# A LIPID INTERMEDIATE IN THE BIOSYNTHESIS OF A TEICHOIC ACID

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The teichoic acid in the wall of *Staphylococcus lactis* I3 possesses a repeating unit (I) in which α-*N*-acetylglucosamine 1-phosphate is attached through its phosphate to the 3-hydroxyl of D-glycerol 1-phosphate, and D-alanine is attached to the 6-hydroxyl of the aminohexose; the points of attachment of this repeating unit to the polymer chain are the terminal phosphate on glycerol and the 3-hydroxyl of the aminohexose [1]. A particulate enzyme system, presumably fragmented membrane, synthesizes polymer lacking alanine residues from the nucleotides CDP-glycerol and UDP-*N*-acetylglucosamine; CDP-glycerol provides the D-glycerol 1-phosphate component and UDP-*N*-acetylglucosamine provides *N*-acetylglucosamine 1-phosphate as an intact unit [2]. As the biosynthesis of other bacterial wall polymers has recently been shown to proceed through polyisoprenoid pyrophosphate intermediates, it seemed possible that teichoic acids might be formed similarly. This report indicates that related intermediates participate in the biosynthesis of the wall teichoic acid in *S. lactis* I3; aspects of the structure of the intermediates are discussed.

When UDP-[$^{14}$C-acetyl]-*N*-acetylglucosamine and CDP-glycerol were incubated with the particulate enzyme preparation required for the biosynthesis of teichoic acid [2] and the mixture examined on paper in ethanol-M-ammonium acetate, pH 3.8 (7.5 : 3 v/v), radioactive material remained at the origin, but extraction of this area with chloroform-methanol (2 : 1 v/v) removed a considerable amount of radioactive products. Repetition of the experiment in the absence of CDP-glycerol, when teichoic acid biosynthesis no longer occurred, yielded an increased amount of labelled extractable material; the material could also be extracted directly from the incubation mixture with butan-1-ol. The amount of labelled lipid increased with time; after 45 min the incorporation was equivalent to 1–2% of the total substrate, and paper chromatography of the extracted lipid in isobutyric acid-N-ammonium hydroxide (5 : 3 v/v) revealed a single radioactive product ($R_F$ 0.95). Hydrolysis of this in 0.1N-HCl for 15 min at 100° (conditions for the hydrolysis of sugar 1-phosphate linkages) gave *N*-acetylglucosamine.

Addition of UMP to the incubation mixture reduced the formation of labelled lipid to 34% of its original value, whereas UDP had relatively little effect. On the other hand, it has not yet been possible to demonstrate a reversal of the formation of the lipid from UDP-*N*-acetylglucosamine in the presence of an excess of UMP, in a manner analogous to that described [3] for peptidoglycan intermediates. As the incorporation of *N*-acetylglucosamine from nucleotide into the lipid fraction might be explained, at least in part, by the occurrence of the related transfer of *N*-acetylglucosamine to lipids containing muramic acid in peptidoglycan biosynthesis [4], a study was made using the doubly labelled [$^{32}$P]UDP-[$^{14}$C-acetyl]*N*-acetylglucosamine in which $^{32}$P is attached to the acetamido sugar. In the absence of other nucleotides the enzyme preparation converted this into lipid containing both $^{32}$P and $^{14}$C. The results (table 1) indicate that about 90% of the radioactive material formed represents a lipid in which an intact *N*-acetylglucosamine 1-phosphate unit has been transferred. This would support the view that the lipid represents an intermediate in the synthesis of teichoic acid rather than of peptidoglycan. Moreover, incubation of the enzyme prepa-



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Table 1
Incorporation of $^{14}C$ and $^{32}P$ into lipid intermediate.

| Without incubation with UMP | | | With prior incubation with UMP followed by washing | | |
|---|---|---|---|---|---|
| mμ moles incorporated into lipid | | | mμ moles incorporated into lipid | | |
| $^{14}C$ | $^{32}P$ | $^{14}C/^{32}P$ | $^{14}C$ | $^{32}P$ | $^{14}C/^{32}P$ |
| (A) 1.27 | 1.13 | 0.89 | 1.24 | 1.21 | 0.98 |
| (B) 1.53 | 1.39 | 0.91 | 1.20 | 1.17 | 0.98 |

Results represent average values after subtraction of values for boiled enzyme controls.
In experiment (A), particulate enzyme (1 ml) was incubated with $MgCl_2$ (20 μmoles) and UMP (5 μmoles) at 37° for 20 min. Cold 0.1M-Tris-HCL, pH 8.0, was added and the mixture was centrifuged at 30.000 *g*. The sedimented particles were washed three times with buffer and then resuspended in buffer to give the original volume. Aliquots (0.25 ml) of this washed enzyme preparation were incubated with $MgCl_2$ (4 μmoles) and [$^{32}P$]UDP-[$^{14}C$-acetyl]-*N*-acetylglucosamine (0.1 μmole) at 37° for 45 min. Similar mixtures were prepared using untreated enzyme. After incubation, the mixtures were extracted 3 times with butan-1-ol. The organic layers were washed once with water and evaporated to dryness *in vacuo*.
Experiment (B) was performed similarly except that a higher concentration (10 μmoles) of UMP was used.

ration with UMP, followed by exhaustive washing before addition of the doubly labelled UDP-*N*-acetylglucosamine, gave a product in which the ratio $^{14}C:^{32}P$ was identical to that in the nucleotide. As the reaction

lipid P + UDP-*N*-acetylmuramylpeptide ⇌
lipid P-P-*N*-acetylmuramylpeptide + UMP

in the biosynthesis of peptidoglycan is freely reversible [4], such a procedure would remove any lipid P-P-*N*-acetylmuramylpeptide acceptor from the system and thereby preclude the formation of radioactive lipid intermediates for peptidoglycan synthesis.

In experiments where the substrate was CDP-[$^{14}C$]glycerol, incorporation of radioactivity into the lipid was much lower (0.1% of the added substrate) than with the uridine precursor, and was not markedly affected by the simultaneous addition of UDP-*N*-acetylglucosamine. The labelled lipid was formed rapidly, and thereafter, the concentration remained constant. Attempts to accumulate this lipid by the addition of sodium deoxycholate or Triton X-100 were unsuccessful. Incubation at 10° rather than 37° slightly increased the amount of labelled lipid from CDP-[$^{14}C$] glycerol when both nucleotides were present.

Reasonable amounts of glycerol-labelled lipid were obtained by butanol extraction of a large-scale incubation mixture containing CDP-[$^{14}C$]glycerol and unlabelled UDP-*N*-acetylglucosamine. Hydrolysis of this material with 0.1N-HCl for 15 min at 100°, followed by paper chromatography in propan-1-ol-aq. $NH_3$ (sp. gr. 0.88)-water (7 : 1 : 2 v/v), gave a radioactive area corresponding to the unit glycerol-phosphate-*N*-acetylglucosamine ($R_{\text{Glycerol 1-phosphate}}$ 3.4).

When a large-scale reaction mixture containing UDP-[$^{14}C$-acetyl]*N*-acetylglucosamine was incubated with the enzyme, diluted with cold Tris buffer and then centrifuged at 30.000 *g*, most of the radioactive lipid sedimented with the enzyme. After repeated washing with buffer to remove residual nucleotide, the labelled particles were resuspended in Tris and then incubated with unlabelled CDP-glycerol. Phenol extraction of the reaction mixture, followed by paper chromatography of the aqueous layer, showed the presence of radioactive teichoic acid, characterized by the degradation scheme previously described [2]. The amount of labelled teichoic acid increased with time after the addition of CDP-glycerol; a simultaneous and equivalent decrease in the amount of radioactive lipid extractable with butanol was observed.

Attempts to demonstrate directly polymer synthesis from butanol-extracted crude or purified lipid were less conclusive. On only one occasion, when a large quantity of labelled crude lipid extract was used, was radioactivity detected in a fraction characterized as teichoic acid by chemical degradation. However, the amount of incorporation observed in this case represented less than 0.1% of the total radioactive lipid added. Presumably problems of solubility and disorientation prevent a more convincing demonstration of the utilization of this material.

Various methods of purification of the lipid intermediate formed from UDP-[$^{14}C$-acetyl]*N*-acetylglucosamine have been attempted. The most satisfactory procedure involved chromatography on two successive columns of DEAE-cellulose (acetate form)

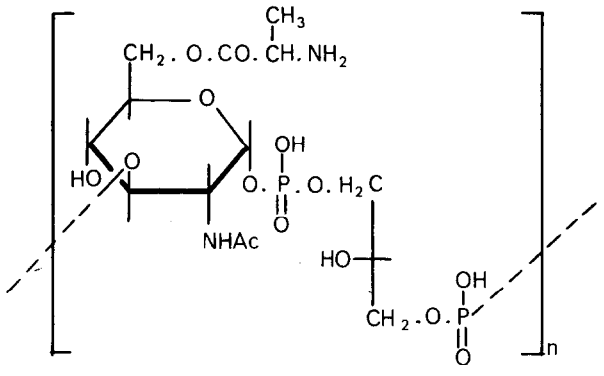

Fig. 1.

[5], using linear gradients of 0–0.4M and 0.15–0.4M-ammonium acetate in methanol. Material giving a sharp peak (A) of radioactivity was eluted at a concentration of about 0.26M-ammonium acetate; a second component (B), much smaller in amount than the first, was eluted at 0.30M-ammonium acetate. Both products were further purified on a column of Sephadex LH20, the elution being carried out with methanol containing 1% of water [6].

Hydrolysis of the major product (A) in 0.1N-HCl for 10 min at 100° gave *N*-acetylglucosamine, identified by paper chromatography in four solvent systems. Hydrolysis with N-NaOH for 1 hr at 100° followed by passage through Dowex 50 ($NH_4^+$ form) resin followed by paper chromatography, gave *N*-acetyl glucosamine 1-phosphate, identified by chromatography in two solvent systems, by hydrolysis to *N*-acetylglucosamine with 0.1N-HCl for 8 min at 100° and by treatment with 50mM-ammonium carbonate buffer, pH 9.4, containing calf intestinal phosphomonoesterase (0.5 mg/ml).

Mild acid hydrolysis (0.1N-HCl for 5 min at 100°) of the minor radioactive product (B) gave a compound that chromatographed in a similar position to *N*-acetylglucosamine in two solvents, but which in butanol-pyridine-water (6:4:3 v/v) had $R_{Glc}$ 0.7.

We conclude that during the biosynthesis of the teichoic acid an *N*-acetylglucosamine 1-phosphate unit is transferred from the nucleotide to a lipid, the detailed structure of which is not yet understood, but which contains the acetamidosugar 1-phosphate residue possibly attached through a pyrophosphate linkage. A second lipid intermediate is formed by transfer of a glycerol phosphate residue from CDP-glycerol to the 3-position on the *N*-acetylglucosamine residue of the first lipid; this then transfers the complete repeating unit to the growing polymer.

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