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# Peptidoglycan Biosynthesis in a Thermosensitive Division Mutant of *Escherichia coli*†

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ABSTRACT: Peptidoglycan biosynthesis during a bacterial division cycle was investigated in the thermosensitive division mutant *Escherichia coli* PAT 84. Synchronous cell division of this organism was initiated by a shift down from restrictive to permissive growth temperature. Cells harvested at different times after a shift down of temperature served as representatives of the various stages during cell division. These cells were made permeable to peptidoglycan nucleotide-sugar precursors by pretreatment with ether and were found capable of catalyzing the polymerization of externally added precursors as well as the covalent attachment of the newly synthesized peptidoglycan strands to those preexisting in the cell wall. Differences were observed in the rate of peptidoglycan synthesis and in the extent of peptide side-chain cross-linkage at the various stages of division. Nonseptate filaments, formed at the restrictive temperature, incorporated significantly more peptidoglycan which was more cross-linked than in normally dividing cells grown at the permissive temperature. Quantitative analyses of the carboxypeptidase and transpeptidase reactions in cells at different stages of division were performed and the inhibitory effect of a number of β-lactam antibiotics was investigated. Of special significance was the finding that low doses of penicillin or growth at restrictive temperature, which did not affect transpeptidation, partially inhibited the carboxypeptidase activity. This inhibition was paralleled by an increase in incorporation of newly synthesized peptidoglycan into the preexisting cell wall. We therefore propose that carboxypeptidase activity regulates the availability of peptidoglycan precursor(s) for attachment to the preexisting peptidoglycan by transpeptidation.

The division of the rod-shaped *Escherichia coli* cell involves ingrowth of the cytoplasmic membrane and the peptidoglycan (murein) of the wall to form the septum which subsequently becomes the new polar caps of the daughter cells (Rogers, 1970; Slater and Schaechter, 1974; Burdett and Murray, 1974b). For this process to occur, a considerable number of hydrolytic and synthetic reactions which modify the morphology of the peptidoglycan layer must take place. The timing of such reactions, their triggering, and the coordination of their sequence during a cell division cycle require very precise regulation (Cooper and Helmstetter, 1968; Donachie and Begg, 1970; Shockman et al., 1974). The location and pattern of growth of peptidoglycan in intact cells of *E. coli* have been studied by following the incorporation of radioactively labeled amino acids into the cell wall peptidoglycan (Hoffmann et al., 1972; Ryter et al., 1973; Schwarz et al., 1975). These studies have indicated that in the cell there are distinct zones of peptidoglycan growth and that the rate of peptidoglycan synthesis fluctuates during the division cycle of the cell. No information was, however, obtained on the biochemical regulation of septum formation during cell division and on the chemical nature of the peptidoglycan synthesized during septum formation.

The sequence of reactions leading to the formation of peptidoglycan was elucidated by studying, in cell-free systems, the incorporation of the radioactively labeled nucleotide precursors UDP-GlcNAc and UDP-MurNAc-L-Ala-D-*i*-Glu-*meso*-DAP-D-Ala-D-Ala[1] (UDP-MurNAc-pentapeptide) into peptidoglycan (for a review see Strominger, 1970). The last stage of peptidoglycan biosynthesis, the incorporation of newly synthesized peptidoglycan strands into the preexisting wall structure, was found to be catalyzed mainly by the penicillin-sensitive transpeptidation reaction (Mirelman et al., 1972, 1974a,b).

Previous investigations with cell-free systems were done under conditions in which the natural controls of the enzymatic reactions which perform peptidoglycan synthesis were partially lost, presumably as a result of the mechanical disruption of the cells.

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[1] Abbreviations used are: DAP, 2,6-diaminopimelic acid; ETB, ether-treated bacteria.

We now report on a novel system for *E. coli* cells, in which the cellular control of peptidoglycan biosynthesis is apparently preserved and is similar to that found in intact cells. In this system, cells were made permeable to exogenous nucleotide-sugar peptidoglycan precursors by treatment with ether (Vosberg and Hoffmann-Berling, 1971). Using this system and the thermosensitive filament-forming division mutant *E. coli* PAT 84 (Hirota et al., 1968; Ricard and Hirota, 1973), it became possible to study peptidoglycan biosynthesis at the different stages of septum formation. In the mutant a shift down from the restrictive to permissive growth temperature triggered a synchronous resumption of septum formation (Burdett and Murray, 1974a; Schwarz et al., 1975). Cells harvested at different times after growth at the restrictive and permissive temperatures were used as representatives of the various stages in the formation of the new septum in dividing cells. The results are discussed in relation to the regulation of cell surface growth and division.

## Methods and Materials

*Bacteria and Growth Conditions.* The thermosensitive *E. coli* mutant PAT 84 which forms multinucleated, nonseptated filaments at 42 °C (Hirota et al., 1968) was used throughout this study. Cells were grown in L-broth (Lennox, 1955) supplemented with thymine (50 μg/ml), 2,6-diaminopimelic acid (DAP) (4 μg/ml), and L-lysine (20 μg/ml). The pH was adjusted to 7.3 with NaOH. The number of cells was monitored with a particle counter (Coultronics, France, S.A.) or by measuring the turbidity of the culture with a Klett-Summerson spectrophotometer (filter no. 54). Cells (1500 ml) grown with aeration at 30 °C to about $6 \times 10^7$ cells/ml (cells at this stage were designated as A) were transferred to 42 °C for 60 min and then shifted down again to 30 °C (stage $T_0$). Cell samples harvested 10, 20, 30, and 45 min after temperature shift down were designated $T_{10}$, $T_{20}$, $T_{30}$, and $T_{45}$, respectively.

*Preparation of Ether-Treated Bacteria (ETB).*[1] Cell samples (200 ml) taken from the growing cultures were immediately mixed with a cold solution of basic medium (80 ml) composed of KCl (80 mM), Tris (40 mM) (pH 7.4), $MgCl_2$ (7 mM), EGTA (2 mM) ([ethylenebis(oxoethylenenitrilo)]tetraacetic acid), spermidine·3HCl (0.4 mM), and sucrose (0.5 M). The cells were collected by sedimentation in the cold (9000*g*, 15 min), resuspended in 2 ml of the basic medium, and treated with ether under the conditions specified by Vosberg and Hoffmann-Berling (1971). After removal of the ether layer, the cells in the aqueous medium were sedimented (7000*g*, 8 min) and the pellet was resuspended in basic medium at a concentration of approximately 10 mg of protein/ml ($10^{10}$ cells/ml) and stored at −20 °C.

*Pulse Labeling of Peptidoglycan in Intact Cells with [$^3$H]DAP.* The peptidoglycan was labeled essentially as described previously (Ryter et al., 1973). Cells of *E. coli* PAT 84 were grown at 30 °C as described above to a cell density of about $2 \times 10^8$. Twenty milliliters of culture was rapidly filtered (Sartorius membrane filter 0.6 μm, Gottingen, Germany) and the cells on the filter were washed twice with 100 ml of growth medium lacking DAP, warmed either to 30 or 42 °C depending on the temperature at which DAP incorporation was to take place. The washed cells were resuspended in 20 ml of L-broth containing 600 μCi of [$^3$H]DAP (specific activity 23 Ci/mmol, Service de Molecules Marques (C.E.A.), France). After incubation for 4 min at 30 or 42 °C, cells were filtered rapidly on membrane filters and resuspended in 20 ml of growth medium lacking DAP; 10-ml samples were added to an equal volume of 8% sodium dodecyl sulfate and heated at 100 °C for 30 min. The remaining cells (10 ml) were added to an equal volume of 10% trichloroacetic acid ($Cl_3CCOOH$) and kept in ice for 30 min.

*Biosynthesis of Peptidoglycan.* Ether-treated bacteria (ETB) (1 mg of protein) were incubated in Tris buffer (50 mM) (pH 8.3), $NH_4Cl$ (50 mM), $MgCl_2$ (20 mM), ATP (10 mM), and 2-mercaptoethanol (0.5 mM) in a final volume of 200 μl. Incubation mixtures also routinely contained UDP[$^{14}$C]GlcNAc (0.9 cpm/pmol, 90 000 cpm) and unlabeled UDP-MurNAc-pentapeptide (100 nmol). In some experiments UDP-MurNAc-*meso*-[$^{14}$C]DAP-pentapeptide (4 cpm/pmol, 80 000 cpm) served as the labeled substrate and UDP-GlcNAc (100 nmol) was the unlabeled substrate.

The enzymatic release of the C-terminal D-alanine moiety of the pentapeptide, which is a measure for the activities of DD-carboxypeptidase and transpeptidase, was assayed with UDP-MurNAc-D-[$^{14}$C]-Ala-pentapeptide (9 cpm/pmol; 160 000 cpm) as the labeled substrate, which was added together with or without UDP-GlcNAc (100 nmol).

Reaction mixtures were routinely incubated at 30 or 42 °C for 60 min, after which 1 ml of 4% sodium dodecyl sulfate or 4 ml of cold $Cl_3CCOOH$ (5% in 0.05 M NaCl) was added. The cells in sodium dodecyl sulfate were then heated for 30 min in a boiling water bath; those in $Cl_3CCOOH$ were kept in ice for 30 min. The insoluble peptidoglycan, collected on membrane filters (0.45 μm, Millipore Co.), was extensively washed as described (Vosberg and Hoffmann-Berling, 1971), and the radioactivity incorporated was measured by counting the filters.

UDP-MurNAc-pentapeptide, UDP-MurNAc[*meso*-$^{14}$C]DAP-pentapeptide (specific activity 4 cpm/pmol), UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide (specific activity 9 cpm/pmol), in which the labeled D-[$^{14}$C]Ala is at the C terminus, and UDP-MurNAc-L-Ala-D-*i*-Glu-*meso*-DAP (UDP-MurNAc-tripeptide) were prepared as described (Izaki et al., 1968; Gorecki et al., 1975). UDP[$^{14}$C]GlcNAc (Radiochemical Centre, Amersham, 260 Ci/mol) was routinely mixed with unlabeled UDP-GlcNAc (Sigma Co., St. Louis, Mo.) to a specific activity of 0.9 cpm/pmol).

*Radioactivity Measurements.* Radioactivity in the insoluble sodium dodecyl sulfate or $Cl_3CCOOH$ material, collected on filters, was measured in a scintillation counter (Packard Tri-Carb 3003) as described (Ryter et al., 1973), with an efficiency of 74% for $^{14}$C and 52% for $^3$H. In experiments where UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide was used as substrate, the clear filtrate was collected in tubes and analyzed on the amino acid analyzer for free [$^{14}$C]alanine (Mirelman et al., 1972).

*Determination of Degree of Peptide Cross-Linkage in the Peptidoglycan Synthesized by ETB Preparations.* Separate tubes were incubated under the conditions described above except that the labeled substrate UDP[$^{14}$C]GlcNAc was of a higher specific activity (40 cpm/pmol, $1.8 \times 10^5$ cpm). Following the incubation, the reaction mixtures were treated with sodium dodecyl sulfate or $Cl_3CCOOH$ and filtered on membrane filters as above. The labeled insoluble peptidoglycan on the membrane filters was extensively washed with ammonium acetate (20 ml, 0.05 M) and placed in vials together with 1 ml of ammonium acetate (0.05 M). The vials containing the filters with $Cl_3CCOOH$ insoluble material were heated in a boiling water bath for

15 min to destroy autolytic enzymes. After cooling, hen egg white lysozyme (100 μl of 1 mg/ml) (Worthington, Freehold, N.J.) was added and the vials were incubated at 37 °C for 18 h with occasional shaking. Aliquots (10 μl) from the digests were taken for the determination of labeled solubilized peptidoglycan fragments. The amount of cross-linked and un-cross-linked fragments present in the digests was computed from the amount of radioactivity in the fragments that migrated after paper chromatography in 1-butanol–acetic acid–water (4:1:5, v/v; Whatman No. 3MM, 72 h). Radioactive compounds were located by autoradiography (Kodak, x-ray film XP-54) and the radioactive spots were cut and counted (Mirelman et al., 1974a).

*Analytical Procedures.* Amino acid analysis of the nucleotide-sugar precursor preparations, of cells and cell walls, was done in a Beckman 120 C amino acid analyzer as described (Mirelman and Sharon, 1967) after acid hydrolysis (6 N HCl, 105 °C, 20 h) and evaporation. Radioactive amino acids and peptides were determined with a Packard Tri-Carb flow analyzer scintillation spectrometer 3022 connected directly to a medium column (17 cm) of the amino acid analyzer and run with the first buffer (citrate, pH 3.25). In this system, the excess of unmodified nucleotide eluted after 9 min whereas [$^{14}$C]alanine is eluted after 24 min (Mirelman et al., 1972). Protein in intact bacteria and ETB preparations was determined by the Lowry technique (Lowry et al., 1951) using bovine serum albumin (Sigma) as standard.

*Materials.* Ampicillin and 6-aminopenicillanic acid (6-APA) were gifts from Beecham Research Laboratories, Betchworth, England. Amidinopenicillanic acid FL 1060 (Mecillinam) was a gift from Leo Pharmaceutical, Denmark. Vancomycin and cephalothin (Keflin) were gifts from Ely Lilly Co., Indianapolis, Ind. D-[$^{14}$C]Alanine, L-[$^{14}$C]lysine, and *N*-acetyl-D-[$^{14}$C]glucosamine were from the Radiochemical Centre, Amersham, England. All other chemicals utilized were commercial products of the highest purity available. The different alanine peptides used were crystalline compounds prepared by Dr. I. Schechter, Weizmann Institute.

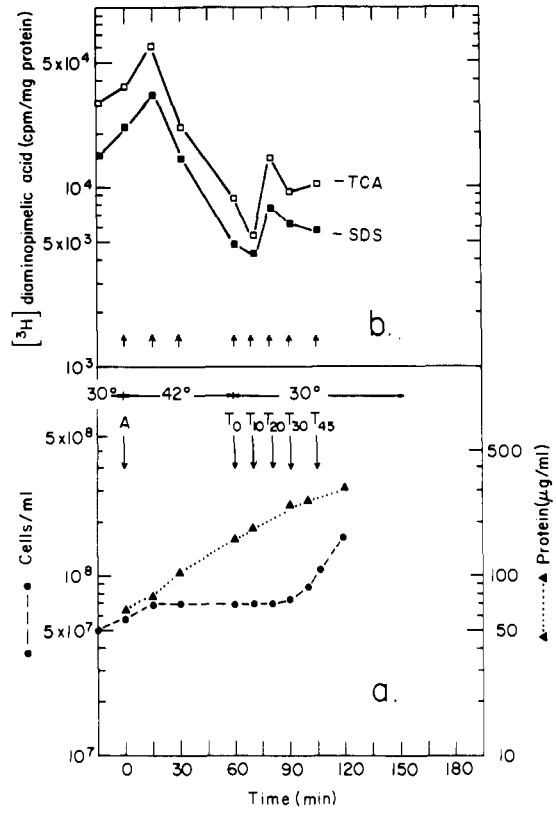


FIGURE 1: (a) Growth of *E. coli* PAT 84 in L-broth, before, during, and after temperature shifts (●–●). Arrows indicate the time of temperature shift up to 42 °C (A) and also the times at which samples of growing cells were harvested for the preparation of ether-treated bacteria. $T_0$ is the time of shift down to 30 °C. $T_{10}$, $T_{20}$, etc., indicate the times at which other cell samples were harvested for the preparation of ETB (see also under Materials and Methods). Protein content (micrograms per milliliter) of intact cells is illustrated as ▲···▲. (b) Incorporation of *meso*-[$^3$H]diaminopimelic acid by intact cells pulsed for 4 min with [$^3$H]DAP at different times during their growth. For details of incorporation see Materials and Methods. Incorporation as determined in sodium dodecyl sulfate insoluble and $Cl_3CCOOH$ insoluble material is given in counts per minute of [$^3$H]DAP/milligram of cell protein.

## Results

*Experiments with Intact Cells.* In accord with earlier findings (Hirota et al., 1968; Ricard and Hirota, 1973) the thermosensitive mutant *E. coli* PAT 84 (DAP$^-$, Lys$^-$) grew and divided normally when incubated in L-broth at 30 °C. A shift up to 42 °C stopped cell division but had no effect on protein synthesis which continued in a linear pattern (Figure 1a). When the culture was returned to 30 °C, a sharp increase in cell number was observed after about a 30-min lag.

Growth temperature had a marked effect on the amount of [$^3$H]DAP that was incorporated into the cell wall peptidoglycan of intact cells (Figure 1b). Measurements of incorporation by pulse labeling for 4 min with [$^3$H]DAP at different times during growth indicated that less incorporation occurred in cells grown at 42 °C for 60 min than at 30 °C. After temperature shift down a further drop in incorporation was observed during the first 10 min, followed by a peak of incorporation between 15 and 30 min, just before the sharp rise in cell number. In intact cells, the amount of [$^3$H]DAP that was incorporated into the $Cl_3CCOOH$ insoluble material was considerably higher than that found in the sodium dodecyl sulfate insoluble material, the difference between the two being most significant 20 min after temperature shift down (Figure 1b).

*Peptidoglycan Biosynthesis in Ether-Treated Bacterial Cells.* Incorporation of UDP[$^{14}$C]GlcNAc into the $Cl_3CCOOH$ or sodium dodecyl sulfate insoluble material of ETB cells was totally dependent upon the addition of UDP-MurNAc-pentapeptide and of $Mg^{2+}$ (Table I). Incorporation was almost linear up to 60 min with a $K_m$ for UDP-GlcNAc of $5 \times 10^{-6}$ M (data not shown). About 50% less incorporation was found in experiments where UDP-MurNAc pentapeptide containing L-lysine, obtained from *Micrococcus luteus* (Mirelman et al., 1972), was added instead of that containing *meso*-diaminopimelic acid. Diaminopimelic acid, L-lysine, and *N*-acetylglucosamine were not incorporated to any appreciable extent into the preexisting peptidoglycan. In cells grown at 30 °C, incorporation into the $Cl_3CCOOH$ insoluble material was always higher than into the sodium dodecyl sulfate insoluble material (Table I and Figure 2). Large differences in the amount of peptidoglycan determined by the two methods were found in cells grown at 30 °C (cell stage A) and in cells 30 min after a temperature shift down from 42 °C ($T_{30}$), whereas only a small difference was seen in cells at the time of temperature shift down ($T_0$). The excess radioactivity found in the $Cl_3CCOOH$ insoluble material over that in the sodium do-

Table I: Incorporation of Substrates by Ether-Treated Bacterial Preparations of Cells Grown at 30 °C.

| Substrates[a] | | Antibiotic ($\mu$g/ml) | Incorporation (pmol/mg of protein per 60 min) | |
|---|---|---|---|---|
| Labeled | Unlabeled | | $Cl_3CCOOH$ Insoluble | Na Dodecyl Sulfate Insoluble |
| UDP[$^{14}$C]GlcNAc | | | 31 | 13 |
| UDP[$^{14}$C]GlcNAc | UDP-MurNAc-pentapeptide | | 435 | 269 |
| UDP[$^{14}$C]GlcNAc | UDP-MurNAc-tripeptide | | 48 | 12 |
| UDP-MurNAc-*meso*-[$^{14}$C]DAP-pentapeptide | | | n.d. | 18 |
| UDP-MurNAc-*meso*-[$^{14}$C]DAP-pentapeptide | UDP-GlcNAc | | n.d. | 245 |
| UDP-MurNAc-*meso*-[$^{14}$C]DAP-pentapeptide | UDP-GlcNAc | Ampicillin (0.5) | n.d. | 266 |
| UDP-MurNAc-L-[$^{14}$C]Lys-pentapeptide | UDP-GlcNAc | | 298 | 128 |
| UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide | UDP-GlcNAc | | 287 | 34 |
| UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide | UDP-GlcNAc | Ampicillin (100) | 445 | 38 |
| UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide | | | 21 | 16 |

[a] Incubation mixtures in a final volume of 200 $\mu$l contained Tris buffer (50 mM) (pH 8.3), $NH_4Cl$ (50 mM), $MgCl_2$ (20 mM), ATP (10 mM), 2-mercaptoethanol (0.5 mM), and ETB (cells stage A; ~1 mg of protein). Labeled substrates added were as follows according to the different experiments: UDP[$^{14}$C]GlcNAc (0.9 cpm/pmol, 90 000 cpm); UDP-*meso*-[$^{14}$C]DAP-pentapeptide (4 cpm/pmol, 80 000 cpm), UDP-MurNAc-L-[$^{14}$C]Lys-pentapeptide (20 cpm/pmol, 80 000 cpm), UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide (9 cpm/pmol, 90 000 cpm). Unlabeled substrates were added as indicated, UDP-MurNAc-pentapeptide (100 nmol) and UDP-GlcNAc (100 nmol). Reaction mixtures were incubated at 30 °C for 60 min. For isolation of sodium dodecyl sulfate and $Cl_3CCOOH$ insoluble material, see text; n.d. = not determined.

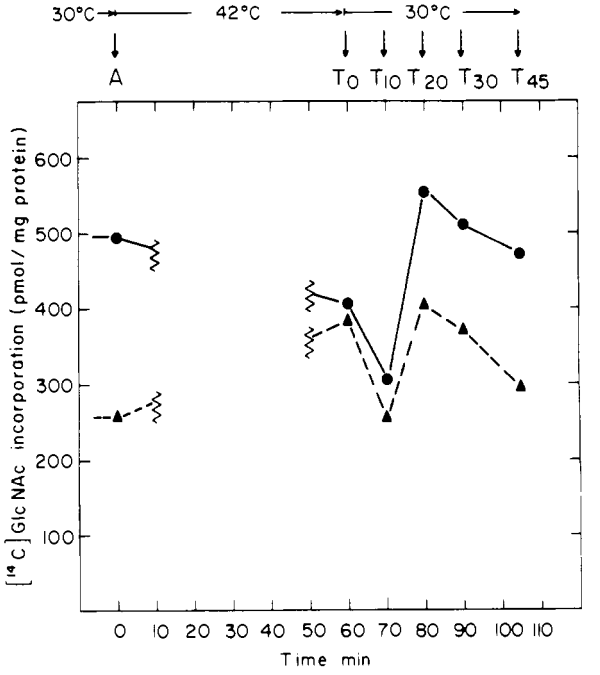


FIGURE 2: Incorporation of labeled UDP[$^{14}$C]GlcNAc in the presence of unlabeled UDP-MurNAc-pentapeptide by the various ETB preparations. The incorporation was measured in the sodium dodecyl sulfate insoluble peptidoglycan sacculus (▲- - -▲) or in the $Cl_3CCOOH$ insoluble material (●—●). For details of incorporation see Materials and Methods.

decyl sulfate insoluble material was entirely solubilized by treatment of the $Cl_3CCOOH$ insoluble material with sodium dodecyl sulfate (4%, 100 °C, 30 min). This solubilized material was nondialyzable and chromatographically immobile in 1-butanol–acetic acid–water (4:1:5) and in isobutyric acid–ammonia (1 M) (5:3). It is most likely a polymer (Mirelman et al., 1974a) since on digestion with lysozyme it was converted (78%) into low molecular weight glycopeptide fragments, the pattern of which is described below.

The amount of peptidoglycan synthesized by the various ETB cell preparations (in picomoles/milligram of cell protein) depended on the growth temperature prior to treatment with ether, on the stage reached in the division cycle, and on the temperature at which incorporation into the ETB was carried out. A shift up in growth temperature to 42 °C, for 60 min, lowered the amount incorporated into $Cl_3CCOOH$ insoluble material by about 20%, whereas the amount incorporated into the sodium dodecyl sulfate insoluble material increased by 50% (Figure 2). A further decrease in incorporation into both sodium dodecyl sulfate and $Cl_3CCOOH$ insoluble material was observed during the first 10 min after temperature shift down. The incubation temperature of the ETB cells also had a marked influence on the amount of peptidoglycan synthesized, but the effect was small in ETB preparations from cells grown at 42 °C (Figure 3b).

*Effect of β-Lactam Antibiotics on the Synthesis of Peptidoglycan by ETB Cells.* Incorporation of MurNAc[$^{14}$C]DAP-pentapeptide or [$^{14}$C]GlcNAc into peptidoglycan of ETB at both 30 and 42 °C was enhanced by low concentrations of ampicillin (0.5 $\mu$g/ml) (Tables I and II; Figures 3 and 4) irrespective of whether it was determined in $Cl_3CCOOH$ or sodium dodecyl sulfate insoluble material. Although enhancement was small in proportion to total synthesis (~15%), it was significant and highly reproducible.

A small enhancement in the amount of GlcNAc incorpo-

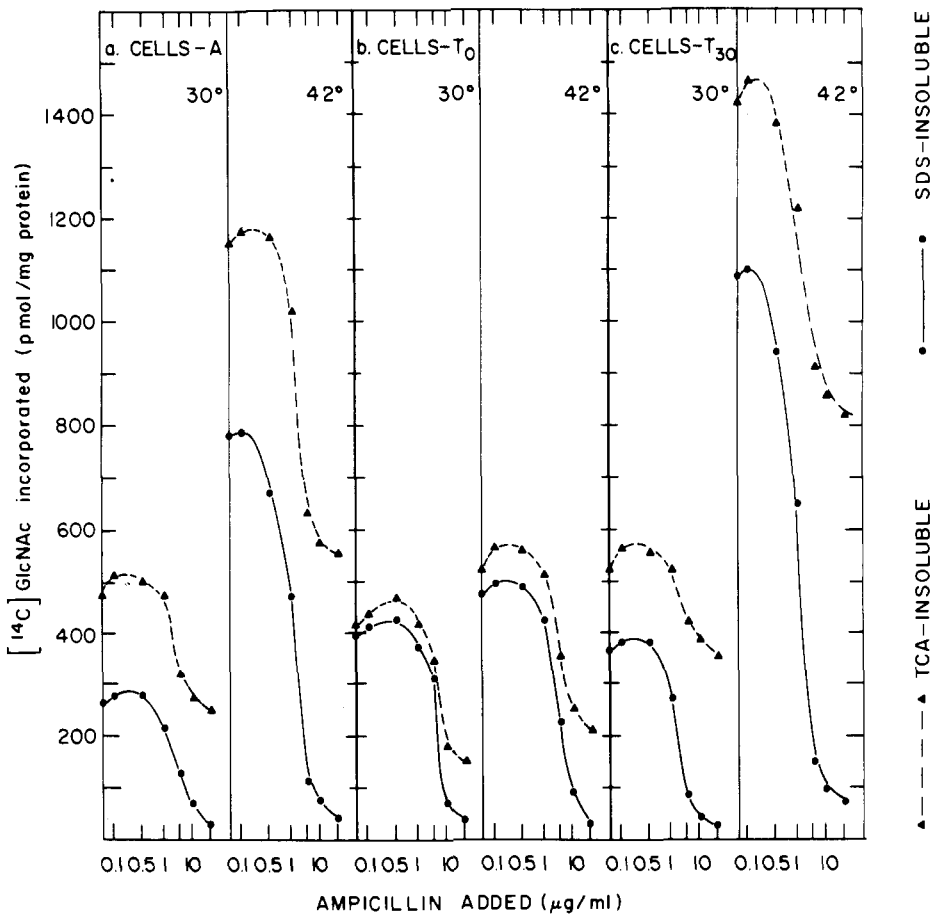


FIGURE 3: Effect of increasing concentrations of ampicillin on the rate of incorporation of labeled UDP[$^{14}$C]GlcNAc peptidoglycan precursor by ETB preparations. The rate of incorporation was measured in the sodium dodecyl sulfate insoluble (●—●) or in the $Cl_3CCOOH$ insoluble material (▲- - -▲) at 30 or 42 °C. ETB preparations were (a) from dividing cells harvested just before shift up to 42 °C (time A), (b) at the moment of shift back to permissive temperature ($T_0$), and (c) 30 min after shift back to permissive temperature. For details on the assay see Materials and Methods.

Table II: Effect of Peptides and Antibiotics on the Incorporation of UDP[$^{14}$C]GlcNAc into the Sodium Dodecyl Sulfate Insoluble Material.[a]

| Compd Added | Final Concn (μg/ml) | [$^{14}$C]GlcNAc Incorpd (pmol/mg of Protein) | % of Control |
|---|---|---|---|
| None | | 320 | 100 |
| D-Ala | 500 | 315 | 98 |
| D-Ala-D-Ala | 500 | 430 | 134 |
| L-Ala-D-Ala | 500 | 335 | 104 |
| D-Ala-L-Ala | 500 | 315 | 98 |
| D-Ala-D-Ala-D-Ala | 500 | 345 | 107 |
| Ampicillin | 0.5 | 375 | 117 |
| 6-APA | 1.0 | 365 | 114 |
| Cephalothin | 1.0 | 320 | 100 |

[a] Incubation mixtures in a final volume of 200 μl contained: Tris buffer (50 mM) (pH 8.3), $NH_4Cl$ (50 mM), $MgCl_2$ (20 mM), ATP (10 mM), 2-mercaptoethanol (0.5 mM), and ETB preparation (1 mg of protein) from cells grown at 30 °C (A). UDP[$^{14}$C]GlcNAc (0.9 cpm/pmol, 90 000 cpm) and UDP-MurNAc-pentapeptide (100 nmol) were added. Reaction mixtures were incubated for 60 min at 30 °C after which 2 ml of sodium dodecyl sulfate (4%) was added and the solution heated (100 °C, 15 min). The insoluble material was filtered on Millipore filters (0.45 μm) and the radioactivity determined as described.

rated into peptidoglycan was also observed with low concentrations of 6-APA and no inhibition of incorporation into sodium dodecyl sulfate insoluble material was observed at concentrations of up to 50 μg/ml (Table II, Figure 4). Cephalothin did not enhance incorporation of cell wall precursors at any concentration, but 50 μg/ml caused about 50% inhibition. Enhancement of incorporation into peptidoglycan was also observed in the presence of di- or tripeptides of D-alanine (Table II).

High concentrations of ampicillin (50 μg/ml) almost completely (~95%) inhibited the incorporation of [$^{14}$C]GlcNAc into sodium dodecyl sulfate insoluble material, but only about 50% of the incorporation into the $Cl_3CCOOH$ insoluble material was inhibited by the same concentration of ampicillin (Figure 3).

*Incorporation of UDP-MurNAc-*D-[$^{14}$C]*Ala-Pentapeptide.* The amount of C-terminal [$^{14}$C]alanine released into the medium by the transpeptidase and carboxypeptidase activities in the various ETB preparations incubated in the presence of unlabeled UDP-GlcNAc varied considerably (Figure 5); omission of UDP-GlcNAc from the incubation mixtures markedly decreased this release. High release was observed in experiments with cells grown at 30 °C (cells A or $T_{30}$), whereas in cells grown at 42 °C ($T_0$) or briefly after a shift down ($T_{10}$, $T_{20}$), the release of [$^{14}$C]alanine was small.

A marked increase in the release of [$^{14}$C]alanine was observed when ETB cells were disrupted by sonication (for 1 min) before the assay (Table III). Such preparations released large amounts of C-terminal D-alanine from UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide even in the absence of

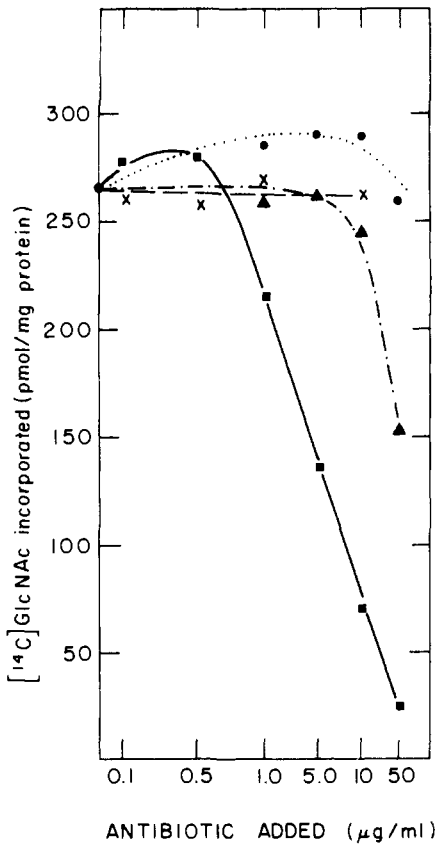


FIGURE 4: Effect of increasing concentrations of several β-lactam antibiotics on the incorporation of the labeled UDP[$^{14}$C]GlcNAc peptidoglycan precursor by ETB preparations of cells harvested at the time of shift up to 42 °C (A). Incorporation at 30 °C was measured in the sodium dodecyl sulfate insoluble material in the presence of ampicillin (■—■), 6-aminopenicillanic acid (●···●), cephalothin (▲···▲), and amidinopenicillanic acid FL 1060 (x···x).

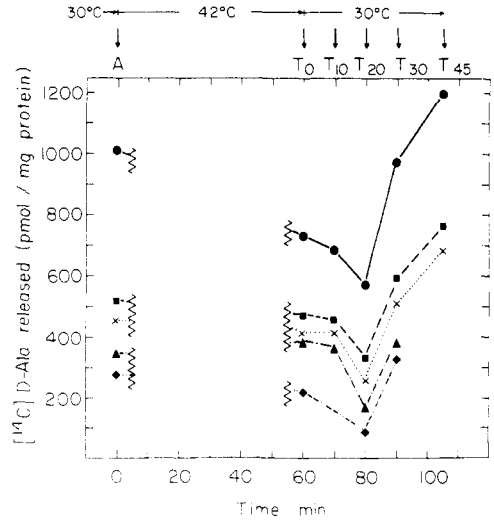


FIGURE 5: The release of [$^{14}$C]alanine by the various ETB preparations incubated (at 30 °C) with UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide and UDP-GlcNAc in the absence or presence of ampicillin: no ampicillin added (●—●); 0.1 μg/ml (■- - -■); 0.5 μg/ml (x···x); 1.0 μg/ml (▲- - -▲); 10 μg/ml (♦- - - - - -♦).

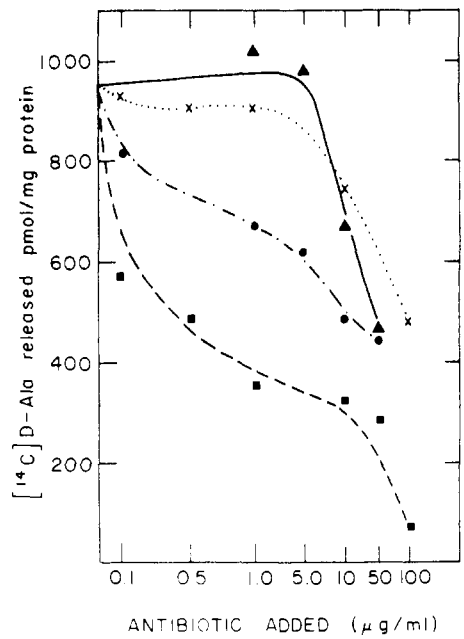


FIGURE 6: Effect of increasing concentrations of several β-lactam antibiotics on the release of [$^{14}$C]alanine by ETB preparations from cells harvested 30 min after growth temperature shift down (cells $T_{30}$). Incubations (at 30 °C) were carried out with UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide and UDP-GlcNAc as described under Methods. Ampicillin (■- - -■); Cephalothin (×···×); amidinopenicillanic acid FL 1060 (▲—▲); 6-amino penicillanic acid, (●- · -●).

UDP-GlcNAc. The incubation temperature also had a large effect on the amount of [$^{14}$C]alanine released. When UDP-MurNAc-L-Ala-D-*i*-Glu-L-Lys-D-Ala-D-[$^{14}$C]Ala was added to the incubation mixture of ETB cells instead of the commonly used DAP-containing UDP-MurNAc-pentapeptide, about 20% less [$^{14}$C]alanine was released.

As shown in Table III, low concentrations of ampicillin (0.5 μg/ml) consistently had a marked inhibitory effect on the release of [$^{14}$C]alanine. The least inhibitory effect was observed in ETB preparations from cells grown at 42 °C ($T_0$ cells) and assayed at 42 °C and in cells harvested several minutes after temperature shift down. The greatest inhibitory effect on the release of [$^{14}$C]alanine at low concentrations of ampicillin (0.5 μg/ml), 80% inhibition, was observed in experiments where UDP-GlcNAc was omitted from the incubation mixture.

An approximation of the relative activity of each of the enzymatic reactions which caused the release of C-terminal D-alanine was derived from the four types of experiments given in Table III. The value for the penicillin-sensitive DD-carboxypeptidase was computed from the difference between the amounts of free [$^{14}$C]alanine obtained in the absence or presence of 0.5 μg/ml of ampicillin in reactions where UDP-GlcNAc was added (difference between results of experiments I and II). The values for the transpeptidase were calculated from the amounts of [$^{14}$C]alanine released in the presence of 0.5 μg/ml of ampicillin in reactions carried out in the presence or absence of UDP-GlcNAc (difference between results of experiments II and III). The free [$^{14}$C]alanine released in experiment III represents the DD-carboxypeptidase activity which was not inhibited by low concentrations of ampicillin (approximately 20% at 30 °C; see IV-III) and which is most likely a side reaction of the endopeptidase present in the cells (Strominger, 1970). The difference between the carboxypeptidase activity calculated from experiments I and II and IV is perhaps due to the fact that in the absence of added UDP-GlcNAc, no synthesis of peptidoglycan strands occurred in experiment IV and, therefore, there was also no competition with C-terminal

Table III: Enzymatic Activities of Ether-Treated Bacterial Preparations That Cause the Release of C-Terminal [$^{14}$C]Alanine from UDP-MurNAc-D-[$^{14}$C]Ala-Pentapeptide.

| Growth Conditions of Cells[a] | Assay[b] | Treatment of Cells[c] | UDP-GlcNAc Added[b] | Ampicillin (0.5 μg/ml) Added | [$^{14}$C]Ala Released (pmol/mg of Protein) at Reaction Temp. 30 °C | 42 °C | | Reaction Involved in Release of [$^{14}$C]Ala (pmol/mg of Protein) 30 °C | 42 °C |
|---|---|---|---|---|---|---|---|---|---|
| 30 °C (cells A) | I | Ether | + | − | 1010 | 3485 | (I − II) = Carboxypeptidase (ampicillin sensitive) | 545 | 1115 |
| | II | Ether | + | + | 465 | 2370 | | | |
| | III | Ether | − | + | 75 | 950 | (II − III) = Transpeptidase | 390 | 1420 |
| | IV | Ether | − | − | 330 | 1975 | | | |
| | V | Ether + sonication | − | − | 1330 | 3045 | | | |
| | VI | Ether + sonication | − | + | 265 | 730 | | | |
| 42 °C (cells $T_0$) | I | Ether | + | − | 735 | 1390 | (I − II) = Carboxypeptidase (ampicillin sensitive) | 295 | 250 |
| | II | Ether | + | + | 440 | 1140 | | | |
| | III | Ether | − | + | 135 | 775 | (II − III) = Transpeptidase | 305 | 365 |
| | IV | Ether | − | − | 410 | 1120 | | | |
| | V | Ether + sonication | − | − | 2020 | 2970 | | | |
| | VI | Ether + sonication | − | + | 345 | 1040 | | | |
| 30 °C (cells $T_{30}$) | I | Ether | + | − | 975 | 3895 | (I − II) = Carboxypeptidase (ampicillin sensitive) | 465 | 1055 |
| | II | Ether | + | + | 510 | 2840 | | | |
| | III | Ether | − | + | 210 | 835 | (II − III) = Transpeptidase | 300 | 2005 |
| | IV | Ether | − | − | 545 | 1810 | | | |

[a] Ether-treated bacterial preparations were from *E. coli* PAT 84 cells harvested at time of temperature shift up (A), 60 min later, at time of temperature shift down ($T_0$), and at 30 min after temperature shift down ($T_{30}$). [b] Incubation mixtures in a final volume of 200 μl contained Tris buffer (50 mM) (pH 8.3), $NH_4Cl$ (50 mM), $MgCl_2$ (20 mM), ATP (10 mM), 2-mercaptoethanol (0.5 mM), UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide (specific activity 9 cpm/pmol, 160 000 cpm), and UDP-GlcNAc (100 nmol), added where indicated. Two milliliters of sodium dodecyl sulfate (4%) was added after the reaction and the solution heated (100 °C, 15 min). Insoluble material was removed on Millipore filters and the filtrate analyzed for [$^{14}$C]alanine as described. [c] Treatment of intact cells with ether was as described under Materials and Methods. Several samples of the same ether-treated cells were also sonicated in the cold for 1 min and then tested for carboxypeptidase activity, as described above.

[$^{14}$C]alanine released by transpeptidation.

At high concentrations of ampicillin (100 μg/ml) almost complete inhibition of [$^{14}$C]alanine release was observed (Figure 6). The effect of other β-lactam antibiotics on the release of [$^{14}$C]alanine is also shown in Figure 6. Whereas 6-APA and ampicillin displayed an increasing inhibitory effect with increasing concentrations, cephalothin and FL-1060 inhibited [$^{14}$C]alanine release only at high concentrations (>10 μg/ml). No significant effect on the release of [$^{14}$C]alanine was observed in experiments in which unlabeled D-alanine (500 μg/ml) or D-Ala-D-Ala (500 μg/ml) was added to the reaction mixtures.

*Formation of Peptide Cross-Linkages.* The extent of peptide cross-linkage formation in the peptidoglycan synthesized in the absence and presence of ampicillin (0.5 and 100 μg/ml) at various stages of septum formation is shown in Table IV and in Figure 7 (see also Methods and Materials). Chromatographic analysis of lysozyme digests of the labeled peptidoglycan synthesized by the ETB preparations indicated that cells grown at 30 °C had a lower ratio of cross-linked glycopeptide fragments ($C_3 + C_4$; Weidel and Pelzer, 1964) vs. un-cross-linked ($C_5 + C_6$) fragments than that found in cells grown at 42 °C. A marked difference in this ratio was also observed in the lysozyme digests of $Cl_3CCOOH$ or sodium dodecyl sulfate insoluble peptidoglycan (Table IV). Low concentrations of ampicillin (0.5 μg/ml) enhanced the proportion of cross-linked fragments both in the sodium dodecyl sulfate and $Cl_3CCOOH$ insoluble material of all ETB cell preparations (Table IV). High concentrations of ampicillin (100 μg/ml) completely inhibited the formation of cross-linked fragments in the $Cl_3CCOOH$ insoluble peptidoglycan (Figure 7) and since no incorporation of [$^{14}$C]GlcNAc into sodium dodecyl sulfate insoluble material occurred under these conditions, no fragments were obtained from the sodium dodecyl sulfate insoluble material.

Practically no incorporation of radioactivity into the sodium dodecyl sulfate insoluble material was detected in experiments where UDP-MurNAc-D-[$^{14}$C]Ala-pentapeptide, labeled at the C-terminal D-alanine, was added together with unlabled UDP-GlcNAc (Table I). However, in these experiments, considerable incorporation was observed in the $Cl_3CCOOH$ insoluble material, which was enhanced 50% by ampicillin (100 μg/ml) (Table I). Chromatographic analysis of lysozyme digests of such $Cl_3CCOOH$ insoluble material produced in the presence of ampicillin (100 μg/ml) afforded a single labeled fragment which migrated with an $R_{C_6} = 1.12$ (see below). Since this fragment was labeled

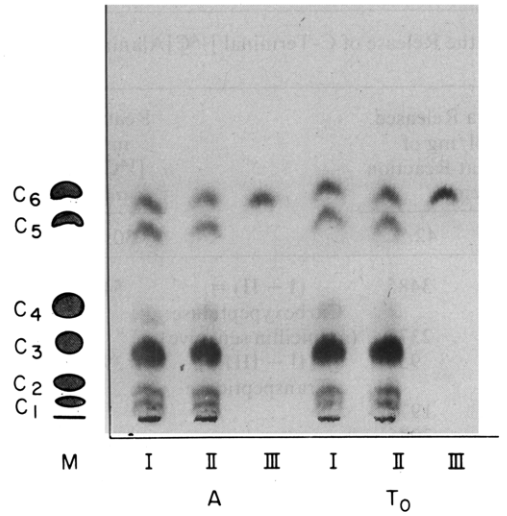


FIGURE 7: Autoradiogram of paper chromatogram of lysozyme digests of [$^{14}$C]GlcNAc labeled peptidoglycan ($Cl_3CCOOH$ insoluble) synthesized by ETB preparations from cells harvested before and after shift back to permissive temperature (A and $T_0$). The chromatogram on Whatman No. 3MM paper was developed for 72 h in 1-butanol–acetic acid–water (4:1:5, upper phase): ETB preparations incubated at 30 °C in the absence of antibiotic (I); incubated in the presence of 0.5 μg/ml of ampicillin (II); and in the presence of 100 μg/ml of ampicillin (III). M-markers of glycopeptide fragments obtained upon lysozyme digestions of *E. coli* cell walls and visualized on the chromatogram after spraying with ninhydrin (Weidel and Pelzer, 1964).

Table IV: Ratio of Cross-Linked ($C_3 + C_4$) to Un-Cross-Linked ($C_5 + C_6$) Glycopeptide Fragments Obtained after Chromatography of Lysozyme Digests.

| ETB Cell Preparation[a] | Extent of Peptide Side-Chain Cross-Linkage: $Cl_3CCOOH$ Insoluble Material[b] + Ampicillin (0.5 μg/ml) | | Na Dodecyl Sulfate Insoluble Material[b] + Ampicillin (0.5 μg/ml) | |
|---|---|---|---|---|
| A | 0.99 | 1.00 | 1.41 | 1.54 |
| $T_0$ | 1.35 | 1.37 | 1.64 | 1.83 |
| $T_{10}$ | 1.28 | 1.38 | 1.63 | 1.77 |
| $T_{20}$ | 1.25 | 1.43 | 1.71 | 1.88 |
| $T_{30}$ | 1.30 | 1.41 | 1.67 | 1.78 |
| $T_{45}$ | 1.26 | 1.24 | 1.65 | 1.74 |

[a] Ether-treated bacterial preparations were made from *E. coli* PAT 84 cells harvested at time of temperature shift up (A), at time of temperature shift down ($T_0$) (60 min later), and at designated times later (10 min = $T_{10}$, 20 min = $T_{20}$, 30 min = $T_{30}$). [b] Incubation mixtures in a final volume of 200 μl contained Tris buffer (50 mM) (pH 8.3), $NH_4Cl$ (50 mM), $MgCl_2$ (20 mM), ATP (10 mM), 2-mercaptoethanol (0.5 mM), UDP-MurNAc-pentapeptide (100 nmol), UDP-[$^{14}$C]GlcNAc (sp act. 40 cpm/pmol, 180 000 cpm), and ETB (~1 mg of protein). Incubations were done at 30 °C for 60 min after which 2 ml of $Cl_3CCOOH$ (10%) or 2 ml of sodium dodecyl sulfate (4%) was added. Insoluble material was filtered on Millipore filters (0.5 μm). The digestions with lysozyme were done as described under Materials and Methods.

exclusively in the C-terminal D-alanine moiety, it is most likely the disaccharide–pentapeptide GlcNAc-β(1→4)MurNAc-L-Ala-D-*i*-Glu-*meso*-DAP-D-Ala-D-[$^{14}$C]Ala. Lysozyme digests of the $Cl_3CCOOH$ insoluble material formed in the absence of ampicillin afforded two labeled glycopeptide fragments; the first (ca. 40% of the radioactivity) migrated identically as the $C_3$ fragment and the second fragment as the above disaccharide–pentapeptide.

Chromatographic analysis of lysozyme digests of the high molecular peptidoglycan obtained after heating of the [$^{14}$C]GlcNAc labeled $Cl_3CCOOH$ insoluble material with sodium dodecyl sulfate (see above) showed that the cross-linked fragments $C_3 + C_4$ comprised only 22% of the total counts. The most abundant glycopeptide fragment in the [$^{14}$C]GlcNAc labeled digest was $C_6$ (60%). In addition, the digests contained the disaccharide–pentapeptide (10% of total counts) which had a migration slightly faster than $C_6$ ($R_{C_6}$ 1.12) (see above).

## Discussion

The synthesis of peptidoglycan carried out by the ether-treated *E. coli* PAT 84 cells from externally added nucleotide precursors resembles in many respects the synthesis which occurs in intact cells (Figure 1b) or in synchronous cultures of *E. coli* (Hoffmann et al., 1972). Labeled peptidoglycan strands synthesized by the ETB preparations became covalently bound to the preexisting peptidoglycan of the cell wall and accounted for approximately 15–25% of the preexisting cell wall peptidoglycan; its synthesis fluctuated during formation of the septum, with a rise 10 to 15 min before cell division (Figure 2). Thus, upon the addition of a labeled nucleotide precursor to preparations of ether-treated cells from bacteria synchronized for the formation of a new septum by a shift down to permissive growth temperature, the peptidoglycan that was incorporated into the cell wall represented the cell's capability of incorporation at the instant of conversion to a permeable state.

In all the experiments with ether-treated bacteria there was a marked difference in the amount of newly synthesized peptidoglycan found in the $Cl_3CCOOH$ insoluble and the sodium dodecyl sulfate insoluble material of cells grown at 30 °C (cells A) as compared to those grown at 42 °C ($T_0$) (Figures 2 and 3). The difference can be explained by taking into consideration that in the $Cl_3CCOOH$ insoluble material all the newly synthesized macromolecular peptidoglycan was present (Figure 8) (Izaki et al., 1968), whereas in the sodium dodecyl sulfate insoluble material only the peptidoglycan that became covalently linked to the preexisting peptidoglycan of the cell wall sacculus remained (Leutgeb and Schwarz, 1967; Mirelman et al., 1972).

In the ether-treated cells, the covalent attachment of newly synthesized peptidoglycan to the preformed cell wall, the release of C-terminal D-alanine, and the formation of peptide cross-linkages were all completely inhibited by ampicillin (90% at 50 μg/ml). These findings show that in *E. coli*, as in gram-positive organisms (Mirelman et al., 1972; Mirelman and Sharon, 1972; Ward and Perkins, 1974; Schrader and Fan, 1974), the penicillin-sensitive transpeptidation is the main reaction by which newly synthesized peptidoglycan strands are attached to preexisting cell wall peptidoglycan. In contrast to our findings with cocci (Mirelman et al., 1972; Mirelman and Sharon, 1972), however, only a small fraction, if any, of the preexisting peptidoglycan strands of *E. coli* became elongated by the penicillin-insensitive transglycosylation. Similar findings to those observed by us with *E. coli* have been reported in *Bacillus licheniformis* (Ward and Perkins, 1974).

Treatment of the $Cl_3CCOOH$ insoluble material with hot sodium dodecyl sulfate yielded, in addition to intact cell sacculi, a labeled, high molecular weight soluble peptidoglycan. This product was mostly un-cross-linked at its peptide side chains, since lysozyme digestion yielded mainly the

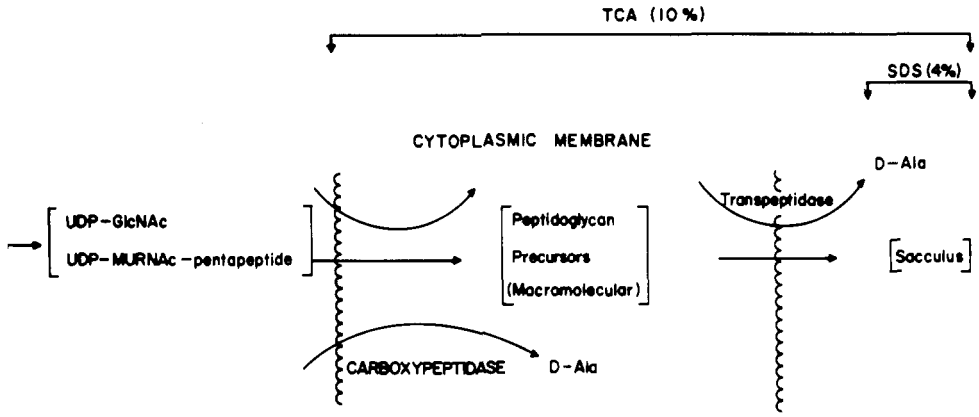


FIGURE 8: Schematic model of principal reactions which participate in the synthesis and attachment of newly synthesized peptidoglycan strands of preexisting ones in the *E. coli* cell wall. The sodium dodecyl sulfate insoluble material accounts only for newly synthesized peptidoglycan bound covalently to the preexisting cell wall. The macromolecular peptidoglycan intermediates remain in the cell upon treatment with $Cl_3CCOOH$, and are predominantly un-cross-linked at its peptide side chains.

disaccharide-tetrapeptide $C_6$ (Weidel and Pelzer, 1964). This polymer may represent peptidoglycan intermediates and may be similar to the "gap material" observed at the sites of division in *E. coli* (Burdett and Murray, 1974a), part of which may eventually become bound by transpeptidation to the sacculus (Mirelman et al., 1974b). Interestingly, very little sodium dodecyl sulfate soluble peptidoglycan was obtained both in ETB preparations and in intact cells grown at restrictive temperature (Figures 1 and 2) and, similarly, very little "gap material" was detected by electron microscopy in these same filaments (Burdett and Murray, 1974a).

The present study has also shown that the activity of the ampicillin-sensitive DD-carboxypeptidase was lower than that of the transpeptidase in cells of *E. coli* PAT 84 grown at the restrictive temperature (Table III). A possible reason for the inability of this type of mutant to form septa at the restrictive temperatures is thus suggested: the extent of peptide side-chain cross-linkage in the cell wall during biosynthesis could be regulated by controlling the number of pentapeptide side chains in the peptidoglycan precursors which serve as donors for the transpeptidation reaction. The increase in the ratio of transpeptidase to carboxypeptidase activity observed in cells grown at the restrictive temperature (1.4, Table III) as compared to that in dividing cells (0.7, Table III) must result in a net increase in the amount of pentapeptide side-chain moieties in the newly synthesized precursor strands of the filament cells (Figure 8). This local increase in the amount of substrate donor for the transpeptidase may account for the increase in incorporation of newly synthesized peptidoglycan into the preexisting cell walls and for the higher extent of peptide cross-linkages observed in our experiments.

Pulse labeling experiments with intact filaments grown at restrictive temperature also showed a marked (~40%) increase in the extent of cross-linkage as compared to that found in cells grown and pulsed at permissive temperature (U. Schwarz and D. Mirelman, unpublished observations). Indications that filamentous *E. coli* cells may have more cross-linked peptidoglycan were also obtained by Kamiryo and Strominger (1974).

The lower activity of the DD-carboxypeptidase in the ether-treated filaments is apparently not the result of temperature sensitivity of the enzyme since mechanical disruption of the filaments by sonication causes the appearance of carboxypeptidase activity (Table III). This indicates that the enzyme is present and can be released but in the "intact" filament it is partially inactive. In *E. coli,* at least three enzymatic activities have been identified which are inhibited by penicillins: (a) the DD-alanine carboxypeptidases which hydrolyze C-terminal D-Ala-D-Ala peptide bonds; (b) the endopeptidase activity (cross-link splitting enzyme) which is thought to be another manifestation of DD-carboxypeptidase; and (c) the transpeptidases which catalyze the cross-linkage reaction. Several in vitro studies with membrane preparations and with isolated enzymes have shown that the inhibition of most of the DD-carboxypeptidase activity occurred at concentrations of penicillin (0.5 $\mu$g/ml) far below the growth inhibitory concentration for *E. coli,* whereas the inhibition of transpeptidase required 100-fold more antibiotic (Strominger, 1970; Nguyen-Disteche et al., 1974; Strominger et al., 1974).

Our results show that low concentrations of ampicillin, which inhibit primarily the DD-carboxypeptidase activity (~80% at 0.5 $\mu$g/ml; Table III), enhance the rate by which peptidoglycan is incorporated into the sacculus (Figure 3). The explanation for this increase in incorporation in essence is identical with the one given for the effect of temperature (see above). Due to the almost exclusive inactivation of the DD-carboxypeptidase (in this case by low concentrations of ampicillin), more of the newly synthesized peptidoglycan strands become available for transpeptidation. Furthermore, an increase in incorporation was also observed upon the addition of di- or tripeptides of D-alanine which presumably compete for the carboxypeptidase with the natural peptidoglycan pentapeptide (Nguyen-Disteche et al., 1974; Pollock et al., 1974).

The effects of other $\beta$-lactam antibiotics on our system are not completely clear and more work is required in order to really understand their mode of action (Figures 4 and 5). 6-Aminopenicillanic acid seemed to function similarly to ampicillin (Nguyen-Disteche et al., 1974; Strominger et al., 1974) but with a lower inhibitory efficiency. Cephalothin did not enhance incorporation of newly synthesized peptidoglycan into the wall at low concentrations and its inhibitory effect on both incorporation and C-terminal D-Ala release occurred only at high concentrations, suggesting that it primarily affects transpeptidation (Nguyen-Disteche et al., 1974). As previously found by Park and Burman (1973), amidinopenicillin FL-1060 did not affect peptide cross-linkage formation. Furthermore, a noticeable inhibitory effect on the release of D-alanine (Figure 6) was only detected at

concentrations far above those inhibiting growth. Unfortunately, no notable differences in the nature of the newly synthesized peptidoglycan were detected with our system in the presence of FL-1060.

From the fluctuation of peptidoglycan biosynthesis in both intact cells and in the ETB (Figures 1 and 2), one can assume that, during the cell cycle, fluctuations also may exist in the activities of enzymes which degrade peptidoglycan. Any interference with the delicate balance of biosynthesis and hydrolysis either by β-lactam antibiotics or by the effect of growth at restrictive temperature (as in *E. coli* PAT 84) can cause a disarray in the enzymic sequence which may manifest itself in one of the commonly observed morphological alterations of the cell (Greenwood and O'Grady, 1973a,b). Our findings suggest that the DD-carboxypeptidase has a role in regulating not only the ultimate extent of peptide cross-linkage in the wall (that function could be done by the endopeptidase) but for actually regulating the amount of peptidoglycan that is incorporated at any time during the cell division cycle into the growing cell wall.

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