A Lipid Intermediate in the Synthesis of a Poly-(*N*-Acetylglucosamine 1-Phosphate) from the Wall of *Staphylococcus lactis* N.C.T.C. 2102

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1. The enzymic synthesis of the wall polymer poly-(*N*-acetylglucosamine 1-phosphate) in *Staphylococcus lactis* N.C.T.C. 2102 was studied by using UDP-[acetyl-¹⁴C]*N*-acetylglucosamine and the corresponding nucleotide containing ³²P. 2. Labelled material was extracted from the particulate enzyme preparation with butan-1-ol. Pulse-labelling experiments indicated that this material contained an intermediate in the biosynthesis. 3. The lipid intermediate was partially purified, and chemical and enzymic degradation showed that it was composed of *N*-acetylglucosamine 1-pyrophosphate in labile ester linkage to an organic-soluble alcohol, possibly a polyisoprenoid alcohol. The methanolysis of sugar 1-pyrophosphate derivatives, including nucleoside diphosphate sugars, is discussed in relation to degradation products obtained from the lipid. 4. The lipids from the particulate enzyme preparation probably contained another compound in which *N*-acetylglucosamine 1-phosphate is attached to an organic-soluble alcohol; this may participate in the biosynthesis of another polysaccharide. 5. The function of the lipid intermediate in polymer biosynthesis is discussed.

The biosynthetic pathways for the production of several bacterial wall and membrane polymers have been studied in considerable detail during recent years. In several cases it has been shown that sugar residues and related components are transferred from nucleotide precursors through lipid intermediates to polymer chains. Thus lipids containing a polyisoprenoid structure have been shown to participate in the synthesis of peptidoglycans (Higashi, Strominger & Sweeley, 1967), lipopolysaccharides (Wright, Dankert, Fennessey & Robbins, 1967) and a membrane mannan (Scher, Lennarz & Sweeley, 1968). The occurrence of a related lipid intermediate in the synthesis of a teichoic acid has also been demonstrated (Douglas & Baddiley, 1968), although the very small amounts available have so far prevented the full characterization of the lipophilic part of the molecule.

The wall of *Staphylococcus lactis* N.C.T.C. 2102 contains a polymer with a repeating unit of *N*-acetylglucosamine α -1-phosphate; linkage between the units in the chain involves the phosphate group and the 6-hydroxyl group on the sugar of a neighbouring unit as shown in Fig. 1 (Archibald, Baddiley, Button, Heptinstall & Stafford, 1968). The synthesis of the polymer, which in many respects

resembles a teichoic acid, has been studied by using a particulate enzyme system, the effective component of which is probably fragmented cytoplasmic membrane (Hussey, Brooks & Baddiley, 1969; Brooks & Baddiley, 1969). It was found that UDP-*N*-acetylglucosamine donated *N*-acetylglucosamine 1-phosphate residues to the '*N*-acetylglucosamine' end of the polymer chain and that synthesis was inhibited by UMP. It was concluded that polymerization involved the reversible step:

This work shows that the acceptor is a lipid phosphate, and that the product in the above step is a lipid pyrophosphate attached to the 1-position of *N*-acetylglucosamine. The structure of the intermediate and the mechanism of the biosynthetic route are discussed.

MATERIALS AND METHODS

UDP-*N*-acetylglucosamine, *N*-acetylglucosamine, di-phenyl phosphate, calf intestinal phosphomonoesterase (alkaline phosphatase), β -glucosidase and inorganic pyrophosphatase were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. [¹⁴C]Acetic anhydride and

Fig. 1. Poly-(*N*-acetylglucosamine 1-phosphate) from walls of *Staphylococcus lactis* N.C.T.C. 2102. Ac, $\text{CH}_3\cdot\text{CO}$.

diphenyl [^{32}P]phosphate were purchased from The Radiochemical Centre, Amersham, Bucks., and DEAE-cellulose was purchased from H. Reeve Angel and Co. Ltd., London E.C.4. Sephadex LH-20 was purchased from Pharmacia (G.B.) Ltd., London W.13. Samples of the 3, 4, 6-triacetates of methyl 2-acetamido-2-deoxy- α (and β)-D-glucopyranoside were kindly given by Dr J. G. Buchanan; a sample of a bacterial phosphodiesterase was kindly given by Dr D. Farr.

Analytical methods. Radioactive material was located on paper chromatograms with a radiochromatogram scanner (Baird-Atomic Inc., Cambridge, Mass., U.S.A.). Quantitative measurements were carried out in a Packard 4000 series Tri-Carb Liquid Scintillation Counter with a scintillant of the following composition: toluene (A.R.), 2l.; 2,5-diphenyloxazole, 8g.; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.2g. For soluble samples the scintillant was mixed with Triton X-100 (2:1, v/v) (Patterson & Greene, 1965).

Paper chromatography. This was carried out on Whatman no. 1 paper that had been washed with 2M-acetic acid, in the following descending solvent systems: *A*, M-ammonium acetate-ethanol, pH 3.6 (5:2, v/v) (Paladini & Leloir, 1951); *B*, propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (7:1:2, by vol.) (LeCocq & Ballou, 1964); *C*, propan-1-ol-ethyl acetate-water (7:1:2, by vol.) (Baar & Bull, 1953). Sugars and polyols were detected with alkaline AgNO_3 (Trevelyan, Procter & Harrison, 1950) and phosphates with the molybdate reagent (Hanes & Isherwood, 1949).

Preparation of enzyme. The organism, *Staphylococcus lactis* N.C.T.C. 2102, was grown and the enzyme prepared as described previously (Brooks & Baddiley, 1968).

Preparation of substrates. UDP-*N*-acetylglucosamine labelled with ^{14}C and ^{32}P was prepared as described previously (Brooks & Baddiley, 1969).

Preparation of methyl *N*-acetylglucosaminides. These were prepared through the corresponding 3, 4, 6-triacetates of methyl 2-acetamido-2-deoxy- α (and β)-D-glucopyranoside by the method of Hardy, Buchanan & Baddiley (1963).

RESULTS

Formation of lipid intermediate from nucleotide precursor. The rate of incorporation of labelled *N*-acetylglucosamine 1-phosphate into extractable lipids in the particulate enzyme preparation is given in Fig. 2. This is compared with the rate of

Fig. 2. Rate of formation of lipid intermediate and polymer. The incubation mixture contained UDP-[acetyl- ^{14}C]*N*-acetylglucosamine (0.1 μmole ; 2.7×10^6 c.p.m./ μmole), MgCl_2 (10 μmoles) and particulate enzyme suspended in 0.1M-tris-HCl buffer, pH 8.5 (0.15 ml.), in a final volume of 0.17 ml. Incubation was carried out at 37° for the times indicated. The reaction was stopped by heating at 100° for 2 min. Lipids were extracted by shaking three times with an equal volume of butan-1-ol, washing the combined extracts with an equal volume of water and removing the solvent *in vacuo*. Polymer was extracted from the residual enzyme with 80% (w/v) phenol, as described previously (Brooks & Baddiley, 1969). x, Incorporation of ^{14}C into polymer; ●, incorporation of ^{14}C into lipids.

incorporation of isotope into polymer, and it is seen that both follow similar courses. After 1 hr. about 4% of the radioactivity of the system represented lipid and about 17% represented polymer.

The incorporation of both *N*-acetylglucosamine and phosphate from the nucleotide into the lipid fraction was demonstrated by using UDP-*N*-acetylglucosamine containing ^{14}C in the acetyl and ^{32}P in the phosphate group attached to the 1-position of the *N*-acetylglucosamine residue. The results are given in Table 1. The $^{14}\text{C}/^{32}\text{P}$ atomic ratio in the lipid was appreciably higher than the theoretical 1:1 required for the transfer of an intact *N*-acetylglucosamine 1-phosphate residue. The experiment in which the enzyme preparation was incubated with UMP and then washed before extraction of the lipids was designed to eliminate the transfer of an *N*-acetylglucosamine residue from the nucleotide to those lipid intermediates containing muramic

Table 1. *Incorporation into lipids of ^{14}C and ^{32}P from UDP-N-acetylglucosamine*

In Expt. A, particulate enzyme suspended in 0.1 M-tris-HCl buffer, pH 8.5 (0.15 ml.), was incubated with MgCl_2 (10 μmoles) and [^{32}P]UDP-[acetyl- ^{14}C]N-acetylglucosamine (0.1 μmole ; Brooks & Baddiley, 1969) in a final volume of 0.17 ml. at 37° for 1 hr. The reaction was stopped by heating at 100° for 2 min. The polymer and lipid were extracted and separated, and their radioactivity was measured as described in Fig. 2. In Expts. B and C, particulate enzyme in 0.1 M-tris-HCl buffer, pH 8.5 (0.15 ml.), was incubated with MgCl_2 (10 μmoles) and the stated amounts of UMP (final vol. 0.17 ml.) at 37° for 30 min. (B) and 1 hr. (C). After incubation, cold 0.1 M-tris-HCl buffer, pH 8.5 (about 25 ml.), was added and the enzyme was sedimented by centrifuging at 27 750 g for 15 min. at 0°. The sediment was washed twice with the buffer and then resuspended in it to give the original volume; MgCl_2 (10 μmoles) and [^{32}P]UDP-[acetyl- ^{14}C]N-acetylglucosamine (0.1 μmole) were added (final vol. 0.17 ml.) and the mixture was incubated at 37° for 1 hr. The reaction was stopped by heating at 100° for 2 min. Lipids and polymer were extracted and separated, and their radioactivity was determined as described in Fig. 2. Results are average values after subtraction of values for boiled controls.

Expt.	Amount of UMP (μmoles)	Radioactivity (c.p.m.)		Atomic ratio $^{14}\text{C}/^{32}\text{P}$	
		ratio $^{14}\text{C}/^{32}\text{P}$		ratio $^{14}\text{C}/^{32}\text{P}$	
		In lipid	In nucleotide	In lipid	In polymer
A	0	17.9:1	6.0:1	3.0:1	1:1
B	0.75	13.9:1	6.6:1	2.1:1	1:1
C	6.0	12.9:1	7.0:1	1.9:1	1:1

acid that are involved in the synthesis of peptidoglycan. The transfer of N-acetylglucosamine residues in this way could be responsible, at least in part, for the high values of the $^{14}\text{C}/^{32}\text{P}$ ratio; incubation with UMP would reverse the first stage (see eqn. 2) of transfer of a muramylpeptide residue from the corresponding nucleotide to lipid (Anderson, Matsushashi, Haskin & Strominger, 1967), thereby removing from the lipids all muramylpeptide-pyrophosphate-lipid capable of accepting an N-acetylglucosamine residue. Although this treatment reduced the $^{14}\text{C}/^{32}\text{P}$ ratio it was still greater than 1:1.

Analysis of labelled lipids. Larger amounts of particulate enzyme preparation were used to study the structure of labelled material arising from UDP-N-acetylglucosamine. In a typical experiment the incubation mixture contained 2.25 ml. of enzyme suspension and 1.5 μmoles of labelled nucleotide. Lipids were extracted with butan-1-ol as described in Fig. 2, the extract was washed with water and evaporated *in vacuo*. Hydrolysis was carried out with 0.1 M-hydrochloric acid in 50% (v/v) methanol for 2 min. at 100°. The clear solution was decanted from lipid material that had been

deposited during the hydrolysis; it was evaporated *in vacuo* several times with water and then chromatographed in solvent A. Radioactive areas were cut out and products were eluted with water. The following were identified:

(1) Inorganic pyrophosphate. This co-chromatographed with authentic material in solvent A. On treatment with inorganic pyrophosphatase (0.1 ml., 6 units) at pH 7 in 0.05 M-tris-hydrochloric acid buffer for 1.5 hr. at 78° it gave inorganic orthophosphate.

(2) N-Acetylglucosamine. This was identified by co-chromatography with authentic material in solvents A and C.

(3) Methyl N-acetyl- β -D-glucosaminide. An authentic sample of this glycoside co-chromatographed with the product in solvents A and C. N-Acetylglucosamine gave no methyl glycoside under identical conditions with 50% (v/v) methanolic hydrochloric acid. However, the β -glycoside was formed under these conditions from UDP-N-acetylglucosamine and from N-acetylglucosamine 1-phosphate. In a similar experiment UDP-glucose gave glucose, methyl β -D-glucopyranoside (R_{Glc} 1.5 in solvent C) and an unidentified product (R_{Glc} 0.194 in solvent C). The configuration of the glucoside was established by hydrolysis with β -glucosidase.

(4) N-Acetylglucosamine 1-pyrophosphate. This product had an R_F of 0.49 relative to N-acetylglucosamine 1-phosphate in solvent A. On hydrolysis in 0.1 M-hydrochloric acid for 10 min. at 100° it gave N-acetylglucosamine, whereas in 0.1 M-hydrochloric acid in 50% (v/v) methanol for 2 min. at 100° it gave N-acetylglucosamine and its methyl β -glycoside. A sample of the pyrophosphate was incubated for 4 hr. at 37° with a phosphodiesterase from a *Bacillus* sp. (see the Discussion section) in 10 mM-imidazole-hydrochloric acid buffer, pH 7.2, that was 0.5 mM with respect to cobalt chloride; after chromatography in solvent A the only labelled product was N-acetylglucosamine 1-phosphate. Treatment of this phosphate for 3 hr. at 37° with intestinal phosphomonoesterase (1 mg./ml.) in 50 mM-ammonium carbonate, pH 9.2, that was 1 mM with respect to magnesium chloride gave N-acetylglucosamine.

(5) N-Acetylglucosamine 1-phosphate. This was a minor product and was identified by co-chromatography with authentic material in solvents A and B. It gave N-acetylglucosamine on hydrolysis in 0.1 M-hydrochloric acid for 10 min. at 100°.

(6) An unidentified product. This was a minor product and had an R_F of 1.0 relative to N-acetylglucosamine in solvent A and 0.64 in solvent C.

Methanolysis of poly-(N-acetylglucosamine 1-phosphate). Polymer containing ^{14}C was treated

with 0.1M-hydrochloric acid in aqueous 50% (v/v) methanol for 5min. at 100°. After evaporation several times, paper chromatography in solvent A revealed *N*-acetylglucosamine, its 6-phosphate and a product that ran just ahead of the 6-phosphate. This product and the 6-phosphate were hydrolysed by the phosphomonoesterase (1mg./ml.) at 37° for 4hr. in 0.1ml. of 50mM-ammonium carbonate (pH 9.5) that was 1mM with respect to magnesium chloride. The *N*-acetylglucosamine 6-phosphate gave *N*-acetylglucosamine, whereas the other product gave methyl *N*-acetyl- β -D-glucosaminide.

Purification of the lipid. The procedure was similar to that used by Dankert, Wright, Kelley & Robbins (1966) for the lipid intermediate in lipopolysaccharide biosynthesis. Labelled lipids were prepared by the method described in Fig. 2 and Table 1, by using 2.25 ml. of enzyme preparation that had been pretreated with UMP. The resulting solution of lipids in butan-1-ol (1ml.) was fractionated on a column (1cm. $\times$ 30cm.) of DEAE-cellulose; after extensive washing of the column with methanol containing 1% (v/v) of water, material was eluted with the same solvent containing ammonium acetate in a concentration gradient rising from 0 to 0.4M. At about 0.1M-ammonium acetate a little *N*-acetylglucosamine was recovered, whereas the major labelled fraction was eluted at 0.24M-ammonium acetate. This material was rechromatographed on Sephadex LH-20 in methanol containing 1% (v/v) of water. A single major peak of radioactive material was observed containing ^{14}C and ^{32}P in the atomic ratio 1.4:1.

Utilization of the lipid. Attempts to demonstrate the utilization of unpurified lipids in defatted

enzyme systems were unsuccessful, although similar experiments on the synthesis of peptidoglycan have been successful (Dietrich, Colucci & Strominger, 1967). However, pulse-labelling studies have shown that labelled UDP-*N*-acetylglucosamine transferred its labelled sugar phosphate residue first to lipids and this was then transferred from lipids to polymer. These experiments are described in Table 2; they show that both ^{14}C and ^{32}P , introduced into lipids from the nucleotide, appear in the polymer after further incubation with unlabelled nucleotide. After the second incubation, a decrease in c.p.m. of both ^{14}C and ^{32}P occurred in the lipid fraction, whereas an increase in both was observed in the polymer. Quantitative interpretation of the results was difficult in view of the simultaneous formation of the lipid that receives only *N*-acetylglucosamine from the nucleotide. Thus the $^{14}\text{C}/^{32}\text{P}$ atomic ratio in the lipid increased from 2.1:1 after the first incubation to 2.7:1 after the second incubation, whereas the value for the polymer changed only from 0.9:1 to 1.1:1 (based on a value of 1:1 for the nucleotide). An additional difficulty in the quantitative studies arose through the irreversible adsorption of variable amounts of UDP-*N*-acetylglucosamine on the enzyme preparation: in an experiment carried out as described in Table 2, in which incubation with labelled nucleotide for 2min. was followed by the washing procedure and phenol extraction, a paper-chromatographic study of the aqueous layer showed that up to 15% of the radioactivity in the particles was due to adsorbed nucleotide. This would tend to mask the course of the isotopic label in the pulse-labelling experiments.

Effect of antibiotics, detergents and solvents on

Table 2. Pulse-labelling of lipid and polymer

Particulate enzyme in 0.1M-tris-HCl buffer, pH 8.5 (0.3 ml.), was incubated with MgCl_2 (20 μmoles) and UMP (1.5 μmoles) in a final volume of 0.32 ml. for 1 hr. at 37°. It was then diluted with 0.1M-tris-HCl buffer, pH 8.5 (about 25 ml.), at 4° and the enzyme was collected at 27750 g during 15 min. at 0°. The pellet was washed twice with buffer and then resuspended in it to give the original volume. The suspension was mixed with MgCl_2 (20 μmoles) and [^{32}P]UDP-[acetyl- ^{14}C]-*N*-acetylglucosamine (0.2 μmole) in a final volume of 0.34 ml. and then incubated at 37° for 1 hr. A sample (0.17 ml.) was removed, heated for 2 min. at 100°, and the lipids and polymer were extracted as described in Fig. 2. The remainder of the suspension was diluted with buffer (about 25 ml.) at 4° and centrifuged at 27750 g for 15 min., and the pellet was washed twice with buffer (about 25 ml. each) and again suspended in it to give the original volume. To it was added MgCl_2 (10 μmoles) and UDP-*N*-acetylglucosamine (0.1 μmole) to give a final volume of 0.17 ml. The mixture was incubated at 37° for 1 hr. and then heated for 2 min. at 100°. Lipids and polymer were extracted and examined by the method given in Fig. 2.

Expt.	Isotope in polymer (c.p.m.)		Isotope in lipid (c.p.m.)		Atomic ratio $^{14}\text{C}/^{32}\text{P}$	
	^{14}C	^{32}P	^{14}C	^{32}P	Polymer	Lipid
Initial incubation with labelled UDP- <i>N</i> -acetylglucosamine	8805	1355	1760	112	0.9:1	2.1:1
Subsequent incubation with unlabelled UDP- <i>N</i> -acetylglucosamine	11300	1433	561	28	1.1:1	2.7:1

lipid synthesis. The action of a number of antibiotics, detergents and solvents on the incorporation of isotopic label into both lipids and polymer was examined. In these experiments, incorporation into lipids would include a contribution to lipid intermediates in the biosynthesis of peptidoglycan; results are given in Table 3. It is noteworthy that the antibiotics, all of which inhibit peptidoglycan synthesis, had little effect on both lipid and polymer biosynthesis. Triton X-100 inhibited polymer formation but produced an enhanced incorporation of isotopic label into lipids. Alcohols were strongly inhibitory through irreversible damage to the enzyme system.

Analysis of the particulate enzyme system. The enzyme preparation presumably represents fragmented cytoplasmic membrane together with ribosomal material, and would thus be expected to be composed of protein, lipids, carbohydrates, RNA and membrane teichoic acid. It is known (unpublished work by G. H. Stafford) that the membrane teichoic acid of the organism is a poly(glycerol phosphate) with few or no sugar residues. The nature of other carbohydrate components was examined by using the phenol extraction procedure (Burger & Glaser, 1964); the aqueous layer from this experiment was washed with chloroform and the dried residue hydrolysed with 0.5M-sulphuric acid at 100° for 3 hr. After removal of acid with barium carbonate the products were examined in solvent C. Galactose, mannose and glycerol were observed, together with a smaller amount of *N*-acetylglucosamine.

DISCUSSION

Previous work (Brooks & Baddiley, 1969) has shown that the biosynthesis of the wall component poly-(*N*-acetylglucosamine 1-phosphate) in *Staphylococcus lactis* N.C.T.C. 2102 occurs by the transfer of *N*-acetylglucosamine 1-phosphate residues from UDP-*N*-acetylglucosamine to the 6-hydroxyl group of an *N*-acetylglucosamine residue at the end of the growing polymer chain; chain extension occurs at the 'sugar-terminal' rather than the 'P-terminal' end of chains (Hussey *et al.* 1969; Brooks & Baddiley, 1969). In the present studies it was found that, during this synthesis with cell-free particulate enzyme preparations, lipid components of the preparation accepted both *N*-acetylglucosamine and phosphate from doubly labelled UDP-*N*-acetylglucosamine (Table 1). The labelled lipids could be extracted from the particulate material with butan-1-ol, and nucleotide containing ¹⁴C in its acetyl group and ³²P in the phosphate group attached to the 1-position of the *N*-acetylglucosamine contributed both of these groups to the polymer and to the lipid. The amount

of ¹⁴C in the lipids increased slowly with time, whereas the amount in the polymer increased more rapidly (Fig. 2); this is consistent with other evidence that the nucleotide contributes *N*-acetylglucosamine residues but not phosphate to lipids involved in the biosynthesis of other cell components.

The ¹⁴C/³²P atomic ratio in the lipid extract was considerably greater than 1:1, the value required for the transfer of an intact *N*-acetylglucosamine 1-phosphate residue from nucleotide to lipid (Table 1); presumably *N*-acetylglucosamine residues without phosphate were also being transferred to lipids. This could be explained if the lipid component of the enzyme system contained *N*-acetylmuramylpeptide-lipids associated with peptidoglycan synthesis but which lacked *N*-acetylglucosamine residues; these lipids would accept *N*-acetylglucosamine but not phosphate from the nucleotide. In studies on the nature of lipid intermediates participating in the biosynthesis of the wall teichoic acid of *Staphylococcus lactis* I3 (Douglas & Baddiley, 1968), this difficulty was overcome by making use of the reversibility (Anderson *et al.* 1967) of the reaction:

Thus preincubation of the enzyme preparation with UMP, followed by thorough washing, removed lipids bearing *N*-acetylmuramylpeptide residues that would have been capable of accepting *N*-acetylglucosamine residues. By using such enzyme preparations, lipid was obtained in which the ¹⁴C/³²P atomic ratio was 1.9:1, and it follows that, although some lipid containing *N*-acetylmuramylpeptide had been removed, the mixture included a component in which *N*-acetylglucosamine had been transferred from the nucleotide without the simultaneous transfer of phosphate.

The demonstration that the lipid mixture contained a component that was a true intermediate in the biosynthesis of the poly-(*N*-acetylglucosamine 1-phosphate) was attempted in two ways. In the first experiments the freeze-dried particulate enzyme preparation was treated at a low temperature (−15°) with chloroform-methanol (1:1, v/v)

or butan-1-ol in an effort to obtain a lipid-free preparation that would utilize added labelled lipid (cf. Dietrich *et al.* 1967; Pieringer & Kunnes, 1965; Pieringer, 1968). However, the system was unable to withstand treatment with the organic solvent, and irreversible denaturation occurred. In the second investigation a pulse-labelling technique was used (Table 2). By using UDP-*N*-acetylglucosamine labelled in the acetyl group and in the phosphate group at the 1-position on the *N*-acetylglucosamine residue it was shown that label from the nucleotide was transferred first to the lipids, and subsequently from lipids to the polymer. As in the previous experiments, it was found that, although the $^{14}\text{C}/^{32}\text{P}$ ratio in the nucleotide was the same as that in the polymer, there was an excess of ^{14}C in the lipids, suggesting the accumulation of a lipid containing *N*-acetylglucosamine.

Partial purification of the lipid intermediate was achieved by chromatography on DEAE-cellulose and Sephadex LH-20. Although a sharp main peak of radioactivity was observed, the $^{14}\text{C}/^{32}\text{P}$ ratio was 1.4:1, indicating that some of the phosphate-deficient lipid contaminant was still present.

A partial structure for the intermediate was established by controlled hydrolysis in aqueous methanol. However, the extremely small amounts of material available (6ml. of enzyme suspension from each 20l. batch of culture) restricted structural work to the study of those parts of the molecule that could be identified chromatographically by detection of their radioactivity, i.e. the *N*-acetylglucosamine and phosphate residues. In the studies by others on the nature of lipid intermediates participating in the biosynthesis of

peptidoglycans or mannan, useful amounts of particulate enzyme material were prepared by dissolution of large quantities of cells with lysozyme; or in the case of lipids in the biosynthesis of lipopolysaccharides other procedures were available for the easy separation of fragmented membrane from cells. In our experiments the organism was resistant towards the action of lysozyme, and the enzyme preparation had to be made by mechanical disruption of washed cells, followed by differential centrifugation and washing. This method is laborious and inefficient, and cannot easily be adapted to large-scale operation. In the related studies by Higashi *et al.* (1967) 11. of sedimented membrane was used to prepare 4 μ moles of purified lipid from a system in which lipid intermediates accumulated. In our system no accumulation occurred since polymer biosynthesis could not be inhibited specifically.

Hydrolysis of the partially purified lipid under very gentle conditions (0.1M-hydrochloric acid in 50% methanol for 2min. at 100°) gave a number of water-soluble products that accounted for all of the radioactivity. The relative proportions of the different products varied with different batches of lipid, suggesting that a mixture with variable composition was being studied. Products included *N*-acetylglucosamine, its α -1-phosphate and inorganic pyrophosphate, together with a compound that was characterized as *N*-acetylglucosamine 1-pyrophosphate (see Scheme 1). This last compound was hydrolysed to *N*-acetylglucosamine 1-phosphate by a recently discovered bacterial phosphodiesterase (J. Hutchinson, D. Farr, P. D. G. Dean & J. Baddiley, unpublished work). These

Scheme 1. Proposed structure of lipid intermediate. Ac, $\text{CH}_3\cdot\text{C}\cdot\text{O}$.

products are consistent with the structure shown in Scheme 1 for the lipid intermediate.

The linkage between lipid and phosphate was completely hydrolysed under the mild conditions used. Such marked lability is unusual for phosphate esters and strongly suggests the presence of a double bond in the β -position to the phosphate ester grouping. This is consistent with the known acid lability of isoprenoid phosphates, but, although it is likely that the lipid contains a polyisoprenoid phosphate structure, as do the lipid intermediates that participate in the biosynthesis of other bacterial polymers (Higashi *et al.* 1967; Wright *et al.* 1967; Scher *et al.* 1968), insufficient material has been obtained for a study by mass-spectroscopy. The first products of hydrolysis would be the isoprenoid alcohol and its rearrangement products, together with *N*-acetylglucosamine 1-pyrophosphate. Partial hydrolysis of the sugar 1-phosphate linkage would be expected, giving *N*-acetylglucosamine and inorganic pyrophosphate; on the other hand, hydrolysis to give *N*-acetylglucosamine 1-phosphate would be less likely, and we believe that this product indicates that a compound with the structure *N*-acetyl glucosamine 1-phosphate-lipid is also present in the partially purified lipid.

A product formed in significant amounts by the action of aqueous-methanolic acid was methyl *N*-acetyl- β -D-glucosaminide. This arises through acid-catalysed methanolysis of the sugar 1-pyrophosphate linkage. Although the mechanism of the reaction has not been established, it is presumably analogous to that for acid-catalysed hydrolysis of glycosides and sugar 1-phosphates; thus an S_N1 mechanism would involve the intermediate formation of a carbonium ion with the charge shared between C-1 and the ring oxygen atom. Subsequent attack by methanol would be sterically influenced by the acetamido group at the 2-position, giving overall inversion of configuration in the final product. In support of this explanation for the formation of methyl *N*-acetyl- β -D-glucosaminide is the observation that under

comparable conditions it is a major product from UDP-*N*-acetylglucosamine and from *N*-acetylglucosamine 1-phosphate. The poly-(*N*-acetylglucosamine 1-phosphate) reacted similarly, giving the methyl β -glycoside of *N*-acetylglucosamine 6-phosphate, and UDP-glucose gave methyl β -D-glucopyranoside and a small amount of an unidentified product. Scher *et al.* (1968) report without comment the formation of ' α -methyl-mannose' through comparable treatment of their mannose 1-phosphate-lipid. Assuming that their product was, in fact, methyl α -mannoside then this would have been formed in a manner similar to that described here, the absence of overall inversion of configuration at the 1-position in the mannose derivatives being due to the stereochemistry of C-2.

A route for the biosynthesis of poly-(*N*-acetylglucosamine 1-phosphate) involving the lipid intermediate is outlined in Scheme 2. In the first step reaction occurs between a lipid monophosphate and UDP-*N*-acetylglucosamine, whereby an *N*-acetylglucosamine 1-phosphate residue is transferred to the lipid to give the intermediate described above. The *N*-acetylglucosamine 1-phosphate residue is then transferred to the acceptor, the other product being lipid monophosphate. As chain extension occurs at the '*N*-acetylglucosamine' end of the polymer, the accepting group must be the 6-hydroxyl group of the terminal *N*-acetylglucosamine on the polymer. It is not yet known whether the other end of the chain is already attached to peptidoglycan or its precursors, but the tenacity with which this water-soluble polymer adhered to the particulate material suggests that it is. The biosynthetic route is similar in some respects to those proposed for the biosynthesis of peptidoglycans and lipopolysaccharides, except that it requires the transfer of phosphate to give a polymer and a lipid monophosphate, whereas the other syntheses involve transglycosylation giving polymers and lipid pyrophosphate. This major difference is consistent with the failure of bacitracin to inhibit

Scheme 2. Biosynthesis of poly-(*N*-acetylglucosamine 1-phosphate) through a lipid intermediate.

Table 3. *Effect of antibiotics, solvents and detergents on polymer and lipid synthesis*

The incubation mixtures contained UDP-[acetyl- ^{14}C]N-acetylglucosamine (0.1 μmole ; 2.7×10^6 c.p.m./ μmole), MgCl_2 (10 μmoles), particulate enzyme preparation suspended in 0.1 M-tris-HCl buffer, pH 8.5 (0.15 ml.), the addition as indicated and water to a final volume of 0.22 ml. The mixtures were incubated for 1 hr. at 37° and then the reactions were stopped by heating for 2 min. at 100°. The lipids and polymer were extracted as shown in Fig. 2.

Inhibitor	Concentration	Lipid synthesis (as % of control)	Polymer synthesis (as % of control)
None	—	100	100
D-Cycloserine	500 $\mu\text{g.}/\text{ml.}$	102	93
	100 $\mu\text{g.}/\text{ml.}$	104	103
Bacitracin	500 $\mu\text{g.}/\text{ml.}$	105	109
	100 $\mu\text{g.}/\text{ml.}$	107	97
Novobiocin	500 $\mu\text{g.}/\text{ml.}$	99	83
	100 $\mu\text{g.}/\text{ml.}$	113	106
Triton X-100	0.25%	175	56
	0.05%	132	105
Sodium deoxycholate	0.25%	76	76
	0.05%	92	107
Cetyltrimethylammonium bromide	0.25%	98	77
	0.05%	115	111
Butan-1-ol	4.00%	6	8
	1.20%	98	61
Octan-2-ol	4.00%	18	49
	1.20%	10	56
UMP	25 $\mu\text{moles}/\text{ml.}$	35	35
	5 $\mu\text{moles}/\text{ml.}$	59	72

the formation of lipid intermediate or polymer in the present case (Table 3). It is believed that bacitracin specifically inhibits peptidoglycan synthesis by preventing the hydrolysis of lipid pyrophosphate to give the monophosphate and inorganic phosphate (Siewert & Strominger, 1967); as this is not a step in the proposed biosynthetic route for the poly-(N-acetylglucosamine 1-phosphate) the antibiotic would not be expected to act as an inhibitor. Similarly, cycloserine and novobiocin are known to inhibit steps that occur in peptidoglycan synthesis, and their failure to influence the synthesis of poly-(N-acetylglucosamine 1-phosphate) is presumably because no corresponding steps occur in the latter case. The action of Triton X-100 is noteworthy; it inhibits polymer formation at the last step in the biosynthetic route, thereby causing an accumulation of lipid intermediate. The other detergents have less marked action, and the powerful inhibitory effect of the higher alcohols is due to irreversible damage to the enzyme system. The inhibitory action of UMP is reversible and occurs presumably through the reversal of the first step, although this has not yet been demonstrated directly.

The experiments with UDP-N-acetylglucosamine labelled with ^{14}C and ^{32}P indicate that, in addition to the lipid intermediate for the biosynthesis of poly-(N-acetylglucosamine 1-phosphate), a lipid product was formed in which only N-acetylglucosamine had been transferred. The occurrence of a lipid with the structure N-acetylglucosamine 1-phosphate-lipid would also explain the presence of N-acetylglucosamine 1-phosphate among the products of mild acid hydrolysis of the lipid fraction; a part of the N-acetylglucosamine formed in this hydrolysis presumably came from this lipid. It is analogous to the mannose 1-phosphate-lipid that is an intermediate in the biosynthesis of a mannan in the membrane of *Micrococcus lysodeikticus* (Scher *et al.* 1968). Examination of the membrane of *Staphylococcus lactis* N.C.T.C. 2102 indicated that polysaccharides are present and that they contain galactose, mannose and N-acetylglucosamine. Thus it is likely that the lipid participates in the biosynthesis of membrane polysaccharides.

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REFERENCES

- Anderson, J. S., Matsushashi, M., Haskin, M. A. & Strominger, J. L. (1967). *J. biol. Chem.* **242**, 3180.
- Archibald, A. R., Baddiley, J., Button, D., Heptinstall, S. & Stafford, G. H. (1968). *Nature, Lond.*, **219**, 5156.
- Baar, S. & Bull, J. P. (1953). *Nature, Lond.*, **172**, 414.
- Brooks, D. & Baddiley, J. (1969). *Biochem. J.* **113**, 635.
- Burger, M. M. & Glaser, L. (1964). *J. biol. Chem.* **241**, 494.
- Dankert, M., Wright, A., Kelley, W. S. & Robbins, P. W. (1966). *Arch. Biochem. Biophys.* **116**, 425.
- Dietrich, C. P., Colucci, A. V. & Strominger, J. L. (1967). *J. biol. Chem.* **242**, 3218.
- Douglas, L. J. & Baddiley, J. (1968). *FEBS Lett.* **1**, 114.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Hardy, F. E., Buchanan, J. G. & Baddiley, J. (1963). *J. chem. Soc.* p. 3360.
- Higashi, Y., Strominger, J. L. & Sweeley, C. C. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1878.
- Hussey, H., Brooks, D. & Baddiley, J. (1969). *Nature, Lond.*, **221**, 665.
- LeCocq, J. & Ballou, C. E. (1964). *Biochemistry*, **3**, 976.
- Paladini, A. C. & Leloir, L. F. (1951). *Biochem. J.* **51**, 426.
- Patterson, M. S. & Greene, R. C. (1965). *Analyt. Chem.* **37**, 854.
- Pieringer, R. A. (1968). *J. biol. Chem.* **243**, 4894.
- Pieringer, R. A. & Kunnes, R. S. (1965). *J. biol. Chem.* **240**, 2833.
- Scher, M., Lennarz, W. J. & Sweeley, C. C. (1968). *Proc. nat. Acad. Sci., Wash.*, **59**, 1313.
- Siewert, G. & Strominger, J. L. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 767.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Wright, A., Dankert, M., Fennessey, P. & Robbins, P. W. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1798.