosphate) to a product of the turnover of phosphatidylglycerol in *S. aureus* is reported [22]. Figure 4 summarises our present knowledge of the pathway for the biosynthesis of teichoic acid linked to peptidoglycan in *S. aureus* H. The linkage unit-polymer in *Micrococcus* sp. 2102 would be assembled in an analagous manner.

Acknowledgement

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