Determination of Murein Precursors during the Cell Cycle of
Escherichia coli

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A convenient and reliable method has been established that allows a quantitative
determination of m-diamino[3H]pimelic acid-labelled murein precursors in 1 ml culture samples
of Escherichia coli. Prior to separation by reversed-phase high-pressure liquid chromatography
the lipid-linked intermediates were hydrolysed to release the muropeptides. The accuracy for the
measurement of UDP-N-acetylmuramylpentapeptide (UDP-MurNAc-pentapeptide) was
±1.9% (sd), for undecaprenyl-P-P-MurNAc-pentapeptide (lipid I) ±10% (sd) and for
undecaprenyl-P-P-(GlcNAc-β1→4)MurNAc-pentapeptide (lipid II) ±5% (sd). The ratio of
UDP-MurNAc-pentapeptide : lipid I: lipid II was about 300:1:3 for E. coli MC4100. The
relative cellular concentrations of all three precursor molecules were found not to vary
throughout the cell cycle. It is concluded that elongation and division of the murein sacculus is
not controlled by oscillations in the concentrations of these late murein precursors.

INTRODUCTION

Propagation of bacteria is a cyclic process in which enlargement of cell mass and volume is
interrupted by cell division. Numerous metabolic processes have to be co-ordinated in a precise
sequence during the bacterial cell cycle. In particular, cell division has to be synchronized with
DNA replication. In addition, septation is also coupled to growth rate. Cells growing in rich
medium are larger than those growing in poor medium (Helmstetter et al., 1968). It has,
therefore, been proposed that cell division may also be regulated by changes in crucial
metabolite pool sizes (Kubitscheck & Pai, 1988; R. D’Ari, personal communication). Regulation of
enzymic reactions by substrate and/or product concentrations is quite common. Feedback control in multi-step biosynthetic pathways is only one example of such control
circuits.

The most important structural event during cell division is the formation of a cross-wall of
murein, which after completion of cell septation forms the new polar caps of the two daughter
 cells (Nanninga & Woldringh, 1985). Within the cell cycle, synthesis of a cross-wall alternates
with synthesis of cylindrical murein during cell elongation. Both processes have been suggested
to be catalysed by different enzyme systems (Spratt, 1975). Indeed, the structure of the murein
synthesized (Olijhoek et al., 1982; Kraus & Höltje, 1987), the sensitivity towards β-lactam
antibiotics (Schwarz et al., 1969) and the rate of synthesis (Hoffmann et al., 1972; Olijhoek et al.,
1982) have been shown to differ during cell elongation and division. Septum formation has been
found to be controlled and executed by quite a number of proteins (Donachie et al., 1984). The
leading enzyme involved in septation is the penicillin-binding protein (PBP) 3 (Spratt, 1975),

Abbreviations: A2 pm, diaminopimelic acid; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid.

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which is present in the cell in constant amounts throughout the cell cycle (Wientjes et al., 1983). Nevertheless, the activity(ies) of at least one of the enzymic reactions involved in cell division is expected to oscillate during the cycle. However, the regulatory signals are still unknown.

Murein biosynthesis takes place in different compartments of the cell (Höltje & Schwarz, 1985), commencing with the synthesis of the nucleotide precursors UDP-\(\text{N-acetylglucosamine (UDP-GlcNAc)}\) and UDP-\(\text{N-acetylglutamyl-l-Ala-D-Glu-m-A}_{2}\text{pm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide)}\) in the cytoplasm. The activated amino-sugar derivatives are subsequently translocated onto a lipid carrier (bactoprenol) residing in the cytoplasmic membrane. First, undecaprenyl-P-P-MurNAc-pentapeptide (lipid I) and finally the undecaprenyl-P-P-(GlcNAc-\(\beta\)1→4)MurNAc-pentapeptide (lipid II) are formed. In a still unknown manner, the lipid-linked disaccharide pentapeptide is then transferred to the outer leaflet of the cytoplasmic membrane and inserted into the pre-existing murein sacculus. In \textit{E. coli} this takes place in the periplasmic space.

The undecaprenylphosphate carrier lipid is present in the cell in limiting amounts (Rothfield & Romeo, 1971) and is re-used. It also participates in the synthesis of other wall polymers such as lipopolysaccharides and capsular polysaccharides (Rogers et al., 1980). As a branch point in the biosynthetic pathways of various wall components it is an ideal site to coordinate the synthesis of these cell wall structures (Taschner, 1988). In particular, murein synthesis could be regulated by the actual concentrations of the ultimate precursors. It was of major interest to gain information on the concentration of the intermediates of the lipid cycle during the cell cycle.

Therefore, we established a method to determine the relative cellular concentration of UDP-muramylpentapeptide, lipid I and lipid II in synchronized cells of \textit{E. coli}.

**METHODS**

\textbf{Bacterial strain and growth conditions.} \textit{E. coli} \textsc{K}12 MC4100 lysA rel\(^+\) was grown at 28 \(^\circ\)C with agitation in minimal citrate medium (Vogel & Bonner, 1956) supplemented with lysine, methionine, threonine (50 \(\mu\)g ml\(^{-1}\) each), thiamin (4 \(\mu\)g ml\(^{-1}\)) and 0.4\% (w/v) glucose (Wientjes et al., 1985). The exponential mass-doubling time was about 80 min.

\textbf{Synchronization procedure.} An exponentially growing culture (400 ml) was harvested by centrifugation at an optical density at 450 nm of 0.45, which corresponds to about 2.4 \(\times\) 10\(^8\) cells ml\(^{-1}\). The cells were synchronized by the centrifugal elutriation technique of Figdor et al. (1981). The elutriation and synchronous growth were monitored by Coulter counter volume distribution and cell number measurements. To minimize metabolic disturbances, elutriation and collection of the newborn cells were done at 10 \(^\circ\)C (instead of the usual 4 \(^\circ\)C). The time point of septum formation was deduced from the number of constricting cells, which was determined by electron microscopy from at least 200 cells per sample.

\textbf{Labelling of murein precursors and murein.} Small cells (3.5 \(\times\) 10\(^8\) cells ml\(^{-1}\)), obtained by centrifugal elutriation at 10 \(^\circ\)C, were shifted to 28 \(^\circ\)C to start synchronous growth. \([\text{\textit{H}}}\)Diaminopimelic acid (\(A_2\)pm) (meso-2,6-diamino[3,4,5-\(\text{H}\)]pimelic acid; 0.85 TBq mmol\(^{-1}\); CEA) was added to a final concentration of 0.96 MBq ml\(^{-1}\).

After 50 min, samples (1 ml) of the synchronously growing culture were taken at intervals of 8 min and added to 2.5 ml of icecold extraction solvent (n-butanol/6 M-pyridinium acetate pH 4; 4:1, v/v) to isolate the murein precursors. After vigorous shaking with 0.5 g of glass beads (0.17-0.18 mm diameter, Braun) in a vibrator for 30 min at 4 \(^\circ\)C, the samples were centrifuged for 10 min at 2500 g and portions from both phases were withdrawn for quantification of the murein precursors.

Incorporation of \([\text{\textit{H}}}\)A_2\text{pm} into TCA-precipitable material was measured as previously described (Wientjes et al., 1985).

\textbf{Quantification of UDP-MurNAc-pentapeptide.} Portions (0.1 ml) of the aqueous phase were centrifuged to remove insoluble material, freeze-dried to remove traces of organic solvents and dissolved in 0.1 ml H\(_2\)O. Samples were separated by reversed-phase high-pressure liquid chromatography (HPLC) on a column (125 by 4.6 mm) prepacked with Hypersil ODS-C18, 5 \(\mu\)m (Bischoff). The column was eluted at 35 \(^\circ\)C at a flow rate of 1 ml min\(^{-1}\) with 50 mm-NaOH adjusted to pH 4.0 with phosphoric acid. The radioactivity in the fractions (217 \(\mu\)l) was determined (TriCarb 1500 liquid scintillation analyser; Canberra Packard) in 10 volumes of scintillator cocktail (Hydroluma, J. T. Baker Chemicals).

\textbf{Quantification of the murein lipid intermediates.} Portions (1.7 ml) of the organic extraction phase were washed twice with 0.5 ml H\(_2\)O and dried under vacuum. The material was hydrolysed with 0.1 M-HCl for 15 min in a boiling water-bath. After neutralization with 0.1 M-NaOH the samples were freeze-dried, dissolved in 80 \(\mu\)l water and analysed by HPLC. Prior to chromatography the muramyl residues of the muropeptides were reduced to their muramitol derivatives (Glauner et al., 1983). Accordingly, the samples (80 \(\mu\)l) were diluted with an equal volume
of 0.5 M-sodium borate buffer, pH 9, and incubated in the presence of 1 mg sodium borohydride ml⁻¹ for 30 min at room temperature. The reaction was stopped by adjusting the pH to 4–5 with 4 M-phosphoric acid. HPLC-fractionation was done as described above except that the pH of the eluent was 4–5 and the fraction size 300 μl.

**RESULTS**

**Labelling of murein in synchronized cells of E. coli MC4100**

The centrifugal elutriation method has yielded physiologically only slightly disturbed cells with good synchrony, especially when the synchronization was done at 10 °C (Figdor et al., 1982; Olijhoek et al., 1982; Wientjes et al., 1983). In synchronously growing cultures of *E. coli* MC4100 the cell number doubled over a period of about 0.62 times the generation time (as calculated from Fig. 4a). The percentage of constricting cells fluctuated between 1 and 60% (Fig. 4a).

*E. coli* MC4100, which is Dap⁺, was used instead of *E. coli* W7, which is Dap⁻, because the latter strain incorporates exogenously supplied A₂pm in a biphasic manner (Wientjes et al., 1985); this would interfere with the pool size determinations during the cell cycle. Incorporation of [³H]A₂pm in MC4100 was found to be already in steady state 20–30 min after the addition of the amino acid to the medium (Fig. 1).

**Method for the determination of murein precursors**

A precise and reliable procedure was established for the extraction of both the water-soluble and the lipophilic murein precursors. By shaking the cells with glass beads in a vibrator in the presence of the extraction solvent, reproducible extraction of the murein precursors was achieved; these were separated and quantified by high-pressure liquid chromatography.

The determination of the water-soluble precursors was greatly affected by traces of organic solvent in the aqueous phase after the extraction step. Therefore, the samples were routinely dried under vacuum and redissolved in water before HPLC separation (UDP-muramylpentapeptide was shown to be stable under these conditions). Fig. 2 shows the separation of UDP-MurNAc-pentapeptide from other components of the aqueous extract.

For the analysis of the lipid-linked precursors the extracted material was hydrolysed to the free muropeptide residues. The lipid I precursor yields muramylpentapeptide which is identical to the product of hydrolysis of UDP-muramylpentapeptide. Therefore, the water-soluble nucleotide precursors had to be completely removed from the organic solvent phase containing the lipid precursors. This was done by washing the sample twice with water. The first wash contained less than 0.1% of UDP-muramylpentapeptide, which was no longer detectable in the

![Fig. 1](image-url)  
**Fig. 1.** Incorporation of [³H]A₂pm into TCA-precipitable material. A synchronously growing culture of *E. coli* MC4100 in minimal-citrate medium at 28 °C was labelled at t = 0 min with 0.96 MBq [³H]A₂pm ml⁻¹. The radioactivity incorporated into TCA-precipitable material was determined in culture samples (25 μl) at the indicated time points.
Fig. 2. Separation of UDP-MurNAc-pentapeptide from other components of the aqueous extract by reversed-phase HPLC. Chromatography was on a 5 μm Hypersil ODS column (125 × 4.6 mm), using isocratic elution conditions with 50 mM NaOH adjusted to pH 4.0 with phosphoric acid (flow rate, 1.0 ml min⁻¹; column temperature, 35 °C). (a) UV-absorbance at 205 nm. (b) Radioactivity of the fractions (217 μl). Peak A represents UDP-MurNAc-pentapeptide (identified by running an authentic standard).

Fig. 3. Separation of MurNAc-pentapeptide and disaccharide-pentapeptide after hydrolysis and reduction of the extracted lipophilic compounds (see Methods) by reversed-phase HPLC. Chromatographic conditions were as described in Fig. 2 except that the pH was 4.5. (a) UV-absorbance at 205 nm. (b) Radioactivity of the fractions (300 μl). Peak A represents MurNAc-pentapeptide; peak B represents GlcNAc(β1→4)MurNAc-pentapeptide (identified by running authentic standards).
Murein precursors in the cell cycle of E. coli

Fig. 4. Cellular concentration of murein precursors during synchronous growth of E. coli. A synchronized culture of E. coli MC4100 was labelled at $t = 0$ min with $0.96$ MBq $[^3]$H$A_2$pm ml$^{-1}$. (a) Increase in cell number (●) and percentages of cells showing a visible constriction (○) were determined as described in Methods. Samples (1 ml) were withdrawn at the indicated time points and analysed for the relative concentration of (b) UDP-MurNAc-pentapeptide; (c) lipid I and (d) lipid II as described in Methods.

The extraction procedure has been described by Anderson et al. (1967) to be complete for the lipid-linked precursors. For the water-soluble nucleotide precursors we found that at least 99% of a known amount of UDP-MurNAc-pentapeptide added to a sample was recovered. Samples of 1 ml of bacterial culture at a density of about $4 \times 10^8$ cells ml$^{-1}$ were sufficient for the analysis.

The accuracy of the analytical method was determined by running fourfold samples; the standard deviation was 1.9% for the determination of UDP-MurNAc-pentapeptide, 10% for lipid I and 5% for lipid II.
Pool sizes of murein precursors in the cell cycle of E. coli

The concentration of the water-soluble UDP-MurNAc-pentapeptide and the undecaprenyl-phosphate-linked precursors, lipid I and lipid II, were determined every 8 min in a synchronously growing culture of E. coli MC4100 during a period of 96 min covering a complete cell cycle with the septum formation (maximum of constricting cells) in the mid-point of the time span analysed (Fig. 4a). The amount of labelled UDP-MurNAc-pentapeptide decreased exponentially (Fig. 4b). This is easily explained by the dilution kinetics of the labelled A,pm, which is the result of two processes going on during cell growth. First, the specific radioactivity of the [3H]A,pm pool decreases due to the endogenous synthesis of A,pm; second, the amino acid is used up by its incorporation into murein. Taking this into consideration the results indicate that the intracellular amount of UDP-MurNAc-pentapeptide does not vary throughout the cell cycle. The same holds true for the two lipid-linked precursors (Fig. 4c, d). This means that the concentrations of the murein precursors UDP-MurNAc-pentapeptide, lipid I and lipid II are kept constant during the cell cycle.

**Determination of the relative amounts of murein precursors in E. coli**

Our results directly specify the relative amounts of the different precursor molecules. A calculation of the ratios of the murein precursors at different time points in the cell cycle yielded constant values throughout. Since we found that the precursors do not vary within the cell cycle the specific radioactivity of the different compounds at each time point must be the same. Therefore, the proportional numbers reflect the relative pool sizes of the various murein precursors. Under the growth conditions employed in this study the following ratios were determined (mean values of all 13 time points ± standard deviation): UDP-MurNAc-pentapeptide exceeds lipid I by a factor of 277.6 ± 41.2, and lipid II by 89.7 ± 15.97. The ratio of lipid II to lipid I was 3.13 ± 0.35.

**DISCUSSION**

Several control mechanisms of murein biosynthesis influence the cellular concentrations of the nucleotide murein precursors (UDP-MurNAc-peptides and UDP-GlcNAc) and the lipid carrier-linked intermediates. Studying the mode of action of penicillin, Park (1952) observed an accumulation of UDP-derivatives (formerly called Park-nucleotides) in the presence of β-lactams. Later it was proposed that UDP-MurNAc-pentapeptide exerts a feedback inhibition on the first enzyme of the biosynthetic pathway of UDP-muramic acid, the phosphoenolpyruvate:UDP-GlcNAc enolpyruvyl transferase (Venkateswaran et al., 1973). Also, the stringent control mechanism regulates the cellular concentration of UDP-MurNAc-pentapeptide. It has been shown that the relA gene product prevents an accumulation of nucleotide murein precursors during amino acid starvation (Ishiguro & Ramey, 1978).

Unfortunately, a detailed analysis of the concentrations of both the cytoplasmic nucleotide precursors and the lipid-linked intermediates in the cytoplasmic membrane has only partially been accomplished (Mengin-Lecreulx et al., 1982, 1983). In particular, determination of lipid precursors was problematic because of the low intracellular levels and incomplete recoveries during isolation and separation of these compounds.

We succeeded in establishing a convenient and rapid method that allows a reliable and precise measurement of the relative concentration of the UDP-MurNAc-pentapeptide and the lipid intermediates. Thereby, the relative amount of the lipid I precursor could be determined with increased accuracy as compared with earlier results by others (Ramey & Ishiguro, 1978; Dai & Ishiguro, 1988). It was found to be only one-third of the lipid II precursor.

Our method enabled us to determine the relative cellular concentrations of the murein precursors within the cell cycle of E. coli. This was of interest because the pools of murein intermediates may regulate murein synthesis, which is known to vary during the growth cycle of E. coli (Olijhoek et al., 1982). The pools of both the cytoplasmic UDP-MurNAc-pentapeptide and the two undecaprenylphosphate-linked intermediates were found to show no oscillation throughout the cycle. Therefore, these intermediates cannot govern murein synthesis. The
molecular signal(s) that initiate(s) the synthesis of the septum, a prerequisite for cell division, remains unknown.

The suggestion that the known coupling of cell division to growth rate may be achieved by changes in the internal pool sizes of certain metabolites (R. D’Ari, personal communication) remains very attractive. Other promising candidates for such regulatory compounds may be found among the numerous nucleotide derivatives which have been called alarmones (Stephens et al., 1975) and which include such important control factors as cyclic AMP and ppGpp. Only the concentration of cyclic AMP has been analysed during the cell cycle of *E. coli*. It was found not to oscillate, excluding this nucleotide as a regulatory element in cell-cycle-dependent processes (Hölte & Nanninga, 1984). A complete survey of the concentrations of the whole list of nucleotide derivatives during the cell cycle would be of utmost importance for the understanding of the regulation of the bacterial cell cycle. Convenient (two-dimensional thin-layer and HPLC) methods have been described (Bochner & Ames, 1982) which are capable of analysing a great number of different nucleotides in one assay.

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REFERENCES


