# THE BIOSYNTHESIS OF MURAMIC ACID PHOSPHATE IN *BACILLUS LICHENIFORMIS*

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Received 19 November 1976

## 1. Introduction

The walls of many Gram-positive bacteria contain as their major components peptidoglycan and teichoic acids. Although it has long been established that these polymers are covalently linked, only in *Staphylococcus lactis* I 3 has the exact chemical nature of the bond been determined [1]. In this organism the teichoic acid, a polymer of *N*-acetylglucosamine-1-phosphate and glycerol phosphate, is linked through a phosphodiester bond to a muramic acid residue in the peptidoglycan. However, the occurrence of muramic acid phosphate among the products obtained on acid hydrolysis of wall preparations from many Gram-positive bacteria [2] has led to the general conclusion that such residues are the point of attachment for both teichoic acids and the other secondary polymers of the wall.

Until recently biosynthetic studies have utilized membrane preparations to investigate the synthesis of both peptidoglycan and teichoic acids as independent components of the bacterial wall. The development of membrane + wall preparations 'dirty walls' in which the integrity of the bacterial envelope is presumably better maintained, has allowed a study of the concomitant synthesis of both polymers. Using such preparations from *Bacillus licheniformis*, we have previously described the synthesis of covalently linked peptidoglycan and poly(glycerol phosphate) the wall teichoic acid of this organism [3]. Similar preparations were used by Bracha and Glaser [4] to study the synthesis of poly(ribitol phosphate) teichoic acid and peptidoglycan in *S. aureus H.* These authors presented evidence for the occurrence of a linkage unit, involving both glycerol phosphate and *N*-acetylglucosamine residues, interposed between the teichoic acid and peptidoglycan. Simultaneous work by Baddiley and his colleagues has provided both chemical [5,6] and biosynthetic [7,8] evidence for the occurrence of a glycerol phosphate trimer as part of the unit linking the two polymers. In all these biosynthetic studies the nature of the linkage formed between the newly synthesized peptidoglycan and teichoic acid was not investigated.

In this communication we report the isolation and characterization of muramic acid phosphate from membrane + wall preparations of *B. licheniformis* in which the in vitro synthesis of covalently linked teichoic acid and peptidoglycan had occurred. The phosphate moiety is derived from UDP-*N*-acetylglucosamine. Moreover, muramic acid phosphate was only isolated from preparations incubated under conditions for the synthesis of both peptidoglycan and teichoic acid. Therefore, we conclude that the synthesis observed is indeed related to the linkage of these two polymers.

## 2. Materials and methods

*B. licheniformis* 94 was grown, harvested and the membrane + wall preparations isolated as described previously [3] [$^{32}$P]UDP-*N*-acetylglucosamine was isolated from cultures of *M. luteus* grown in peptone (1%, w/v) containing yeast extract (0.1%, w/v), glucose (0.2%, w/v), NaCl (0.5%, w/v), inorganic [$^{32}$P]phosphate (2–5 μCi/ml) and inhibited with vancomycin (50 μg/ml). The specific activity of the purified nucleotide obtained ranged from 2.5–6.6 mCi/mmol. [β-$^{32}$P]CDP-glycerol was synthesized enzymically from [γ-$^{32}$P]ATP, glycerol and CTP [3]. UDP-*N*-acetyl-[$^{14}$C]muramyl-L-alanyl-D-isoglutamyl-

mesodiaminopimelyl-D-alanyl-D-alanine (UDP-[$^{14}$C]-MurAc-pentapeptide) was prepared as described previously as was the preparation or source of all other substrates [9]. Muramic acid phosphate was isolated and purified from acid hydrolysates of *B. licheniformis* walls by procedures similar to those described in this paper. *N*-acetylmuramic acid was purchased from Pfanstiehl Laboratories, Waukegan, Illinois, USA.

Reaction mixtures for the synthesis of peptidoglycan and poly(glycerol phosphate) teichoic acid contained UDP-[$^{14}$C]MurAc-pentapeptide (0.37 mM, 10.7 mCi/mmol) and either UDP-*N*-acetylglycosamine (0.6 mM) and [$^{32}$P]CDP-glycerol (0.2 mM 5 mCi/mmol) or [$^{32}$P]UDP-*N*-acetylglucosamine (0.6 mM, 6.6 mCi/mmol) and CDP-glycerol (0.2 mM) together with membrane + wall preparation (1.0 ml) in a total volume of 1.5 ml. After incubation at 28°C for 30 min the reactions were terminated by the addition of an equal volume of sodium dodecyl sulphate (10%, w/v) (SDS) and the walls were isolated and purified as previously described [3]. Polymeric material was also recovered from the SDS-soluble fraction. After removal of the bulk of the detergent by cooling the fraction to 0°C and centrifuging, the supernatant was chromatographed on a column (1 X 70 cm) of Sephadex G-75 eluted with water. All radioactive material appearing before the main peak of unused or modified nucleotide precursors was pooled and taken as soluble polymer.

Radioactive walls or soluble polymeric material, together with added purified walls of *B. licheniformis* 94 (10 mg) were hydrolysed in 4 N HCl at 100°C for 3 h. After removal of acid by repeated evaporation of water in vacuo the hydrolysed material was chromatographed on a column (1 X 66 cm) of Dowex 50 equilibrated in 0.1 M pyridinium acetate, pH 2.8 and eluted with the same buffer.

Paper chromatography was carried out on either Whatman I or Whatman 3MM paper in the following solvent systems: A, butan-1-ol/acetic acid/water (3:1:1, by vol.), B, propan-1-ol/$NH_3$/water (6:3:1, by vol). Electrophoresis was carried out on Whatman 3MM paper at 70 V/cm in the following buffer systems: C, pyridine/acetic acid/$H_2O$ (1:10:989, by vol) pH 3.5; D, pyridine/acetic acid/$H_2O$ (50:2:948, by vol) pH 6.5 and at 7 V/cm in buffer system, E, 0.25 M formic acid, pH 1.9.

All determinations of radioactivity and analytical methods were as described previously [9].

### 3. Results and discussion

In previous experiments using UDP-MurAc pentapeptide, UDP-*N*-acetylglucosamine and CDP-[$^{14}$C]-glycerol, membrane + wall preparations of *B. licheniformis* were shown to synthesize covalently linked peptideglycan and teichoic acid. The covalent linkage of the two polymers was inferred from the resistance of the radioactive label to extraction by either hot SDS or 80% phenol. However, the use of these particular substrates precluded any investigation of the synthesis of muramic acid phosphate as a possible point of attachment of the teichoic acid or peptidoglycan. In the present investigation UDP-MurAc pentapeptide labelled with [$^{14}$C]muramic acid, together with either [$^{32}$P]UDP-*N*-acetylglucosamine or [$^{32}$P]-CDP-glycerol, have been used as the radioactive precursors. This allowed the identification of muramic acid phosphate among the products obtained upon acid hydrolysis of the newly synthesized material and the derivation of the phosphate moiety to be established. Table 1 shows the incorporation of $^{14}$C into peptidoglycan together with the incorporation of $^{32}$P from the other nucleotide linked precursors present in the incubation mixtures.

The bulk of the $^{32}$P incorporated from [$^{32}$P]CDP-glycerol was shown to be present as 1,3-poly(glycerol phosphate) by ion-exchange chromatography of alkaline hydrolysates followed by paper chromatography of the separated products in solvent B [3]. $^{32}$P from [$^{32}$P]UDP-*N*-acetylglucosamine was also incorporated into the wall both in the presence and absence of CDP-glycerol. The presence of the teichoic acid precursor resulted in a marked inhibition of this incorporation the reasons for which remain unknown.

Figure 1 shows the early fractions obtained on Dowex 50 chromatography of a wall hydrolysate containing newly synthesized material derived from UDP-[$^{14}$C]MurAc-pentapeptide, CDP-glycerol and [$^{32}$P]-UDP-*N*-acetylglucosamine. 67.5% of the applied radioactivity was eluted as a single peak appearing between 190 ml and 230 ml of buffer and was identified as muramic acid. Two minor fractions comprising 1.6 % and 6.6% of the applied radioactivity, were eluted

Table 1
Synthesis of peptidoglycan and teichoic acid by membrane + wall preparations of *B. licheniformis*

| Radioactive precursor[a] | Other additions | nmol incorporated | |
|---|---|---|---|
| | | $^{14}C$ | $^{32}P$ |
| | | Wall | |
| [$^{32}$P]CDP-glycerol | UDP-*N*-acetylglucosamine | 14.7 | 31.8 |
| [$^{32}$P]UDP-*N*-acetylglucosamine | CDP-glycerol | 24.5 | 2.2 |
| [$^{32}$P]UDP-*N*-acetylglucosamine | – | 26.2 | 6.0 |
| | | Soluble polymer | |
| [$^{32}$P]UDP-*N*-acetylglucosamine | CDP-glycerol | 12.8 | 2.9 |

[a]All incubation mixtures contained UDP-[$^{14}$C]MurAc-pentapeptide. Other experimental conditions were as described in the text.

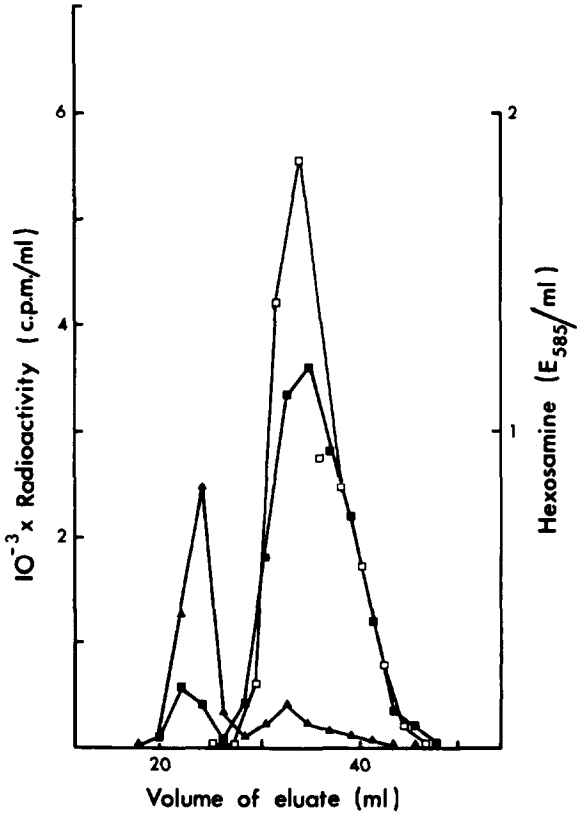


Fig.1. Fractionations on Dowex 50 of hydrolysed walls from *B. licheniformis* which contain peptidoglycan and teichoic acid newly synthesized from UDP-[$^{14}$C]MurAc-pentapeptide, [$^{32}$P]UDP-*N*-acetylglucosamine and CDP-glycerol. Samples (0.05 ml) of the fractions were analysed for $^{14}$C (■), $^{32}$P (▲) and hexosamine (□).

earlier from the column. The second of these co-chromatographed with a peak of amino sugar; the first did not and has not been characterized further. Muramic acid phosphate would be expected to be eluted from the column in the position of the second minor fraction. In contrast, all the $^{32}$P was eluted from the column in two fractions, the majority between fractions 1 and 2 of the $^{14}$C. On paper chromatography in solvent B this was predominantly inorganic phosphate (confirmed by electrophoresis, at pH 6.5) with a small amount of material remaining on the origin of the chromatogram. A second minor fraction, 20% of the total, did however elute with the second $^{14}$C fraction.

Similar separations of $^{14}$C were obtained from hydrolysates of soluble polymer isolated from the above experiment and of walls containing material newly synthesized from UDP-[$^{14}$C]MurAc-pentapeptide and either UDP-*N*-acetyl-glucosamine and [$^{32}$P]CDP-glycerol or [$^{32}$P]UDP-*N*-acetylglucosamine alone. Major peaks of $^{32}$P were also obtained in the position of inorganic phosphate from hydrolysates of material synthesized from [$^{32}$P]CDP-glycerol in the presence of UDP-*N*-acetyl-glucosamine, from [$^{32}$P]UDP-*N*-acetylglucosamine in the absence of CDP-glycerol and from soluble polymer synthesized from [$^{32}$P]UDP-*N*-acetylglucosamine in the presence of CDP-glycerol. In all these cases there was no evidence of a second peak of $^{32}$P but a trail from the major peak of radioactivity was obtained which coincided with the second $^{14}$C peak and represented 12.5%, 2.5% and 9.1% respectively

of the total $^{32}P$ incorporated.

In each of the experiments described, column fractions containing the second peak of $^{14}C$ were pooled and buffer salts were removed by repeated evaporation of water. The pooled material was then subject to paper electrophoresis at pH 1.9. Radioactive material containing both $^{14}C$ and $^{32}P$ having the electrophoretic mobility of muramic acid phosphate, $R_{P_i}$ 0.72, was eluted and further characterized after *N*-acetylation. The eluted material in water (100 μl) was adjusted to pH 8–9 with triethylamine (2 μl), cooled in an ice-bath and acetic anhydride (5 μl) added. After mixing and standing 2–3 h at 4°C the reagents were removed in vacuo and the procedure repeated. The *N*-acetylated material was then subjected to electrophoresis at pH 3.5. Material synthesized from [$^{32}P$]-UDP-*N*-acetyl-glucosamine, UDP-[$^{14}C$]MurAc-pentapeptide and CDP-glycerol gave two peaks of $^{14}C$ of which only the second, $R_{P_i}$ 0.7, the mobility of *N*-acetylmuramic acid phosphate, contained $^{32}P$. The first peak $R_{P_i}$ 0.40 was eluted and identified as *N*-acetylmuramic acid by co-chromatography with authentic material in solvent A. *N*-acetylmuramic acid phosphate was also present in material synthesized from [$^{32}P$]-CDP-glycerol but contained little $^{32}P$. In contrast, the majority of the $^{14}C$ present in the *N*-acetylated material derived from both the soluble polymer and also from material synthesized from [$^{32}P$]UDP-*N*-acetyglucosamine in the absence of CDP-glycerol, remained on the origin after electrophoresis. It did not contain $^{32}P$. In both these latter cases a small amount of material containing both $^{14}C$ and $^{32}P$ did

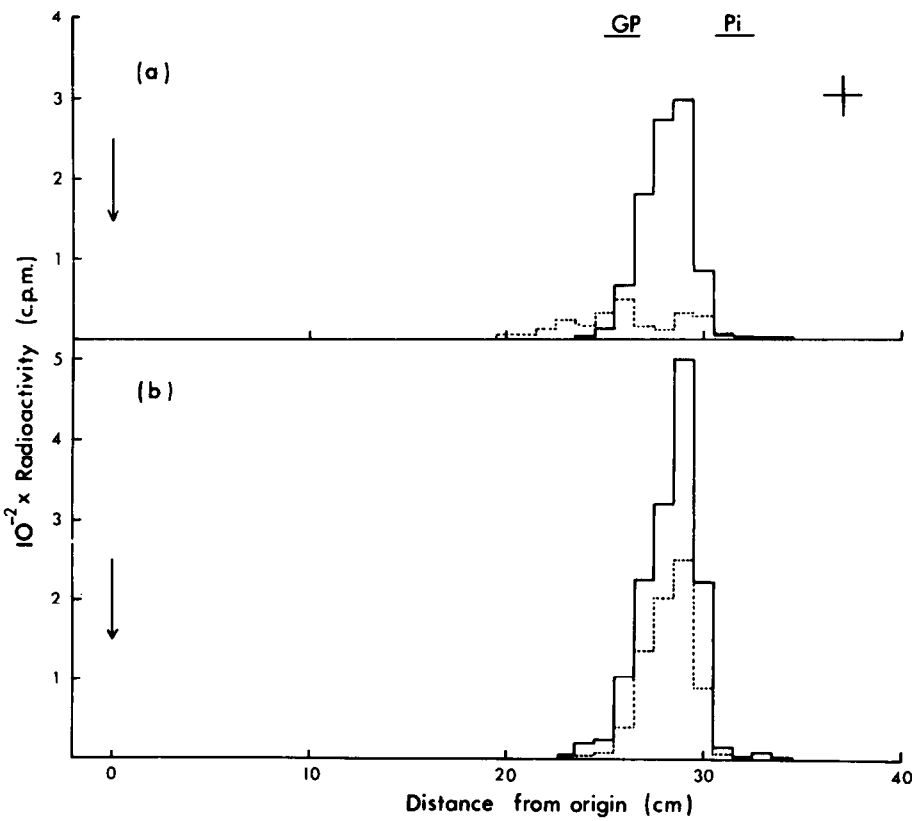


Fig. 2. Separation of *N*-acetylmuramic acid phosphate by electrophoresis at pH 6.5. Strips (1 cm) were cut from the paper for determination of radioactivity as $^{14}C$ (——) and $^{32}P$ (- - - -). *N*-acetylmuramic acid phosphate synthesized in incubation mixtures containing UDP-[$^{14}C$]MurAc pentapeptide and either [$^{32}P$]CDP-glycerol (a) or [$^{32}P$]UDP-*N*-acetylglucosamine (b) is shown. The bar markers indicate the location of α-glycerol phosphate (GP) and inorganic phosphate ($P_i$).

have the mobility of *N*-acetylmuramic acid phosphate. In each of the experiments the areas corresponding to *N*-acetylmuramic acid phosphate were eluted and subjected to further electrophoresis at pH 6.5. Figure 2 shows the separations obtained with material labelled with either [$^{32}$P]UDP-*N*-acetyl-glucosamine or [$^{32}$P]CDP-glycerol in the presence of the other unlabelled nucleotide. In both cases only $^{14}$C-labelled material having the electrophoretic mobility of *N*-acetylmuramic acid phosphate, $R_{P_i}$ 0.92, was present. This contained $^{32}$P derived from UDP-*N*-acetylglucosamine in a molar ratio of *N*-acetyl-[$^{14}$C]muramic acid to [$^{32}$P]phosphate of 1:1.04, but did not contain $^{32}$P derived from CDP-glycerol. This identity was further confirmed by treatment with acid phosphatase (EC 3.1.3.2) which gave *N*-acetylmuramic acid in both cases and $^{32}$P inorganic phosphate where this was labelled. The yield of purified material was equivalent to 1.14% ([$^{32}$P]UDP-*N*-acetyl-glucosamine) and 0.85% ([$^{32}$P]CDP-glycerol) of the $^{14}$C present in the original hydrolysates. *N*-acetylmuramic acid phosphate was not isolated from either soluble polymer or from material synthesized from UDP-*N*-acetylglucosamine in the absence of CDP-glycerol. In both cases the $^{14}$C and the associated small amount of $^{32}$P, ran as a broad peak, $R_{P_i}$ 0.74, which after treatment with acid phosphatase was identified as *N*-acetylmuramic acid. The nature of this material has not been investigated further.

Thus in *B. licheniformis* the synthesis of muramic acid phosphate is dependent on the presence of CDP-glycerol although the phosphate moiety is not derived from this precursor but from UDP-*N*-acetylglucosamine. This evidence implies that the synthesis observed is related to the attachment of the wall teichoic acid rather than being involved in some non-specific attachment of *N*-acetylglucosamine residues. Moreover, synthesis of muramic acid phosphate is also dependent on the formation of crosslinked peptidoglycan since none was isolated from the soluble polymer. This observation confirms earlier studies when uncrosslinked peptidoglycan synthesized by *B. licheniformis* incubated in the presence of benzylpenicillin, was shown to be devoid of associated phosphate [10].

The use of UDP-*N*-acetylglucosamine as a source of the phosphate residue suggests the presence of some linkage unit which is required for attachment of the teichoic acid, to peptidoglycan. As described in the Introduction recent biosynthetic studies using *S. aureus* [4,7,8,11] and *D. subtilis* W23 ([18]. A. W. Wyke and J. B. Ward unpublished observations) have described the presence of a linkage unit comprising *N*-acetylglucosamine and glycerol phosphate residues in these organisms. Chemical evidence [5,6] has been obtained for the presence of the glycerol phosphate oligomer and more recently [11] for excess glucosamine (relative to muramic acid) in the teichoic acid glycan complex isolated from *S. aureus*. Thus the possibility exists that these organisms use a similar mechanism to that found in *B. licheniformis*, for the synthesis of muramic acid phosphate and the attachment of the wall teichoic acid.

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