JOURNAL OF BACTERIOLOGY, Aug. 1983, p. 447–453
0021-9193/83/080447-07$02.00/0
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Vol. 155, No. 2

Changes in the Composition of *Escherichia coli* Murein as It Ages During Exponential Growth

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Received 2 February 1983/Accepted 5 May 1983

Escherichia coli murein was specifically labeled with [^{14}C]diaminopimelic acid in the mutant strains W7 (*dap lysA*) and BUG6. Pulse-labeled heat-denatured *E. coli* cells were digested with 2 mg of egg-white lysozyme per ml to degrade the murein completely and free any lipoprotein-bound muropeptide trimers, dimers, and monomers. Pulse-chase experiments showed that the relative percentage of trimers and dimers found in the newly synthesized murein increased somewhat with time at the expense of monomers. The increase in cross-links indicated that the radioactive monomers served as acceptors in multisite transpeptidations occurring after the labeling period. The content of nonreducing monomers (C_7 and C_8) remained unaltered, indicating that the oligosaccharide chain length did not change with time. A gradual conversion of the reducing disaccharide tetrapeptide monomer to its tripeptide analog occurred during chasing. Braun lipoprotein was linked to about 2% of the murein subunits within 30 s of the incorporation of subunits into insoluble murein, and after one-half a generation of chase, lipoprotein-associated muropeptides had approached the maximum (16% of the total murein subunits). The distribution of muropeptides was similar in lipoprotein-linked and lipoprotein-free murein, showing that the enzyme that links Braun lipoprotein to murein does not discriminate between monomers, dimers, and trimers. No evidence for a chasable, soluble polymer of murein was found in our experiments. Hence, our data support the idea that new murein is incorporated directly into the sacculus without first existing as a soluble intermediate (Mett et al., J. Biol. Chem. 255:9884–9890, 1980).

Although the basic features of cell wall peptidoglycan (murein) structure and biosynthesis in gram-negative bacteria are well established, a detailed knowledge of the process whereby the murein sacculus lengthens and divides during the cell division cycle is lacking. For example, there is little insight concerning how multiple synthetic and lytic activities in *Escherichia coli* cooperate to produce an elongating murein cylinder of a defined diameter or how the new poles of the daughter cells are formed during septation. Ryter et al. (13) used a morphological approach (autoradiography) to study the topography of murein growth in *E. coli*, providing knowledge that seems basic for an understanding of the process at the molecular level. They reported that new murein is first assembled in a central zone and later redistributed over the whole cylinder. However, using the same approach with cells starved for diaminopimelic acid (DAP) to deplete their intracellular pools of murein precursors, we have recently obtained evidence that the cylindrical murein in *E. coli* is synthesized by a diffuse (multisite) process without prior insertion into a central zone (L. G. Burman, J. Reichler, and J. T. Park, J. Bacteriol., in press).

Mett et al. (10) have interpreted data from pulse-chase experiments to mean that in *E. coli* an initial soluble low-cross-linked product is attached to preexisting murein by transpeptidation reactions, using as donors the abundant pentapeptides found in the soluble polymer. This molecularly oriented model is compatible with zonal, as well as multisite, models of growth.

Sufficient complexity exists in *E. coli* murein to allow the possibility that modifications or changes in structure occur with time. In addition to the bisdisaccharide peptide dimers (C_3 and C_4) and monomers disaccharide tripeptide (C_5) and disaccharide tetrapeptide (C_6) characterized by Weidel and Pelzer (16), the structural variation is increased further by monomers C_7 and C_8 of Weidel which have been shown by Holtje et al. (their X and X′) to be the nonreducing counterparts of the disaccharide peptide monomers in which muramic acid is present as a 1,6-

anhydrosugar (6). Another modification is the covalent linkage of approximately every 10th DAP to the carboxy-terminal lysine of the lipoprotein, as shown by Braun and Rehn (2). Finally, Gmeiner recently reported that about 5% of the muropeptides in *E. coli* murein were present in the form of peptide-cross-linked trisdisaccharide peptide trimers (5), indicating that three neighboring glycan chains of the *E. coli* sacculus can be directly interconnected via one tripeptide bridge.

We were interested in the possibility that changes in the structure of murein with time might provide clues to the specific biochemical reactions involved in the elongation of the sacculus and in the initiation and completion of septation. Therefore, we performed pulse-chase experiments with radioactive DAP and monitored in detail the structure of *E. coli* murein during synthesis and maturation, including the attachment of Braun lipoprotein to the cell wall peptidoglycan. Other results suggested that any soluble polymers of murein associated with growing cells were products of autolysis rather than being late-stage intermediates destined for incorporation into the murein sacculus.

MATERIALS AND METHODS

Organisms, growth, and labeling conditions. *E. coli* W7 (*dap lysA*) cultures were grown in minimal glucose medium, starved for DAP, pulse-labeled with radioactive DAP, and chased strictly according to a recently developed protocol (Burman et al., in press).

In some experiments, the conditional division mutant *E. coli* K-12 BUG6 (12) was used. It was grown in L broth (8) containing 0.2% glucose and 50 μg of lysine per ml. BUG6 had normal morphology and cell division at 30°C but grew as nonseptate filaments immediately upon a shift to 42°C. Synchronous division occurred after the filaments were shifted back to 30°C, with the most rapid formation of septa evident 10 to 20 min after the shift. The murein of septating cells was labeled during this period. The label used in all experiments was [^{14}C]DAP (0.16 to 0.21 μCi/ml, 0.10 to 0.13 μg/ml); it was added to the cultures at an optical density of 0.5. BUG6 was labeled with radioactive DAP for 10 min. The label could not be effectively chased upon the addition of unlabeled DAP because of the large intracellular pool of DAP. "Chased" BUG6 cells thus contained some newly labeled material in addition to the old material.

Processing and analysis of samples. Samples of labeled bacteria were taken at intervals, added to 2 volumes of water at 97 to 99°C, and kept at that temperature for 30 min. Such prolonged heating was found to be necessary for the complete inactivation of murein-degrading enzymes. The bacteria were then washed twice with 22°C water with a Beckman Microfuge B table centrifuge (10,000 rpm, 5 min). This procedure also rendered the *E. coli* murein accessible to digestion by hen egg-white endo-*N*-acetylmuramidase (lysozyme). The digestion of the heat-treated bacteria with lysozyme (2 mg/ml in 0.05 M ammonium acetate buffer, pH 7.0, for 15 to 24 h at 37°C) resulted in the complete (>99%) degradation of murein to monomers, dimers, trimers, and lipoprotein-linked subunits as judged by paper chromatography. In some experiments, *Chalaropsis* muramidase was used for the digestion of murein (5). Although 4 to 13% of the incorporated radioactivity of BUG6 was in the form of lysine, this did not interfere with the analysis of its murein. The murein was selectively released by lysozyme in the form of its typical degradation products.

The products of lysozyme digestion were separated by descending chromatography for 24 to 28 h, using Whatman 3MM paper with isobutyric acid–1 M ammonium hydroxide (5:3, vol/vol) as the solvent. They were located with reference dimers (C_3 and C_4) and monomers (C_5 and C_6) which were detected by ninhydrin color reaction. Trimers and nonreducing monomers (C_7 and C_8), present in small quantities, were usually located on the basis of their known R_f values relative to the reference samples.

The radioactive material moving near the front (R_f = 0.9) and mobilized by chromatography of intact (undigested) pulse-labeled bacteria represented bactoprenol-linked muropeptides. After lysozyme digestion, additional radioactivity located in this region was trypsin sensitive and represented lipoprotein-linked muropeptides (see below). Within 1 min of chasing, bactoprenol-linked radioactivity was no longer present, and the radioactivity in lysozyme-digested samples which had an R_f of 0.9 consisted entirely of lipoprotein-linked muropeptides. The lipoprotein-linked muropeptides were analyzed further by chromatography in isobutyric acid–1 M ammonium hydroxide solvent after liberation from lipoprotein by digestion of the material that had an R_f of 0.9 with 1 mg of trypsin per ml in 0.05 M ammonium acetate (pH 7.0) for 12 to 24 h at 37°C. Before treatment with trypsin, the lipoprotein-linked muropeptide fraction was eluted from the paper with a solvent composed of isobutyric acid, 1 M ammonium hydroxide, and water (5:1:3).

Enzymes and chemicals. Trypsin and hen egg-white lysozyme were obtained from Sigma Chemical Co., St. Louis, Mo., and *Chalaropsis* muramidase was obtained from Miles Laboratories, Inc., Elkhart, Ind. 2,6-[*U*-^{14}C]DAP (*meso* and some LL; 315 mCi/mmol) and [*U*-^{3}H]DAP (50 Ci/mmol) were purchased from Services des Molecules Marquees, Commissariat à l'Energie Atomique, Gif-sur-Yvette, France.

RESULTS

Assay of trimers. By using hot-water-extracted cells as the substrate and high concentrations of lysozyme (2 mg/ml for 16 h at 37°C), we obtained a practically complete breakdown of high-molecular-weight murein (Fig. 1A). In a parallel digest, 10 μg of a commercial preparation of *Chalaropsis* muramidase per ml left a small residue of murein that did not migrate during chromatography (Fig. 1B). Both enzymes released not only the expected amounts of monomer and dimer muropeptides but also a minor peak moving almost half as fast as the large peak identified as peptide-cross-linked bisdisaccharide dimer. Using the same solvent but silica gel thin-layer plates instead of paper, Gmeiner identified the product released by *Chalaropsis* mura-

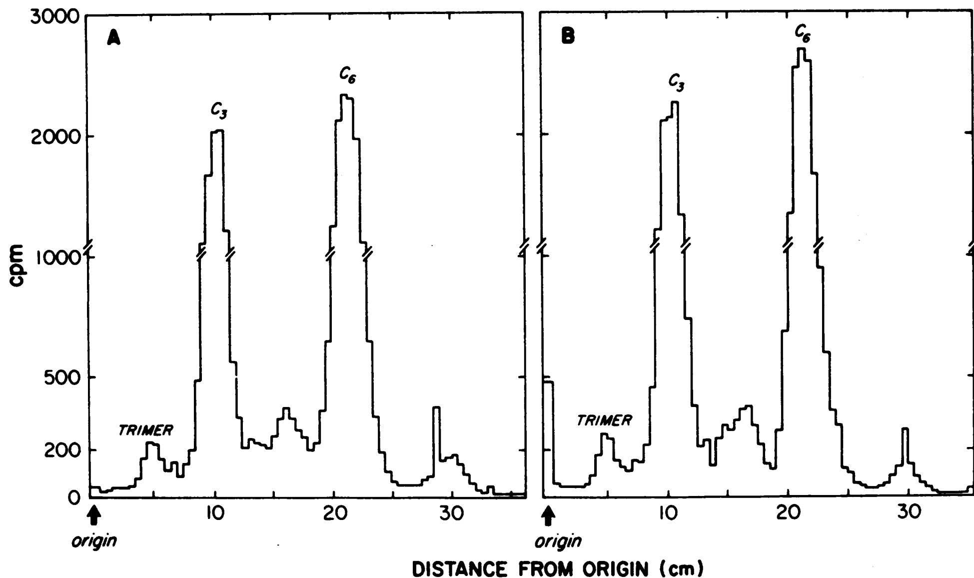

FIG. 1. Paper chromatography of the residue plus muropeptides liberated from pulse-labeled *E. coli* W7 peptidoglycan by lysozyme (2 mg/ml; A) and *Chalaropsis* muramidase (10 μg/ml; B). The cells were pulse-labeled for 2 min. The radioactivity in individual 0.5-cm strips of paper was counted. The region near the solvent front of the chromatogram which contains bactoprenol-linked and lipoprotein-linked muropeptides is not shown.

midase having an R_f of 45% that of dimer as trimer (5). We therefore conclude that 2 mg of hen egg-white lysozyme per ml will completely digest *E. coli* murein of heat-treated cells in 16 h at 37°C and can be used to release the trimers from *E. coli* sacculi.

In the following experiments, lysozyme was routinely used for digestion of *E. coli* murein after heating the bacteria for 30 min at 97 to 99°C and, provided that digestion was efficient (defined as less than 1% of the radioactivity remaining at the origin after paper chromatography), the material moving about half as fast as the reference dimer was defined as trimer. Incomplete digestion by lysozyme was seen with aged lysozyme preparations and large cell samples (5 to 10 ml of culture), particularly of stationary-phase bacteria, when incompletely digested oligomers falsely increased the trimer content by two- to three-fold. In such cases, the lysozyme digestion was repeated before further analysis.

Fate of trimers. The content of the trimers, dimers, and monomers of *E. coli* murein was followed during [^{14}C]DAP pulses and during chases. All three classes of fragments were present in lysozyme digests already at 0.5 min of labeling of strain W7, the shortest period tested. Trimers represented about 5% of the murein label in pulses and 7% after prolonged chases (Fig. 2), showing that they were formed rapidly and that additional label was recruited into the trimers during further growth of the cells. In BUG6, the trimer content was about 4%, but again as with W7, the percentage increased somewhat during chasing (Table 1). Furthermore, no difference was detected in the trimer content of murein synthesized by BUG6 growing as filaments and BUG6 septating after the transfer of filamenting cells to the nonrestrictive temperature. Thus, in BUG6 at least, it appears that trimers are formed in cylindrical, and probably also in septal, murein or under conditions believed to represent septal synthesis (9).

Dimers. In strain W7 growing in minimal glucose medium, as noted above, dimers were present after 0.5-min [^{14}C]DAP pulses. Dimers represented about 43% of the radioactivity released by lysozyme from these pulse-labeled cells and increased to about 50% after 15 min of chasing (Fig. 2). In normal and nonseptate (filamentous) BUG6 cells growing in L broth, about 34% of the label in pulse-labeled murein was present in dimers, whereas synchronously septating BUG6 cells contained 40% of the label in dimers. The radioactive dimer content in the murein of BUG6 growing as filaments at the restrictive temperature was 34% during short pulses (Table 1) but increased to about 40% with time, indicating that cross-linkage increased slowly as the murein aged. This presumably is due in part to labeled monomers (see below) serving as acceptors in later high-cross-linkage transpeptidation reactions and in this way becoming part of the dimers. Thus, cylindrical murein increased its cross-linkage during maturation, whereas septal murein apparently

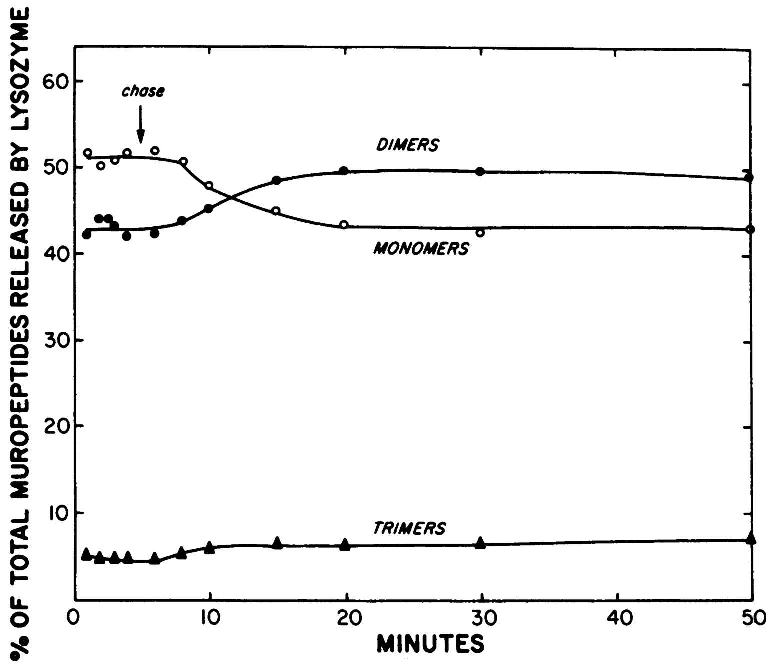

FIG. 2. Distribution of [14C]DAP in trimer, dimer, and monomer muropeptides in *E. coli* W7 murein during pulse-labeling and chasing.

achieved a maximum degree of cross-linkage simultaneously with its synthesis. Other workers have also observed high cross-linkage during septation (11).

Monomers. The percentage of monomers in *E. coli* sacculi mirrored that of dimers; i.e., monomers were most abundant in pulse-labeled material (51%) and dropped by almost 10% during chasing (Fig. 2). Since little or no loss of label occurred during chases, the decline of radioactivity in the monomer fraction must reflect their conversion to dimers and trimers (see above).

The monomer fraction in lysozyme digests of murein from *E. coli* consists of disaccharide tripeptide (C_5), disaccharide tetrapeptide (C_6), the corresponding nonreducing muropeptides (C_7 plus C_8) with internal muramic acid 1,6 anhydro linkages (6), and presumably trace amounts of reducing and nonreducing disaccharide pentapeptides. We have examined the fate of the four identifiable monomers in pulse-labeled and chased cells (Fig. 3). During pulses, the C_5/C_6 ratio was low and relatively constant (0.08 to 0.09), but during the chase with unlabeled DAP, C_5 increased at the expense of C_6 so that 15 min later the C_5/C_6 ratio was 0.19. During prolonged chasing, the ratio continued to rise and reached about 0.4 at two generations of chasing. Thus, of the [14C]DAP found almost immediately in murein as disaccharide tetrapeptide, about 30% was gradually converted to the tripeptide form within two generations. The percentage of the label in nonreducing disaccharide peptides (C_7 and C_8) was between 3 and 4% (Fig. 3) and did not appear to change during a chase period of 100 min (data not shown). These disaccharide fragments released by lysozyme are believed to represent the ends of glycan chains in *E. coli* created by the controlled action of a murein transglycosylase (6). The average glycan chain length of about 30 disaccharides derived from the content of nonreducing disaccharides found in our experiments agrees with that estimated by a different method by Schindler et al. (14). If the amount of [14C]DAP in nonreducing muropeptides is directly related

TABLE 1. Peptidoglycan composition in normally growing, filamentous, and septating *E. coli* BUG6 cells labeled for different periods of time

Muropeptides	Peptidoglycan composition[a] in:					
	Filamentous cells			Septating cells	Normal cells	
	2–6 min	20–25 min	40 min	10 min	2–6 min	40 min
Trimers	4	4	4	4	4	5
Dimers	34	37	40	40	35	40
Monomers	62	59	56	56	62	56

[a] Percentage of total [14C]DAP released in soluble form by lysozyme.

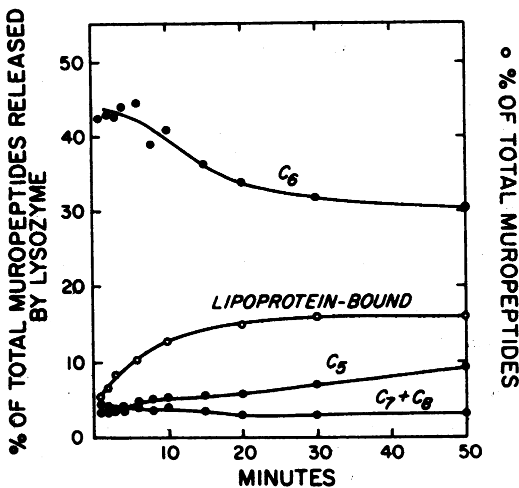

FIG. 3. Distribution of [^{14}C]DAP in monomers C_5, C_6, and C_7 plus C_8 and of total lipoprotein-linked muropeptides in *E. coli* W7 murein during pulse-labeling (0 to 5 min) and chasing (5 to 50 min).

to glycan chain length, the result shown in Fig. 3 would indicate that chain length is determined at the time of incorporation into the murein sacculus and remains unchanged thereafter.

Linkage of Braun lipoprotein to murein. Lipoprotein (2) was attached to new peptidoglycan strands soon after their incorporation into the sacculus. After 30-s and 2-min pulses, almost 3 and 7% of the [^{14}C]DAP-labeled subunits, respectively, were attached to the lipoprotein as judged by an analysis of lysozyme digests. A maximum of about 16% of the subunits eventually became covalently bound to the lipoprotein (Fig. 3). Thus, the linkage of lipoprotein to *E. coli* muropeptides did not occur before or concomitantly with their polymerization. However, the attachment process was initiated soon after murein synthesis and was essentially complete about one-half generation later.

The lipoprotein-linked muropeptides released by lysozyme were further analyzed after removing the lipoprotein with trypsin. Chromatography of the resulting muropeptides, which would contain lysine attached to DAP (2, 3), gave a series of well-defined bands almost identical to those obtained with a lysozyme digest of murein. The four bands observed moved slightly faster than known trimer, C_3, C_6, and C_7 plus C_8 samples in the isobutyric acid–1 M ammonium hydroxide (5:3) solvent system. Although the bands were not characterized further, it seems clear that lipoprotein molecules were linked to trimers and dimers as well as to the reducing monomer and the corresponding nonreducing monomer (disaccharide tripeptide). The observed percentages for the different lipoprotein-bound muropeptides were similar to those found for muropeptides from lipoprotein-free regions of the cell wall peptidoglycan (Table 2). Thus, neither trimers, dimers, nor monomer muro peptide was preferred as the acceptor during lipoprotein attachment to *E. coli* murein.

Absence of a soluble polymeric murein precursor. In the experiments with *E. coli* W7, it was found to be essential to adhere to the pulse-chase protocol previously developed. Otherwise, either autolysis caused by premature exhaustion of DAP or inefficient pulse-labeling and chasing because of the remaining pools of unlabeled DAP and murein precursors were likely to occur. The pulse-chase procedure used avoided filtration and washing of the bacteria immediately before or after the labeling period, techniques which were considered as possible sources of artifacts. We tried to isolate the soluble large-molecular-weight, chromatographically immobile murein of Mett et al. (10) by chromatography of the supernatant from cultures of *E. coli* that had been heated for 30 min at 97 to 99°C after being labeled with [^{14}C]DAP for 0.5 to 5 min. In 16 determinations from eight experiments, the average amount of soluble polymer found was 0.26%. The amount varied from 0 to 0.56%. This amount was about equivalent to that reported by Mett et al. for polymer soluble in sodium dodecyl sulfate and much less than was isolated from cells ruptured in a French press (10). Heating of the pulse-labeled cells in 5% sodium dodecyl sulfate (95°C for 30 min) and pelleting the murein sacculi by high-speed centrifugation (150,000 × *g* for 5 min in a Beckman Airfuge) did not reveal any soluble murein polymer not released by the hot water treatment.

However, larger amounts of soluble polymer were observed in pulse-labeled cells that had

TABLE 2. Attachment of Braun lipoprotein to different muropeptides of *E. coli* murein

Muropeptides[a]	% Attachment		
	Lipoprotein-bound muropeptides		Lipoprotein-free muropeptides (50-min chase)
	3-min pulse	50-min chase	
Trimers	5.3	10.7	6.3
Dimers	59.1	44.2	39.0
Monomers[b]	35.6	45.0	52.3

[a] Obtained by lysozyme digestion of *E. coli* W7 cells labeled with [^{14}C]DAP and separation of lipoprotein-free and lipoprotein-bound muropeptides. The latter were treated with trypsin (to remove the lipoprotein), followed by chromatography to separate the muropeptides thus liberated.

[b] Includes C_5, C_6, C_7, and C_8, or for the lipoprotein-bound muropeptides their lysine-containing derivative.

been stored for 30 to 60 min on ice before heating and, in particular, upon washing such samples with water. In two additional experiments, soluble polymer was assayed after labeling with high-specific-activity [^{3}H]DAP at the concentration used by Mett et al. to demonstrate "nascent" murein (11 ng/ml). This amount of DAP is only sufficient to allow murein synthesis to continue for 1 min in strain W7 starved for DAP. Mett et al. (10) labeled W7 cells for 20 min after 20 min of DAP deprivation (which in our hands is almost sufficient for a complete depletion of preexisting pools of DAP and DAP-containing precursor). We performed 5-min pulses after 29 and 37 min of DAP deprivation and obtained 0.9 and 1.7% soluble polymer, respectively, i.e., severalfold more than in experiments using 100 to 130-ng amounts of [^{14}C]DAP per ml with W7 cells deprived of DAP for 25 min (see above). All of these observations suggest that soluble polymer is a product of autolysis rather than being nascent murein.

Since bactoprenol-linked radioactivity was not extracted by hot water, we treated pulse-labeled W7 bacteria with 0.1 N hydrochloric acid for 10 min at 100°C in an attempt to reveal any bactoprenol-linked nascent oligosaccharide. However, in contrast to the bactoprenol-linked disaccharide pentapeptide label (lipid intermediate), which was released by acid hydrolysis of hot-water-extracted cells and which in 0.5- to 1-min pulses equalled the cell content of label in insoluble murein, no slowly moving or chromatographically immobile radioactivity was released from pulse-labeled bacteria by mild acid hydrolysis. Thus, there can be very little label present as bactoprenol-linked oligosaccharides that is not also attached to the murein sacculus.

The most convincing evidence that the soluble murein fraction was not a normal precursor of the murein sacculus came from pulse-chase experiments. In contrast to bactoprenol-linked disaccharide pentapeptide, a known precursor which was chased into insoluble murein in less than 2 min, the soluble murein polymers did not disappear during a chase but declined slowly over a 2-h period.

The above data indicate that the elongation peptidoglycan strands were cross-linked directly to the murein sacculus before they were released from the bactoprenol.

DISCUSSION

The present experiments indicate that in *E. coli* newly synthesized peptidoglycan strands are immediately attached to the preexisting murein and subsequently undergo changes involving increased cross-linkage and loss of alanine. The percentage of nonreducing monomers C_7 and C_8 present in "mature" murein did not differ from that found in newly inserted material. This is in agreement with the result of De Pedro and Schwarz, (4), who found that the final oligosaccharide chain length was determined within 3 min of incorporation, the shortest time tested. Our data indicate that the oligosaccharide chain length was determined already within 30 s, suggesting that chain length is probably fixed at the time of polymerization of the peptidoglycan.

During chasing, there was a gradual conversion of at least 20% of the radioactive monomers into dimers and trimers, resulting in an increased cross-linkage of mature murein as compared with new murein. The observed increase in dimers confirms the earlier result of De Pedro and Schwarz (4). If, as we believe, dimers and trimers were continuously being opened, the actual postsynthesis monomer-to-dimer conversions may be much greater than 20%. Increased cross-linkage occurred also in the cylindrical murein of BUG6 with time, whereas its septal murein had a high cross-linkage from the beginning. The significant conversion of monomers to dimers that occurs during chasing (Fig. 2) and aging of the cylindrical BUG6 murein (Table 1) requires that a sizable fraction of the pulse-labeled (old) monomers are used as acceptors in transpeptidations from new glycan chains. It can be estimated that a murein sacculus contains about 10^6 dimers. If there were a single growth zone where a borderline between new and old murein existed, only about 1,000 radioactive monomers (the number of free monomers in a length of murein equivalent to the circumference of the sacculus) could be incorporated into dimers to increase the radioactivity of dimers during a chase. This would increase the radioactivity in dimers only 0.1%. On the order of 100 or 200 borderlines per cell between old and new murein strands each representing a growth site is suggested by the increase of 20%. These data thus confirm the multisite mode of elongation of the sacculus suggested by autoradiography experiments (15; Burman et al., in press).

The relatively constant percentages of label in the cross-linked muropeptides after the initial rise observed as murein ages during growth of *E. coli* (Fig. 2) do not prove, however, that the interpeptide cross-links are stable. A considerable amount of continuous opening of old trimer or dimer cross-links and formation of new trimer or dimer cross-links as new strands are inserted is probably obscured by these numbers since we have recently obtained evidence for a labile situation among murein trimers and dimers in growing *E. coli* (Burman et al., in press).

One of the most striking changes during the maturation of murein revealed by the present experiments was a severalfold increase in C_5 (disaccharide tripeptide monomer) at the ex-

pense of C_6 (disaccharide tetrapeptide monomer) (Fig. 3). The tripeptides are formed by the action of a periplasmic (1) LD-carboxypeptidase (carboxypeptidase II) which removes the terminal D-alanine from the tetrapeptide (7). An increase in carboxypeptidase II activity at the time of division (1) suggested that C_5 content is related to septation.

However, in the present experiments C_5 was present in filamenting, as well as septating, BUG6. Thus, the physiological role of carboxypeptidase II remains unclear.

A new and somewhat unexpected finding in our experiments was that after murein was assembled, dimers and trimers, as well as monomers, of *E. coli* murein were linked to Braun lipoprotein (Fig. 3). Thus, the addition of Braun lipoprotein to the sacculus was secondary to cell wall peptidoglycan incorporation into the sacculus, and the linking process appeared to be nonspecific in terms of site of linkage and to have no controlling effect in terms of oligosaccharide chain length or dimer and trimer content. The fraction of muropeptides linked to lipoprotein is less than 3% after 0.5 min of labeling (data not shown). The percentage of muropeptides linked to lipoprotein increased rapidly with time and plateaued at about 16% after a chase of one-half generation (Fig. 3). Our data for the rate of binding of lipoprotein to new murein differ somewhat from those that Braun and Wolff (3) obtained under different conditions. We both conclude, however, that lipoprotein is not present on a precursor of murein and is added to it gradually as the murein ages.

Mett et al. (10) have characterized a soluble, chromatographically immobile peptidoglycan fraction isolated from pulse-labeled *E. coli* and have suggested that it is a late-stage intermediate in the synthesis of the murein sacculus. There are several reasons why we believe that this material is not a true intermediate. First, the soluble material was unique in having a high content of pentapeptide suggestive of a precursor, but it seemed abnormal as a precursor in that 25% of its muropeptide content was bound to lipoprotein. Since, as just discussed, most lipoprotein is not attached to murein until minutes after the incorporation of new murein strands into the sacculus, it seems most unlikely that nascent peptidoglycan would contain any lipoprotein, let alone as much as is found in the mature sacculus. Second, as described above after the pulse of [^{3}H]DAP, the cells were completely devoid of DAP for almost 20 min, and this situation provided ample opportunity for autolysis to release newly incorporated material and allowed time for the addition of lipoprotein. Third, and the most compelling reason for believing that the soluble polymer was not a normal late-stage intermediate, both we and Mett et al. (10) found that the soluble material did not chase rapidly into insoluble murein. Instead of chasing completely in less than 2 min, we found that most of the label was still present in the soluble peptidoglycan fraction a generation later. Thus, we believe that soluble polymer is a by-product of autolysis and that under normal conditions new strands of murein are linked to the sacculus concomitantly with their formation.

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