Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 4200–4204, November 1976
Microbiology

Oligomeric intermediate in peptidoglycan biosynthesis in *Bacillus megaterium*

(cell wall/disaccharide pentapeptide dodecamer/peptidoglycan morphogenesis/undecaprenol-linked synthesis/muramidase)

ELAINE FUCHS-CLEVELAND AND CHARLES GILVARG

Princeton University, Department of Biochemical Sciences, Princeton, New Jersey 08540

Communicated by B. D. Davis, August 10, 1976

ABSTRACT **An oligomeric intermediate in the biosynthetic pathway of peptidoglycan was isolated from *B. megaterium*. The oligomer has been identified as [disaccharide(pentapeptide)]$_{12}$ pyrophosphoryl undecaprenol.**

In almost all bacteria, the basic precursor unit of peptidoglycan is GlcNAc-β-(1→4)-MurNAc, substituted with a pentapeptide side chain on the lactate moiety of the muramic acid. The biosynthesis of this structure is well established; however, little information exists on the mechanism of the transfer and assembly of these subunits from their lipid carriers into the growing cell wall. There are two processes by which a peptidoglycan subunit can be incorporated into the cell wall. One is via transpeptidation; the other is via transglycosylation. In a cell-free system, transglycosylation has been shown to occur by addition of disaccharide pentapeptide subunits at the reducing end of a growing chain (1).

It is unknown whether the enzymatic reactions that lead to peptidoglycan synthesis occur between a single incoming subunit and the growing wall. Alternatively, monomers might be used to form an oligomeric intermediate, which could then be transferred as a unit to the wall. Oligomers have been observed in studies *in vitro* and in experiments in which antibiotics perturb cell wall biosynthesis in cells. For instance, oligomeric glycopeptides are produced by cell-free peptidoglycan-synthesizing systems in which the transpeptidase reaction is absent (2). Similarly, incubation of exponentially growing cells with penicillin G results in the formation of long, uncrosslinked peptidoglycan chains (3, 4). However, no peptidogylcan oligomers have ever been observed under normal conditions *in vivo*.

It is crucial to our understanding of bacterial wall assembly to know whether, *in vivo*, monomeric lipid precursors are incorporated directly into the wall or are first incorporated into oligomeric intermediates which are then transferred as units to the sacculus. In an effort to answer this question, we have conducted a study *in vivo* of peptidoglycan biosynthesis. Using a double auxotroph of *Bacillus megaterium* which requires both diaminopimelic acid (A_2pm) and lysine for growth, we were able to specifically and intensively label all peptidoglycan precursors with radioactive A_2pm. This technique has revealed an oligomeric intermediate in the biosynthetic pathway of peptidoglycan.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions. All experiments were conducted using *B. megaterium* M46 (A_2pm^-, Lys^-). Properties of this mutant and its conditions for growth have been described (5, 6). A minimal medium supplemented with 5 μg/ml of A_2pm and 10 μg/ml of Lys·HCl was used in all experiments.

Incorporation of [^{3}H]A_2pm into Peptidoglycan. Routinely, the cells from 1 liter of a culture grown to a turbidity of 50 Klett units were collected by centrifugation at 8000 × *g* for 3 min at 32°. Pellets were rapidly suspended in 100 ml of minimal medium supplemented with 100 μg/ml of Lys·HCl and 1% (wt/vol) glucose at 32°. Proper aeration was essential for continued exponential growth and was achieved with a magnetic stirrer and a high ratio of surface to volume. Cells were labeled by incubation with carrier-free [^{3}H]A_2pm, 1.25 μg/ml. Incorporation was terminated with an equal volume of ice-cold 10% trichloroacetic acid (TCA). Cells were then harvested by centrifugation at 0° for 10 min at 12,000 × *g*. The cells were washed with ice-cold 5% TCA, followed by repeated washings with ice-cold water until the supernatants were free of radioactivity.

Sodium Dodecyl Sulfate ($NaDodSO_4$) Extraction of Lipid Intermediates. Washed, labeled cells were resuspended in 5.0 ml of 0.5% $NaDodSO_4$ and incubated at 32° for 5 hr. The cells were then centrifuged at room temperature for 15 min at 12,000 × *g*. The supernatant was removed and centrifuged again as a precaution against particulate contamination. This $NaDodSO_4$ extraction procedure was repeated until no more label appeared in the supernatant. Usually two extractions were adequate. Extracts were combined and placed on a Sephadex G-50 column (2.5 cm diameter, 115 cm height) equilibrated with 0.02 M NaCl in 0.5% detergent. Fractions of 5 ml were collected, and 0.2-ml aliquots were transferred to scintillation vials with 3.0 ml of Hydromix (Yorktown Chemicals). Radioactivity was determined in a Packard Tricarb Scintillation counter.

Hydrofluoric Acid Extraction of Cells. Washed, labeled cells in a polypropylene tube were resuspended in 1 ml of ice-cold 60% HF. After 1 hr, the solution was neutralized by dropwise addition of ice-cold 10 M KOH. Temperature was maintained at 0° through use of a dry ice-acetone bath. Cells were removed by centrifugation, and the supernatant was applied to a Sephadex G-50 column (2.5 cm diameter, 115 cm height) equilibrated with 0.02 M NaCl.

Covalent Attachment of Lysozyme to Sepharose 4B. Lysozyme was covalently linked to insoluble matrices of Sepharose in high yield by cyanogen bromide activation (7).

Preparation of Marker Compounds. [^{14}C]A_2pm-labeled DP_1, DP_2, DP_3, DP_4, and DP_5, as well as a mixture of DP_9-DP_{12}, were prepared from a partial lysozyme-Sepharose digest of labeled, soluble, high-molecular-weight peptidoglycan from penicillin G-treated cells and then purified by gel filtration (E. Fuchs-Cleveland and C. Gilvarg, manuscript in preparation).

Abbreviations: A_2pm, diaminopimelic acid; DP_n, an uncrosslinked glycan oligomer of *n* disaccharide pentapeptide units linked by β-1,4 bonds; LI-1, the lipid intermediate, disaccharide pentapeptide pyrophosphoryl undecaprenol; TCA, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

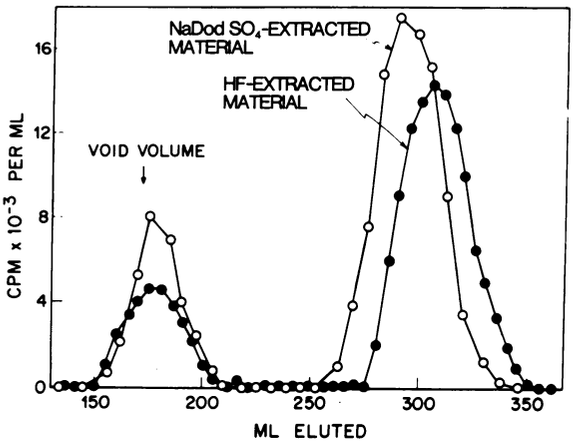

FIG. 1. Sephadex G-50 elution profiles of $NaDodSO_4$- and HF-extracted cell wall intermediates. In each case, cells from 1 liter of a culture grown to Klett 50 were labeled with [^{3}H]A_2pm as described in the *text*. Extractions and gel filtrations were also conducted as described in the *text*. The material in the first peak of the $NaDodSO_4$ extract will be referred to as LI-2. The material in the low-molecular-weight peak of the $NaDodSO_4$ extract will be referred to as LI-1. (●) HF-extracted material; (○) $NaDodSO_4$-extracted material.

(DP_n is an uncrosslinked glycan oligomer of n disaccharide pentapeptide units linked by β-1,4 bonds.)

[^{14}C]A_2pm-labeled lipid intermediate, disaccharide pentapeptide pyrophosphoryl undecaprenol (LI-1), was prepared by incubating logarithmic phase cells in a concentrated growth system supplemented with 200 μg/ml of vancomycin, 100 μg/ml of Lys·HCl, and 1% (wt/vol) glucose for 2 min, followed by the addition of 1.25 μg/ml of [^{14}C]A_2pm. After 5 min, ice-cold 10% TCA was added to stop incorporation of label. Cells were harvested and washed as described. Methods for the $NaDodSO_4$ extraction of LI-1 and its purification by Sephadex G-50 gel filtration are cited in the *text*.

Chemicals. α,ϵ-Diamino[*1,7*-^{14}C]pimelic acid (40 mCi/mmol) was purchased from Calatomic. 2,6-Diamino[*U*-^{3}H] pimelic acid (300 mCi/mmol) was purchased from Amersham-Searle.

RESULTS

Isolation of Lipid Intermediates from *B. megaterium*. With $NaDodSO_4$ as a membrane disrupting agent, lipid intermediates of two distinct molecular weight ranges have been isolated from TCA-washed cells. The Sephadex G-50 elution profiles of these compounds are shown in Fig. 1. The elution position of the low-molecular-weight peak corresponds to MurNAc (pentapeptide) pyrophosphoryl undecaprenol and disaccharide (pentapeptide) pyrophosphoryl undecaprenol. These compounds are the classic lipid intermediates described in detail in the literature (8, 9). The radioactive peak appearing near the void volume of the Sephadex G-50 profile has not been reported previously. It accounts for 20% of the radioactivity extracted.

The possibility that this high-molecular-weight material represented a new intermediate in the biosynthetic pathway of peptidoglycan was tested by investigating the kinetics of the flow of tracer A_2pm through its cellular pool. In the first experiment, infusion of tracer into the UDP-containing intermediates, the undecaprenyl lipid intermediates, and the high-molecular-weight unknown was measured (Fig. 2, left). The flow of tracer into the high-molecular-weight material displayed the same kinetic behavior as the other intermediates in the biosynthetic pathway of peptidoglycan. The order of

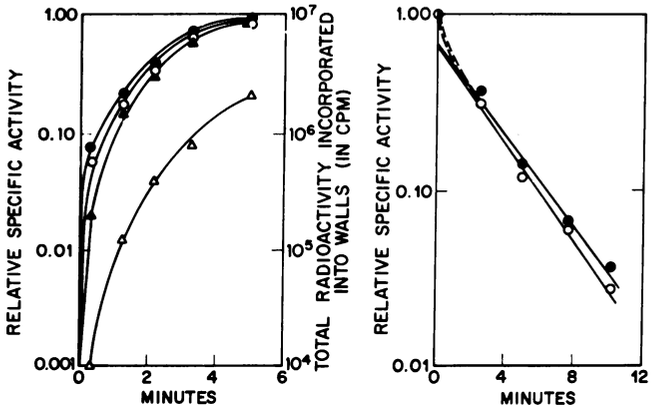

FIG. 2. Kinetics of the appearance and disappearance of tracer into pools of peptidoglycan intermediates. (Left) 1.25 μg/ml of [^{3}H]A_2pm was added to 50 ml of cell suspension at Klett 500 (see *text*). At 0.3, 1.2, 2.1, 3.2, and 5.0 min, 10 ml were removed and placed in an equal volume of ice-cold 10% TCA. Cells were centrifuged and washed as usual. UDP-compounds were isolated from the TCA supernatants, which were first extracted three times with equal volumes of ether to remove the TCA. Any remaining acid was neutralized before the supernatant was concentrated by rotary evaporation. UDP-compounds were then separated from free A_2pm by passing each sample through a Sephadex G-50 column (2.5 cm diameter, 115 cm height). Lipid intermediates were isolated from each of the pellets by 0.5% $NaDodSO_4$ extraction and gel filtration analysis. All gel filtration elution profiles were obtained by liquid scintillation methods. The specific activity per cell was calculated by determining the amount of label in a particular pool at time t versus the total amount of label per cell in the cellular pool at t = 10 min, the steady state value. (●) UDP intermediates (1.8×10^6 cpm, steady state); (○) LI-1 (4.6×10^4 cpm, steady state); (▲) LI-2 (9.2×10^3 cpm, steady state); (△) wall. (Right) pulse-chase experiment was conducted using a 50-ml concentrated growth system; 1.25 μg/ml of [^{3}H]A_2pm was administered for 5 min, after which time a 500-fold excess of unlabeled A_2pm was added. At t = 0, 2.5, 5.0, 7.5, and 10.0 min after the chase, 10-ml aliquots were removed and placed in an equal volume of ice-cold 10% TCA. Pellets were washed and extracted with 0.5% $NaDodSO_4$. LI-1 and LI-2 were separated by gel filtration (see *text*). (●) LI-2; (○) LI-1.

appearance of label in the three pools was consistent with their expected order in the biosynthetic pathway: cytoplasmic precursors, monomeric lipid intermediates, and finally, oligomeric intermediates.

In a second experiment, the kinetics of depletion of tracer from the internal pools of the undecaprenyl lipid precursors and the high-molecular-weight unknown were examined (Fig. 2, right). The pulse-chase experiment indicated that tracer could be chased from the high-molecular-weight material into the wall, since the compound did not accumulate and could not be detected in the medium. Furthermore, the chase kinetics for the unknown paralleled the data for the known disaccharide pentapeptide lipid intermediate. The fact that a discrete pool of high-molecular-weight unknown was observed and that the kinetics of the flow of tracer through this cellular pool were characteristic of known intermediates in peptidogylcan biosynthesis argues strongly that the high-molecular-weight material obtained from $NaDodSO_4$-extracted, TCA-washed cells represents a new intermediate in the biosynthetic pathway of peptidoglycan. The disappearance of label from the monomeric lipid pool at a slightly faster rate than from the high-molecular-weight material was again consistent with the hypothesis that the high-molecular-weight material is an oligomeric link between the monomeric lipid and the intact wall. Knowledge of the structure of this material was therefore desirable.

The observation that a membrane-disrupting agent such as $NaDodSO_4$ was required to release this new intermediate from

Table 1. Beta-elimination of lactyl peptides from $NaDodSO_4$- and HF-extracted high-molecular-weight material

	% Total label appearing as lactyl peptide
$NaDodSO_4$-extracted high-molecular-weight material	0 ± 1
HF-extracted high-molecular-weight material	8 ± 1

[^{3}H]A_2pm-labeled high-molecular-weight compounds from $NaDodSO_4$- and HF-extracted cells were isolated and purified. HF-extracted high-molecular-weight material was incubated with alkaline phosphatase for 1 hr at 37°. Beta-elimination on 5000 cpm aliquots of each sample was conducted as described (10). Samples were incubated in 4 M NH_3 (pH 10.5) for 1 hr at 37°. Ammonia was removed by lyophilization. Lactyl peptides were separated from high-molecular-weight glycopeptides by Sephadex G-50 gel filtration (2.5 cm diameter, 45 cm height). Fractions of 2.0 ml were collected. Aliquots (1.0 ml) of each fraction were transferred to scintillation vials for determination of radioactivity.

TCA-washed cells suggested that the glycopeptide portion of the compound was covalently attached to a membrane component. Beta-elimination studies on the high-molecular-weight intermediate extracted with $NaDodSO_4$ revealed that the reducing end of this compound was blocked (Table 1), and it was therefore quite probable that this high-molecular-weight material was comprised of a hydrophobic moiety in addition to glycopeptide.

The known lipid intermediates in the biosynthetic pathway of peptidoglycan contain a pyrophosphoryl link between glycan and lipid. The possibility that this new intermediate, LI-2, possessed a similar structure was therefore investigated. Since ice-cold concentrated HF is an extremely specific reagent for phosphate-oxygen bond cleavage (11), 60% HF was used to extract the glycan portion of LI-2 from TCA-washed cells. We have shown that prolonged treatment with ice-cold 60% HF has no effect on either crosslinking or β-1,4glycosidic linkages in peptidoglycan (E. Fuchs-Cleveland and C. Gilvarg, manuscript in preparation). Consequently, it was possible to extract the glycan portion of lipid intermediates from labeled, TCA-washed cells with ice-cold 60% HF without disrupting the intact sacculus. The Sephadex G-50 elution profile of the HF-extracted material (Fig. 1) is virtually identical to that obtained from an $NaDodSO_4$ extraction, with the exception of a slight shift of the low-molecular-weight peak to a higher elution volume. This finding is consistent with the knowledge that 60% HF cleaves the phosphate bonds linking undecaprenol to peptidoglycan. Evidence that both extraction procedures yield the same material with respect to the peptidoglycan portion of the molecules was supplied by conducting an $NaDodSO_4$ extraction on an HF-extracted pellet and an HF extraction of an $NaDodSO_4$-extracted pellet. In either case, the second extraction resulted in no further release of radioactivity from the pellet.

The high-molecular-weight material released by HF was not crosslinked (Table 2). It was included in the elution volume of a Sephadex G-75 column (not shown). It eluted as a single sharp peak, just ahead of a [^{14}C]A_2pm-labeled marker, which was shown by beta-elimination analysis to have an average chain length of 10 ± 1 subunits. Beta-elimination of the HF-released material yielded an average glycan length of 12 ± 2 subunits (Table 1), a value consistent with its elution properties on Sephadex G-75.

Table 2. Crosslinking of A_2pm in $NaDodSO_4$- and HF-extracted high-molecular-weight material

	% A_2pm crosslinked
$NaDodSO_4$-extracted high-molecular-weight material	0 ± 1
HF-extracted high-molecular-weight material	0 ± 1
B. megaterium	50 ± 2

[^{3}H]A_2pm-labeled high-molecular-weight compounds from $NaDodSO_4$- and HF-extracted cells were isolated and purified (see *text*). The method for determination of crosslinking was based on the reactivity of free amino groups of uncrosslinked A_2pm residues with nitrous acid (12).

In the interest of precisely defining the homogeneity of the glycan portion of LI-2, a technique for the separation of uncrosslinked peptidoglycan oligomers was devised. The method involves electrophoresis of glycopeptide oligomers on Whatman DE 81 paper (E. Fuchs-Cleveland and C. Gilvarg, manuscript in preparation). DEAE-paper electrophoresis of a partial lysozyme digest of glycopeptide polymers released by penicillin G produces a marked separation of oligomers differing by a single disaccharide pentapeptide unit. The use of a purified oligomer of known size enables the identification of all the resolved peaks from the electrophoretic profile of the lysozyme digest. By this procedure, a [^{14}C]A_2pm-labeled marker, shown by beta-elimination analysis to have an average chain length of 10 ± 1 subunits, was identified as a mixture of DP_9, DP_{10}, DP_{11}, and DP_{12}. This mixture was then used as an internal marker to identify the length of the [^{3}H]A_2pm-labeled LI-2. The electrophoretic profile (Fig. 3) indicates that the glycopeptide moiety of the new intermediate is remarkably homo-

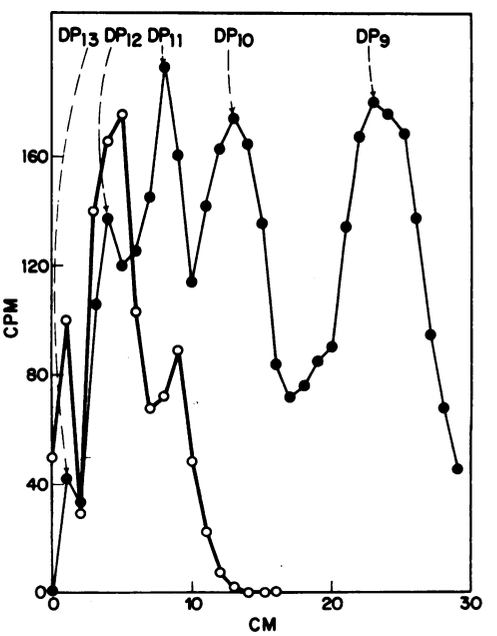

FIG. 3. Determination of the glycan length and homogeneity of LI-2 by DEAE-paper electrophoresis. [^{3}H]A_2pm-labeled LI-2 (○), isolated by 60% HF treatment of TCA-washed cells, was combined with a [^{14}C]A_2pm-labeled mixture of DP_9-DP_{13} (●), and spotted on Whatman DE 81 paper. Electrophoresis was carried out in pyridinium-acetate buffer, pH 3.5, for 16 hr at 3000 V. This paper was cut into 1-cm sections and transferred to scintillation vials for determination of radioactivity.

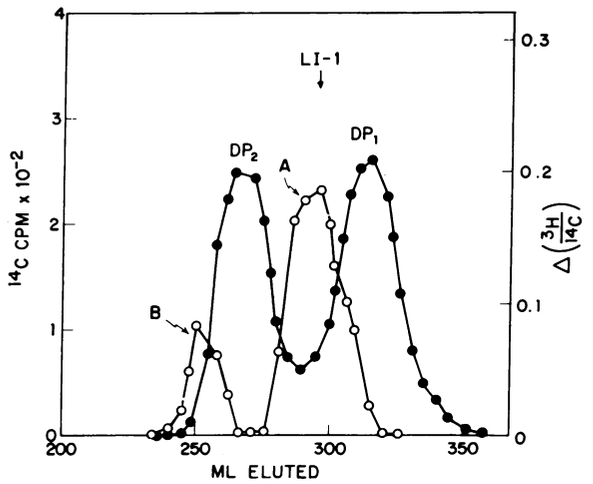

FIG. 4. Sephadex G-50 difference spectrum of a 36-hr lysozyme-Sepharose digestion of a mixture of [^{3}H]A$_2$pm-labeled, NaDodSO$_4$-extracted LI-2 and [^{14}C]A$_2$pm-labeled DP$_4$. Tritium-labeled LI-2 was isolated as described in the *text*. Before digestion with lysozyme, NaDodSO$_4$-extracted LI-2 was passed through a Sephadex G-50 column equilibrated with 0.5% Nonidet P-40. This procedure was necessary since lysozyme is inactive in NaDodSO$_4$. [^{3}H]A$_2$pm-labeled LI-2 was then combined with [^{14}C]A$_2$pm-labeled DP$_4$ in 0.1 M ammonium acetate, pH 6.8, and incubated with lysozyme-Sepharose beads at 32° for 36 hr. The mixture was filtered, and the beads were washed with 1 ml of 1 mg/ml of GlcNAc and 2 ml of 0.5% NaDodSO$_4$ in order to remove all radioactivity from the beads. The digest was then applied to a Sephadex G-50 column (2.5 cm diameter, 115 cm height) equilibrated with 0.02 M NaCl in 0.5% Nonidet P-40. Fractions, 4.0 ml, were collected and radioactivity of 1.0-ml aliquots was determined. (●) Elution profile of ^{14}C label; (○) difference between the ^{3}H:^{14}C ratio at each point on the graph and the ^{3}H:^{14}C ratio obtained for the peak fractions of DP$_1$ and DP$_2$. The total amount of tritium in the sample was 5600 cpm. Peak A contained 410 cpm of tritium; peak B contained 100 cpm of tritium.

geneous, with 65% of the radioactivity appearing as DP$_{12}$ and 35% divided between the flanking DP$_{11}$ and DP$_{13}$ peaks.

To eliminate the possibility that the intact sacculi were trapping intermediates of molecular weight greater than DP$_{13}$, we sheared labeled TCA-washed cells through a French Press and then centrifuged them before their extraction with HF. The Sephadex G-100 elution profile of the extracted material indicated essentially no difference between extracted compounds from cells treated with the French Press and intact cells. In either case, a distinct peak of radioactivity corresponding to LI-2 eluted at the same place in the included volume of the gel. The notable lack of radioactivity eluting between the void volume and the LI-2 peak again confirms the homogeneity of this intermediate.

The characteristics of LI-2 paralleled the behavior expected for an oligomeric form of the established precursor, disaccharide pentapeptide pyrophosphoryl undecaprenol. Digestion of the intermediate with lysozyme should therefore generate the monomeric lipid intermediate as one of the products. Therefore [^{3}H]A$_2$pm-labeled LI-2 was combined with an internal marker, [^{14}C]A$_2$pm-labeled DP$_4$, and incubated with lysozyme-Sepharose beads. The digestion products were analyzed by gel filtration (Fig. 4). The limit digest of uncrosslinked peptidoglycan with lysozyme-Sepharose beads is a mixture of DP$_1$ and DP$_2$, rather than DP$_1$ alone. The ^{3}H-^{14}C difference spectrum indicates the presence of two tritium peaks that migrate just ahead of the DP$_1$ and DP$_2$ peaks. The elution pattern of LI-1 has been shown to superimpose on the second peak (peak A). The first peak (peak B) should therefore correspond to the undecaprenyl

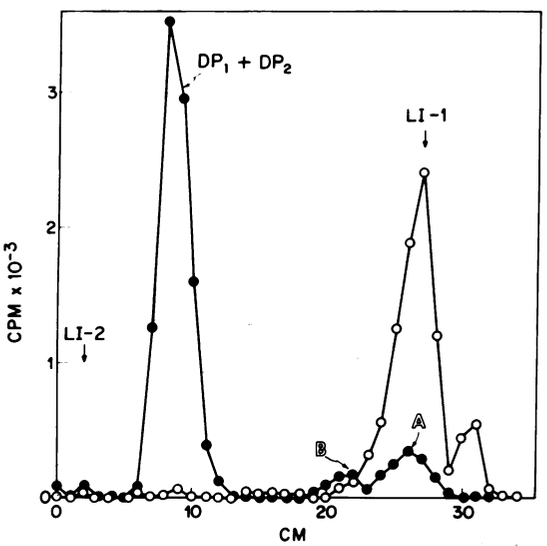

FIG. 5. Electrophoresis of a 36-hr lysozyme-Sepharose digestion of [^{3}H]A$_2$pm-labeled, NaDodSO$_4$-extracted LI-2 (●) and [^{14}C]-A$_2$pm-labeled LI-1 (○). Conditions for the enzymatic digestion and preparation of samples are given in the legend to Fig. 4. Whatman I paper electrophoresis was conducted for 1 hr at 4000 V in pyridinium-acetate buffer, pH 3.5.

pyrophosphoryl analog of DP$_2$. Furthermore, the sum of radioactivity appearing in peak A plus half the label of peak B amounts to 8% ± 1% of the total tritium label. This independent estimate of chain length is consistent with the established length of LI-2 based on the beta-elimination studies.

A second characterization method was then devised for the hydrophobic portion of the new intermediate. A lysozyme-Sepharose bead digest of [^{14}C]A$_2$pm-labeled LI-1 and [^{3}H]A$_2$pm-labeled LI-2 was prepared as described in the legend to Fig. 4. Electrophoresis of this digest on Whatman I paper produced a clear separation of LI-1 from DP$_1$ and DP$_2$ (Fig. 5). The superposition of tritium-labeled peak A with [^{14}C]-A$_2$pm-labeled monomer lipid intermediate confirms that lysozyme digestion of the new intermediate generates a LI-1 molecule. The most probable assignment for peak B in the electropherogram is, again, the undecaprenyl pyrophosphate analog of DP$_2$.

The data accumulated from the above experiments define the following structure for the new lipid intermediate: [disaccharide (pentapeptide)]$_{12}$ pyrophosphoryl undecaprenol.

DISCUSSION

The introduction of a procedure for intense labeling of peptidoglycan intermediates *in vivo* has led to the discovery of a new lipid intermediate in the biosynthesis of the murein sacculus. This intermediate is an oligomeric form of the standard undecaprenyl pyrophosphoryl disaccharide pentapeptide. It consists of an uncrosslinked glycan of 12 disaccharide pentapeptide moieties joined by β-1,4 bonds with a pyrophosphoryl linkage to a C$_{55}$-isoprenoid alcohol at the reducing end of the oligomer.

The obvious lack of oligomeric lipid intermediates of a substantially larger or smaller number of subunits is surprising. The previous conception of formation of lipid-linked disaccharide pentapeptide oligomers by addition of single peptidoglycan precursor units to the reducing end of the growing chain (1) predicted the existence of a spectrum of lipid-linked disaccharide pentapeptide oligomers. The maximum length of this distribution was expected to be dictated by the relative rates

of transglycosylation compared to transpeptidation. If, in fact, such lipid intermediates are used by the cell, their pool sizes must be exceedingly small, since the detection limits of the system are within five molecules per cell.

The kinetics of the movement of tracer through the cellular pool of LI-2 provide evidence that this oligomer is an intermediate in the biosynthetic pathway of peptidoglycan. From the pulse-chase experiments, it is clear that LI-2 is rapidly inserted into the growing wall. In light of our present knowledge of the mechanism of transglycosylation (1), it is apparent that this oligomeric lipid intermediate is not a proper substrate for a transglycosylase-mediated insertion into a wall acceptor site. Therefore, the only plausible mode of its insertion into the wall is via transpeptidation. The fate of LI-2, once it has been covalently attached to the wall, is unknown. Wall-bound LI-2 would presumably maintain its pyrophosphoryl linkage to undecaprenol. Therefore, the potential for continued elongation via transglycosylation would still exist once the oligomer had been inserted into the wall.

The fact that no oligomeric intermediates less than 11 disaccharide pentapeptide units in length were observed raises the question as to how LI-2 is generated. One possibility is that LI-2 molecules can be formed by enzymatic cleavage from the wall. Once freed, LI-2 molecules would be able to move to a new area of active wall synthesis, where they would become attached to the wall via transpeptidation and serve as a site for formation of a new LI-2 molecule by extension through transglycosylation.

This mechanism for preserving and relocating growth primers requres two assumptions. The first is that the 12 disaccharide pentapeptide subunits of the lipid end of a growth point are not in the proper stereochemical or environmental orientation to act as substrates for the transpeptidase. The observation that free LI-2 intermediates are completely uncrosslinked supports this hypothesis. The second requirement is that an *N*-acetylmuramidase must be present in the sacculus to release the LI-2 intermediate from the wall. *N*-Acetylmuramidases have been found in several bacterial species: *Streptococcus faecalis* 9790 (13), *Lactobacillus acidophilus* strain 63 AM Gasser (14), *Arthrobacter crystallopoieties* (15), and *Bacillus thuringiensis* (16). Involvement in the enzymatic cleavage and release of LI-2 intermediates from the wall in order to recycle growing points to active areas of nascent peptidoglycan biosynthesis would provide a rational function for the *N*-acetylmuramidase. However, the mechanism that would make it possible for the muramidase to so precisely define the size of LI-2 is unknown.

The above model for regeneration of LI-2 molecules by *N*-acetylmuramidase cleavage only provides a system for maintaining the number of LI-2 intermediates in a cell. No method for increasing the number of LI-2 molecules has been proposed, although this number must double every generation. Conceivably, this might occur by sequential addition of monomers to an initiating monomer to produce the dodecamer lipid intermediate. Although such precursors were not observed, the detection of twelve separate pools of compounds involved in the formation of a small number of LI-2 molecules per generation might not fall within the sensitivity range of our system. Another possible explanation for the failure to detect these putative precursors would arise if the formation of the LI-2 glycan chain occurred on a carrier protein rather than on the C_{55}-isoprenoid alcohol. This situation would be analogous to fatty acid biosynthesis, where an acyl carrier protein displaces coenzyme A as the matrix upon which the fatty acid is built.

An alternative mechanism for constructing new LI-2 intermediates, which avoids the formation of small oligomeric precursors, involves the direct attachment via transpeptidation of LI-1 molecules to the wall. After the initial attachment at this site, transglycosylation could occur. After sufficient lengthening, muramidase action would result in the liberation of a new dodecamer lipid intermediate. At present, any one of these mechanisms for LI-2 synthesis is plausible.

There are approximately 570 free LI-2 molecules per cell. This pool is relatively small in comparison to the LI-1 pool, which consists of 34,000 molecules per cell. If the model of regeneration of LI-2 molecules by *N*-acetylmuramidase activity is valid, however, the pool size of LI-2 precursor in the wall may be substantially larger than the quantity of free LI-2 intermediates measured.

The necessity of a precisely defined oligomer must be a significant structural feature in peptidoglycan biosynthesis. The importance of this narrow distribution of oligomeric intermediates is not understood. It is unknown whether these intermediates play a generalized role in wall synthesis or a specialized one, such as septation. Deciphering the significance of this new intermediate must await knowledge of the function and mechanism of action of this oligomeric precursor in the assembly of the bacterial cell wall.

This research was supported by USPHS Grant AI-11,756, and a training grant to E.F.C. from the National Institutes of Health.

1. Ward, J. B. & Perkins, H. R. (1973) *Biochem. J.* **135,** 721–728.
2. Ward, J. B. (1974) *Biochem. J.* **141,** 227–241.
3. Tynecka, Z. & Ward, J. B. (1975) *Biochem. J.* **146,** 253–267.
4. Mirelman, D., Bracha, R. & Sharon, N. (1974) *Biochemistry* **13,** 5045–5053.
5. Fukuda, A. & Gilvarg, C. (1968) *J. Biol. Chem.* **243,** 3871–3876.
6. Pitel, P. W. & Gilvarg, C. (1970) *J. Biol. Chem.* **245,** 6711–6717.
7. Arndt-Jovin, D. J., Jovin, T. M., Bahr, W., Frischauf, A. & Marquardt, M. (1975) *Eur. J. Biochem.* **54,** 411–418.
8. Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. USA* **53,** 881–889.
9. Higashi, U., Strominger, J. L. & Sweeley, C. C. (1967) *Proc. Natl. Acad. Sci. USA* **57,** 1878–1884.
10. Tipper, D. J. (1968) *Biochemistry* **7,** 1441–1449.
11. Lipkin, D., Phillips, B. E. & Abreu, J. W. (1969) *J. Org. Chem.* **34,** 1539–1544.
12. Fordham, W. D. & Gilvarg, C. (1974) *J. Biol. Chem.* **249,** 2478–2482.
13. Shockman, G. D., Thompson, J. S. & Conover, M. J. (1967) *Biochemistry* **6,** 1054–1065.
14. Coyette, J. & Ghuysen, J. M. (1970) *Biochemistry* **9,** 2952–2955.
15. Krulwich, T. A. & Ensign, J. C. (1968) *J. Bacteriol.* **96,** 857–859.
16. Kingan, S. L. & Ensign, J. C. (1968) *J. Bacteriol.* **96,** 629–638.