JOURNAL OF BACTERIOLOGY, June 1976, p. 1250-1260
Vol. 126, No. 3

Printed in U.S.A.

Activity of Three Murein Hydrolases During the Cell Division Cycle of *Escherichia coli* K-12 as Measured in Toluene-Treated Cells

BARBARA D. BECK[1] AND JAMES T. PARK*

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received for publication 22 March 1976

The specific activities of three murein hydrolases, carboxypeptidase I, carboxypeptidase II, and amidase were studied with respect to cell division in toluene-treated cells of *Escherichia coli* K-12. Carboxypeptidase I and amidase activities were constant throughout the division cycle in cells of D11/*lac*$^+$*pro*$^+$. Detectable carboxypeptidase II activity varied and was highest at the time of division by a factor of three. Carboxypeptidase II specific activity was also correlated with cell division in BUG 6, a temperature-sensitive division mutant (J. N. Reeve, D. J. Groves, and D. J. Clark, 1970). Fifteen minutes after shifting BUG 6 from 42 C (nondividing conditions) to 32 C (dividing conditions), there was a rapid resumption of cell division, accompanied by a 10-fold increase in the specific activity of carboxypeptidase II. These results demonstrate a correlation between detectable carboxypeptidase II activity and cell division as reflected by activity in toluene-treated cells. The subcellular location of carboxypeptidase II, a soluble enzyme, was found to be periplasmic since it was released by tris(hydroxymethyl)-aminomethane-ethylenediaminetetraacetate treatment and osmotic shock, two methods known to release periplasmic enzymes.

The cell envelope of gram-negative rods, such as *Escherichia coli*, consists of an inner cytoplasmic membrane, a rigid murein layer (from the Latin word "murus" meaning wall), and an outer membrane (16, 43, 52, 63). Between the cytoplasmic membrane and the outer membrane is a compartment termed the periplasmic space (37) that contains soluble proteins such as hydrolytic enzymes and amino acid- and sugar-binding proteins (20, 21, 53). Since this work is a study of certain enzymes that degrade the murein layer, it is appropriate to describe the biochemistry of this layer. *E. coli* murein consists of chains of alternating sugar residues of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) (32, 36, 45, 46, 63). The MurNAc residue has a peptide side chain consisting of L-alanyl-D-glutamyl-γ-*meso*-diaminopimelyl-D-alanine (L-Ala-D-Glu-*m*-Dpm-D-Ala). Some side chains lack the fourth amino acid residue, D-alanine. In addition there is a fifth amino acid, a carboxyl terminal D-alanine, present in the murein precursor, which is lost during the cross-linking and polymerization process. About half of the peptide side chains are cross-linked between D-alanine and the diaminopimelic acid (46, 57). Covalently attached to approximately every tenth diaminopimelic acid residue is a lipoprotein (6-8) of molecular weight 7,500 (7, 25) which extends into the outer membrane. The basic structure of the *E. coli* murein is shown in Fig. 1.

During cell growth, the rigid murein layer expands longitudinally. At the time of cell division in *E. coli*, the murein layer forms a septum, an invagination perpendicular to the surface of the cell (9, 10). Since the murein layer is a dynamic structure, it seems likely that localized degradation of murein by murein hydrolases may play a role in its growth. (In this paper the term "murein hydrolase" refers to an enzyme that specifically degrades the murein or its precursors, and does not necessarily result in solubilization of murein). A murein hydrolase(s) may be specifically involved in septum formation. The formation of a central bulge in *E. coli* cells grown in the presence of a low concentration of penicillin (54) suggests localized weakening of the shape-maintaining murein layer in the septal region owing to the action of a murein hydrolase(s). In addition, the ability of *E. coli* cells to form penicillin-induced bulges is greatest 20 min before cell separation (9, 10) and cells of *E. coli* are also most readily lysed by penicillin at this time (23). Since this is

[1] Present address: Department of Microbiology, University of Massachusetts Medical School, Worcester, Mass. 01605.

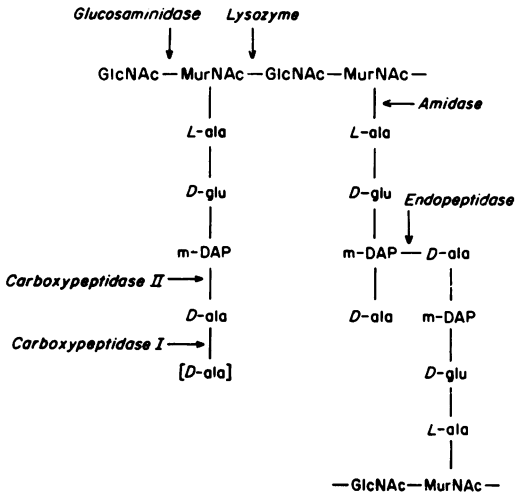

FIG. 1. *Murein hydrolases of Escherichia coli.*

the time of initiation of septum formation (10) a temporal correlation between the activity of a murein hydrolase(s) and septation may exist. To understand the regulation of septum formation, we therefore decided to study the activity of three of the murein hydrolases of *E. coli* as a function of cell size. Such an approach might reveal which, if any, of these murein hydrolases play a role in septum formation.

There are several enzymes that degrade the murein at specific sites (Fig. 1). Three enzymes were chosen for this study. For each enzyme, a radioactive substrate was prepared permitting its specific assay in crude preparations.

(i) Carboxypeptidase I (D-Ala-D-Ala carboxypeptidase; EC 3.4.2), a membrane-bound enzyme (2, 27, 48), cleaves the terminal D-alanine residue from the murein precursor, uridine diphosphate *N*-acetyl-muramyl-L-Ala-D-Glu-*m*-Dpm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide). *E. coli* K-12 has two carboxypeptidase I-containing fractions (41) which differ in their ampicillin sensitivity (42) and their ability to carry out in vitro transpeptidation (48).

(ii) *N*-acetylmuramyl-L-alanyl amidase (amidase; EC 3.5.1), a membrane-bound enzyme, (59) cleaves the bond between *N*-acetylmuramic acid and L-alanine. Amidase can act on polymerized murein (55) and murein fragments (59), but not on the nucleotide-bound precursor (unpublished data). Amidase was originally reported to be a membrane-bound enzyme (59); however, van Heijenoort et al. now report that the majority of amidase activity is soluble and that the lack of demonstrable activity in the 43,000 × *g* supernatant may have been due to high-molecular-weight interfering material (60).

(iii) Carboxypeptidase II, (Dpm-D-Ala carboxypeptidase; EC 3.4.2), a soluble enzyme, cleaves the bond between diaminopimelic acid and D-alanine in UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala (UDP-Mur-NAc-tetrapeptide) (28). In addition, there is an enzyme that cleaves the same bond in polymerized wall (62). It is not known whether the same enzyme releases D-alanine from UDP-MurNAc-tetrapeptide.

In this paper we report the activities of carboxypeptidase I, carboxypeptidase II, and amidase with respect to cell division. In addition, the location of these enzymes in the cell was determined. A preliminary report of these findings has been presented (B. D. Beck and J. T. Park, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1974, G38, p. 26).

MATERIALS AND METHODS

Strains of *E. coli* K-12. D11 (F^-, *strA, proB, his, trp, lac*, λ+, *tsx, thi*) was obtained from H. Boman (4). 3300 Hfr (Hfr, *thi*, rel, *lacI*, λ−) was obtained from M. H. Malamy (3). D11-1 (D11 *lacI* pro^+) was made by mating D11 with 3300 Hfr and selecting for pro^+strA recombinants on E plates with glucose, thiamine, histidine, tryptophan, and streptomycin. Lac^+ recombinants were selected from these on E plates with lactose, thiamine, histidine, and tryptophan. BUG 6 (F^-, *thi, str, gal, mtl*) (51) was obtained from E. W. Green.

Growth conditions. Cells were grown either in L broth (31) or in E medium (61) supplemented with thiamine (1 μg/ml), required amino acids (50 μg/ml), and either 0.5% lactose or 0.5% glucose as a carbon source. Absorbance at 585 nm (A_{585}) was measured with a Zeiss PMQ II spectrophotometer. For cell number determinations, cells were diluted into 0.4% formaldehyde in E medium which had been previously filtered through a 0.2-μm membrane filter (Millipore Corp.) and counted using an ElectroZone celloscope (Particle Data, Inc.) equipped with a 24-μm orifice tube.

Preparation of substrates for murein hydrolase assays. Both radioactive and nonradioactive substrates were prepared in all cases.

Preparation of substrate for assay of amidase activity. *N*-acetyl-muramyl-L-Ala-D-Glu-*m*-Dpm was the substrate for the assay of amidase activity, and was prepared in nonradioative form from UDP-MurNAc-tripeptide. UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm was prepared by cycloserine treatment of cells of *Bacillus subtilis* W23. Thirty liters of cells was grown at 37 C in a medium composed of 2% peptone (Amber Laboratories, Inc., Milwaukee, Wis.), 0.15% yeast extract (Difco, Laboratories, Detroit, Mich.), and 0.45% K_2HPO_4, adjusted to pH 7.2 with HCl. Cells were harvested, yielding a 70-g cell paste which was suspended in 300 ml of the final volume of the following three amino acid mixture (3AA): 0.5% glucose, 0.03% L-glutamic acid, 0.03% L-aspartic acid, 0.018% L-alanine, 0.015% D-cycloserine (Nutritional Biochemicals, Cleveland, Ohio), and 0.8% K_2HPO_4. Four-tenths milliliter of 10 N NaOH was

added per 100 ml and the pH was adjusted to 7.1. Fifty milliliters of this cell suspension was added to 950 ml of prewarmed 3AA mixture in a 2-liter flask and shaken vigorously at 37 C for 90 min. Cells from six such flasks were harvested in 1-liter bottles in an MSE Mistral 6L centrifuge equipped with swinging buckets. The cell pellets were resuspended in 460 ml of cold water, and 40 ml of cold 100% trichloroacetic acid was added. After approximately 20 min, the trichloroacetic acid-soluble material was recovered by centrifugation. Trichloroacetic acid was removed from the extract by three ether extractions. The water phase was neutralized with NH_4OH and concentrated by evaporation under reduced pressure to approximately 15 ml. The extract was applied to a Sephadex G25 (medium) column (2.7 by 94 cm) and eluted with water. The main ultraviolet (UV)-absorbing fractions were pooled, concentrated to <5 ml, and streaked on Whatman 3MM paper (<1 μmol/cm). The chromatogram was run in ethanol: 1 N ammonium acetate, pH 7.5, (5:2) for 48 h. The area containing UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm, the first of two UV-absorbing bands, located 7 to 8 cm from the origin, was cut out and eluted with water (14). The yield was about 200 μmol of nucleotide as determined by the bound *N*-acetylhexosamine test (17).

Preparation of labeled UDP-MurNAc-tripeptide involved the preliminary isolation of UDP-MurNAc-L-Ala-D-Glu (UDP-MurNAc-dipeptide) from lysine-starved *Staphylococcus aureus* H (26). UDP-MurNAc-dipeptide was then enzymatically added to [^{14}C]Dpm to yield UDP-MurNAc-tripeptide. For this reaction, a supernatant from sonically disrupted *E. coli* D11 cells was used as a source of Dpm-adding enzyme. The enzyme was prepared by growing D11 in two liters of L broth at 37 C to midlog phase (A_{585} = 0.7 to 1.0), and harvesting and washing the cells twice with cold 0.2 M KPO_4 buffer, pH 7.2. Four milliliters of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, was used to suspend the cell pellet in a final volume of 5 to 6 ml. The sample was treated with 10-s bursts using a Bronwill Biosonik III or an MSE ultrasonicoscillator until the absorbance decreased by at least 80% and particulate material was removed by centrifugation at 80,000 × *g* for 60 min in a Beckman L3-50 ultracentrifuge. This supernatant fluid contained approximately 100 mg of protein/ml and was used as the source of enzyme. The reaction mixture for preparation of Dpm-labeled MurNAc-tripeptide contained 3 ml of crude enzyme in 0.05 M Tris-hydrochloride, pH 8.0, 224 μmol of adenosine 5'-triphosphate (Sigma Chemical Co., St. Louis, Mo.), neutralized with 5 N NaOH, 0.4 mmol of Tris-hydrochloride, pH 8.0, 0.4 mmol of $MgCl_2$, 0.4 mmol of KCl, 80 μCi of [^{14}C]Dpm (7.7 μCi per μmol) and 5 μmol of UDP-MurNAc-L-Ala-D-Glu in a final volume of 10 ml. The mixture was incubated at 37 C for 120 min, boiled for 3 min, and centrifuged for 15 min at 12,000 × *g*. The supernatant fluid was applied to a Sephadex G25, fine, column (2.7 by 83 cm) (Pharmacia Fine Chemicals, Inc. Piscataway, N.J.) and eluted with water. Fractions were monitored for UV absorbance and radioactivity. The nucleotide-containing fractions were concentrated by evaporation under reduced pressure and chromatographed on Whatman 3MM paper in isobutyric acid: 1 N NH_4OH (5:3) for 72 h (27). The chromatogram was air dried without heat, and the nucleotide, located by autoradiography, was eluted with water. Overall, 1.0 μmol (14 × 10^6 counts/min) of nucleotide was recovered.

MurNAc-L-Ala-D-Glu-*m*-Dpm and MurNAc-L-Ala-D-Glu-*m*-[^{14}C]Dpm were obtained by hydrolysis of UDP-MurNAc-tripeptide in 0.1 N HCl for 8 min at 100 C and subsequent purification by electrophoresis on Whatman 3MM paper in 0.1 N formic acid for 90 min at 25 V/cm.

Preparation of substrate for assay of carboxypeptidase I activity. For measurement of D-Ala-D-Ala carboxypeptidase activity, the nonradioactive substrate UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala-D-Ala was prepared by enzymatic addition of D-Ala-D-Ala (Cyclo Chemical Corp.) to UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm. The conditions were as described above for *m*-Dpm addition except that the mixture contained 53 μmol of UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm and 75 μmol of D-Ala-D-Ala. The UDP-MurNAc-pentapeptide was purified by Sephadex G25 column chromatography followed by chromatography on Whatman 3MM paper in isobutyric acid: 1 N NH_4OH (5:3) for 3 days. The yield was 15 μmol.

Radioactive substrate, UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala-D-[^{14}C]Ala (UDP-MurNAc-pentapeptide) was donated by R. K. Sinha. It was prepared by the enzymatic addition of D-Ala-D-[^{14}C]Ala to UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm. D-Ala-D-[^{14}C]Ala was synthesized by dissolving 1.9 mg of D-[1-^{14}C]Ala, 54.9 μCi/μM (ICN Pharmaceuticals, Inc.), and 2.7 mg of D-alanine-*N*-carboxyanhydride (Fox Chemical Co.) in 0.5 ml of cold 0.5 M sodium borate buffer, pH 10.2, and shaking for a few minutes. The mixture was chromatographed overnight on Whatman 3MM paper in *n*-butanol-acetic acid-water (3:1:1). The chromatogram was dried, and the D-Ala-D-[^{14}C]Ala band was located autoradiographically and eluted, to yield approximately 16 μmol. The D-Ala-D-Ala adding enzyme was prepared from a 2.5 g of paste from cells of *Bacillus megaterium* KM grown in 0.5% yeast extract (Difco), 0.5% phytone (Difco), and 0.3% K_2HPO_4, pH 7.3, and 0.2% glucose added after autoclaving. The paste was frozen, thawed, resuspended in 5.0 ml of water, and the cells were broken by shaking for 6 min with 7.5 g of plastic beads in a Braun homogenizer which was cooled under CO_2. The suspension was centrifuged at 20,200 × *g* for 20 min and the supernatant was used as a source of enzyme. The reaction mixture contained 1.2 μmol of adenosine 5'-triphosphate, neutralized with NaOH, 0.45 mmol of Tris-hydrochloride, pH 8.3, 0.45 mmol of $MgCl_2$, 8.3 μmol of UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm, 8 μmol of D-Ala-D-[^{14}C]Ala, 0.5 mg of penicillin G (Sigma), and 1.0 ml of enzyme in a final volume of 2.7 ml. The nucleotide was purified as described and yielded 1.9 μmol (129 × 10^6 counts/min).

Preparation of substrate for assay of carboxypeptidase II activity. To assay Dpm-D-Ala carboxypep-

tidase activity, the substrate UDP-MurNAc-L-ala-D-glu-*m*-Dpm-D-[^{14}C]Ala (UDP-MurNAc-tetrapeptide) was prepared from UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-[^{14}C]Ala-D-[^{14}C]Ala.

UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-[^{14}C]Ala-D-[^{14}C]Ala was prepared using the same conditions as for Dpm addition except that the mixture contained 3 μmol of UDP-MurNAc-L-ala-D-Glu-*m*-Dpm, 250 μCi of D-[1-^{14}C]alanine (ICN Pharmaceuticals, Inc.), 12.5 μCi per μmol, and 1 mg of D-ampicillin (Doktacillin; Astra, Sweden). The labeled UDP-MurNAc-pentapeptide was purified in the same way as the unlabeled UDP-MurNAc-pentapeptide except that the nucleotide was located by autoradiography. The yield was 1.5 μmol (80 × 10^6 counts/min).

UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala and UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-[^{14}C]Ala were then prepared by carboxypeptidase I treatment of the UDP-MurNAc-pentapeptide. The source of the enzyme was either enzyme purified from *B. subtilis*, donated by J. Umbreit and P. Blumberg, assayed as described (29) or a membrane preparation of D11. The membranes were prepared from spheroplasts (44) from 4 liters of L broth grown cells of D11. The spheroplasts were lysed by dilution in 0.02 M Tris-hydrochloride, pH 8.0, washed three times with 25 ml of Tris buffer at 80,000 × *g*, and resuspended in a final volume of 2 ml of Tris buffer. The membranes contained carboxypeptidase I activity and no carboxypeptidase II activity. A typical reaction mixture contained 1 μmol (50 × 10^6 counts/min) UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-[^{14}C]Ala-D-[^{14}C]Ala, 0.5 mmol of Tris, pH 9.2, 0.05 mmol of $MgCl_2$, and 1.5 ml of membrane suspension in a final volume of 4.6 ml, and was incubated at 37 C for 7 h by which time half of the labeled alanine was released from the nucleotide. A sample was hydrolyzed in 0.1 N HCl for 10 min at 100 C and chromatographed overnight on Whatman 3MM paper in phenol: 0.2% aqueous 8-hydroxyquinoline (4:1) (2) to verify that the mixture contained only UDP-MurNAc-tetrapeptide and no UDP-MurNAc-pentapeptide. UDP-MurNAc-tetrapeptide was purified by the same method as used for UDP-MurNAc-pentapeptide purification.

Enzyme assays. Carboxypeptidase I was assayed according to Izaki and Strominger (28). The reaction mixture contained 0.5 nmol of (20 × 10^3 counts/min) UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala-D-[^{14}C]-Ala, 2 μmol of Tris-hydrochloride, pH 9.2, 0.2 μmol of $MgCl_2$, and 15 μg of protein in a final volume of 20 μl. The mixture was incubated for 15 to 60 min at 37 C, boiled for 2 min to terminate the reaction, and centrifuged to remove insoluble material. Alanine and unhydrolyzed substrate were separated on a column that contained 0.2 ml of Dowex AG1-X2 (Bio-Rad, Laboratories, Richmond, Calif.), 200 to 400 mesh, chloride form. Alanine was eluted with 3 ml of water and the nucleotide subsequently eluted with 3 ml of 1 N HCl. Before reuse the column was washed with water until the eluate was neutral. Samples were dried at 100 C, taken up in 0.1 ml of water, and the radioactivity was determined in 3 ml of cellosolve scintillation fluid (18) in either a Beckman LS 235 or a Nuclear-Chicago Mark I liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). One unit of carboxypeptidase I is that amount of enzyme that will release 1 nmol of D-[^{14}C]alanine from UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala-D-[^{14}C]Ala in 15 min at 37 C.

Carboxypeptidase II was assayed according to Izaki and Strominger (28). The reaction mixture contained 1.8 nmol of (10 × 10^3 counts/min) UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-[^{14}C]Ala and 2 μmol of Tris-hydrochloride HCl, pH 8.0, in a final volume of 20 μl. The amount of enzyme varied according to the method of preparation and contained 10 to 150 μg of protein. The mixture was incubated for 5 to 60 min at 37 C and the reaction was terminated by boiling. Radioactive product (alanine) and unhydrolyzed substrate were separated as described above. One unit of carboxypeptidase II is that amount of enzyme that will liberate 1 nmol of D-[^{14}C]alanine from UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-[^{14}C]Ala in 15 min at 37 C.

Amidase was assayed according to Van Heijenoort and Van Heijenoort (59). The reaction mixture contained 15 nmol (7 × 10^3 counts/min) of MurNAc-L-Ala-D-Glu-*m*-[^{14}C]Dpm, 2 μmol of Tris-hydrochloride, pH 8.0, and 15 μl of toluene-treated cells containing 100 to 500 μg of protein. Product (tripeptide) and substrate were separated electrophoretically (59), and the radioactivity was localized by strip scanning with a Nuclear-Chicago Actigraph III. The radioactive spots were cut out and the radioactivity was determined in toluene scintillation fluid composed of 15.2 g of 2,5-diphenyloxazole (Nuclear-Chicago) and 380 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (Packard Instrument Co., Inc., Downers Grove, Ill.) dissolved in 3.8 liters of toluene. One unit of amidase is that amount of enzyme that will cleave 1 nmol of tripeptide from MurNAc-L-Ala-D-Glu-*m*-[^{14}C]Dpm in 15 min at 37 C.

For an enzyme assay to be valid, enzyme activity must be linear with respect to both time and enzyme concentration. Carboxypeptidase II activity was linear with respect to both time and enzyme concentration in toluene-treated cells of D11 grown at 37 C in E medium plus glucose (Fig. 2). Similar linearity was found for carboxypeptidase I and amidase.

Penicillinase was assayed by the automated method of Lindström and Nordström (34). The reaction mixture contained from 2 to 6 mg of protein in a final volume of 0.5 ml and was incubated for 60 min at 22 C. One unit of enzyme is the amount necessary to produce a reduction in absorbance of 0.01 in 60 min at 22 C.

β-galactosidase was assayed by following the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (Sigma) at 420 nm (47), using a Gilford 240 spectrophotometer equipped with a model 2453 recorder. Protein (2 to 10 μg) from crude cell extract was assayed and the reaction was followed for 4 min at 22 C. One unit of enzyme is that amount of enzyme that will produce an increase in absorbance of 0.01 in 1 min at 22 C.

Specific activity. Specific activity is expressed as total enzyme units per milligram of protein. Protein was determined by the Lowry method (35).

Total units. Total units are defined as the number

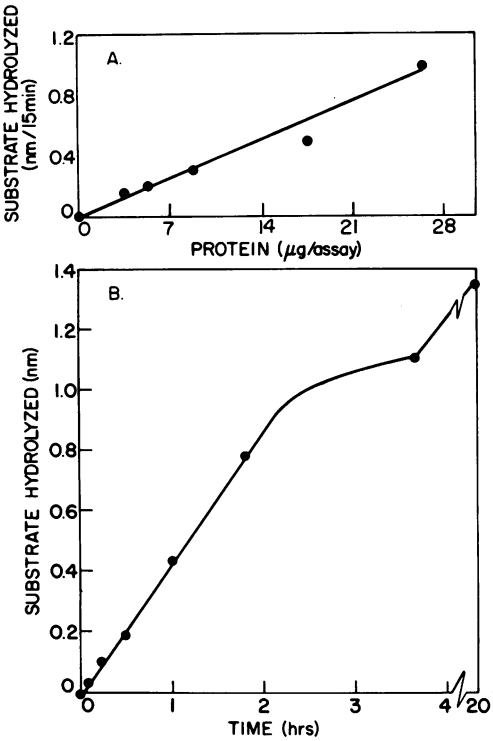

FIG. 2. *Activity of carboxypeptidase II with respect to enzyme concentration and time. (A) D11-1 was grown at 37 C in E medium plus glucose to A_{585} = 0.5. Cells were harvested and toluene treated. Carboxypeptidase II activity was assayed at different enzyme concentrations. (B) The source of enzyme was the same as above. Carboxypeptidase II activity was assayed for different times of incubation. Each assay contained 8.8 μg of protein.*

of enzyme units in cells from 100 ml of culture.

Methods of enzyme preparation. (i) The standard method of toluenization was as follows. Cells were grown in E medium with lactose or glucose as a carbon source, harvested in midlog phase (A_{585} = 0.5), and washed twice with cold E medium. Cells from 100 ml of culture were resuspended in 1 ml of 0.02 M Tris-hydrochloride, pH 8.0, and frozen. For use cells were thawed, and 20 μg of deoxyribonuclease I (Worthington) per ml and 3% toluene were added. Cells were mixed on a Vortex mixer for 10 s and kept at 4 C for 10 to 60 min. Immediately before assay, cells were mixed again.

(ii) Sonically disrupted cells were prepared from cells harvested and washed as described above and subjected to sonic oscillation at 4 C in 10-s bursts with a Bronwill Biosonik III or an MSE ultrasonicator until the absorbance was reduced by 80%.

(iii) Polymyxin B (Sigma) treatment was according to Cerny and Teuber (12).

(iv) The osmotic shock method was that of Neu and Heppel (39).

(v) Tris-ethylenediaminetetraacetate (Tris-EDTA) treatment was as described by Levy and Leive (33) except that 0.4 ml 0.02 M EDTA in 0.1 Tris-hydrochloride was added per 1.0 ml of cell suspension in Tris. The cells were incubated for 6 min at 37 C.

(vi) Lysozyme-EDTA treatment was that of Osborn et al. (44).

Large-scale separation of cells on the basis of size by centrifugation through a sucrose gradient. Cells of D11-1 grown in 5 liters of E medium plus lactose to A_{585} = 0.5 were harvested, washed twice with cold E medium, and suspended in 30 ml of 3% sucrose in E medium. Cells were then applied to the inner edge of a 1.3-liter 5 to 15% sucrose in E medium gradient in an MSE zonal A rotor. The rotor was rotating at 600 rpm in an MSE Mistral 6L centrifuge chilled to 4 C. The cells were overlaid with 100 ml of 1% sucrose in E medium and centrifuged for about 40 min at 2,500 rpm. The speed was lowered to 600 rpm and 30% sucrose was pumped in at the outer edge at the rate of 30 ml/min with a Sigmamotor pump. Thirteen-milliliter fractions were collected with a Gilson Escargot fraction collector. Samples were diluted 1:10 in E medium for absorbancies. Additional samples that contained 0.4% formaldehyde were used for subsequent cell counting. The cells from every two fractions were pooled, washed twice with 0.02 M KPO_4, pH 7.0, resuspended in 0.15 ml of 0.02 M Tris-hydrochloride, pH 8.0, and frozen. Before assay samples were treated with toluene as described above.

RESULTS

Subcellular location of murein hydrolases. The results in Table 1 show that 80% of both carboxypeptidase I and amidase activity was found associated with the 80,000 × *g* membrane pellet of D11-1 indicating that these enzymes were membrane bound. In contrast none of the carboxypeptidase II activity was found in the pellet. The finding of the amidase as primarily membrane bound is in contrast to the work of Van Heijenoort et al. (60) who found that the majority of the amidase was periplasmic.

Carboxypeptidase II was released from L broth-grown cells of D11-1 by some of the stand-

TABLE 1. *Subcellular location of murein hydrolases of D11-1*[a]

Subcellular location	Carboxypeptidase I	Total units of Carboxypeptidase II	Amidase
Supernatant	5 (22%)	101 (100%)	34 (20%)
Membrane	18	0	136

[a] D11-1 was grown in L broth at 37 C to A_{585} = 1.0. Cells were harvested, washed, and sonically disrupted in 0.02 M Tris-hydrochloride, pH 8.0, as described. The sonic extract was centrifuged at 80,000 × *g* for 60 min, the supernatant fluid was decanted, and the pellet was washed twice with Tris buffer and resuspended in 1 ml of Tris buffer. Numbers in parentheses refer to the percentage of enzyme units found in the supernatant fraction.

ard methods used to demonstrate the periplasmic location of a particular protein (Table 2). These methods were osmotic shock (55.4% release) and Tris-EDTA treatment (100% release). No carboxypeptidase II was released by polymyxin B treatment or by lysozyme-EDTA-induced spheroplast formation. However, cells exposed to 0.01 M $CaCl_2$ in 0.03 M Tris, pH 8.0, did release carboxypeptidase II upon lysozyme-EDTA treatment (unpublished data). The total carboxypeptidase II enzyme units recovered from the lysozyme-EDTA treated cells represented less than 50% of the enzyme units recovered from the other samples. A partial explanation for this is that carboxypeptidase II was inhibited by the lysozyme-EDTA solution itself (unpublished data).

The pattern of release of carboxypeptidase II differed from that of penicillinase, another periplasmic enzyme (21, 56). Whereas no carboxypeptidase II was released by polymyxin B treatment or lysozyme-EDTA spheroplast formation, nearly all of the penicillinase was released by these methods. In addition, the release of β-galactosidase, a cytoplasmic enzyme (21), was always low, demonstrating that no gross cellular lysis had occurred.

Sizing of cells by centrifugation through a sucrose gradient. A murein hydrolase specifically involved in cell division might have maximum activity around the time of septum formation or cell separation. It was therefore decided to investigate the activity of carboxypeptidase I, carboxypeptidase II, and amidase in cells of D11-1 at different stages in the cell cycle to determine if the activity of any of these enzymes was correlated with cell division.

Since assay of all three enzymes required many cells, it was necessary to use a method for obtaining large numbers of cells at different stages in the growth cycle. Separation of cells on the basis of size by centrifugation through a sucrose gradient (38) in a zonal rotor seemed a potentially useful method, since midlog cells from 5 liters of culture could be applied to such a gradient.

Figure 3 shows that this method did separate cells of D11-1 on the basis of size. Absorbance per cell, a function of cell mass, increased linearly from 1.2 to 2.4 units between fractions of 27 and 41. Thus the smaller and younger cells were near the top of the gradient and the larger older cells sedimented towards the bottom of the gradient.

To demonstrate that cells of different size

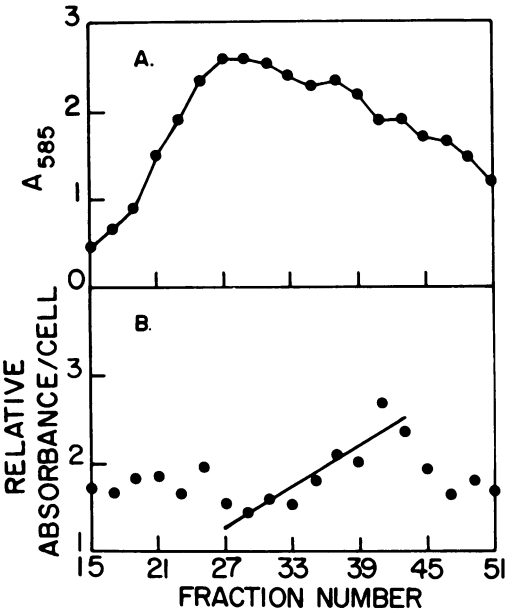

FIG. 3. *Sizing of D11-1 by sucrose gradient centrifugation in MSE zonal rotor. (A) D11-1 was grown at 37 C in E medium plus lactose and fractionated on the MSE zonal rotor as described in Materials and Methods. Absorbance per fraction was determined. Direction of sedimentation was from left to right. (B) Absorbance × 10^9 per cell was determined as described in Materials and Methods.*

TABLE 2. *Extractability of carboxypeptidase II, penicillinase, and β-galactosidase*[a]

Treatment	Total units					
	Carboxypeptidase II		Penicillinase		β-Galactosidase	
	Extract	Residue	Extract	Residue	Extract	Residue
Lysozyme-EDTA	0 (0%)	49	217 (84.3%)	40	314 (10.0%)	3128
Polymyxin B	0 (0%)	192	185 (93.2%)	13	5 (0.2%)	3355
Osmotic shock	40 (55.4%)	32	13 (25.0%)	40	67 (2.7)	2479
Tris-EDTA	129 (100%)	0	174 (92.8%)	13	122 (4.0%)	3070

[a] D11-1 was grown in L broth at 37 C to A_{585} = 1.0. Cells were harvested and treated for release of periplasmic proteins as described. The residues were resuspended in 1 ml of 0.02 M Tris-hydrochloride and disrupted by sonic oscillation. Numbers in parentheses refer to the percentage of total enzyme units released by each method.

classes corresponded to cells of different ages, cells from different regions of the gradient were inoculated into prewarmed medium to check for synchrony. Cell growth resumed immediately (Fig. 4A). The cells from near the top of the gradient (fractions 27 and 28) divided synchronously with a division midpoint of 45 min (Fig. 4B). Since the generation time was 70 min, these cells were 25 min old at the start of the experiment. Cells further down the gradient (fractions 47 and 48) started to divide immediately with a division midpoint of 10 min and thus were 60 min old at the start of the experiment. The cells from fractions 47 and 48 did not show an initial complete doubling in cell number. Presumably the cells had started to divide before inoculation into the prewarmed medium.

Centrifugation through a sucrose gradient in a zonal rotor effectively sized about two-thirds of the population. It was not clear where the smallest cells were located. The absorbance per cell experiment suggested that it was fraction 27 (Fig. 3B), whereas the synchronization experiment suggested that it was prior to fraction 27 and 28 since the cells in this fraction were 25 min old at the start of the experiment (Fig. 4B). Cells at the bottom of the gradient, below fraction 47, were examined microscopically and found to be heterogeneous in size. In addition, many cells had more than one constriction and were longer than two cell lengths, representing cells that did not separate during growth.

Murein hydrolase activity in cells sized by sucrose gradient centrifugation. The specific activity of carboxypeptidase I, carboxypeptidase II, and amidase was determined in toluene-treated cells of D11-1 sized by centrifugation. The specific activity of carboxypeptidase I and of amidase was the same in cells of all sizes (Fig. 5B,D). This indicates that carboxypeptidase I and amidase do not fluctuate during the cell cycle and therefore probably do not play a specific role in septum formation. In addition, the activity of β-galactosidase (Fig. 5A) in the fully induced cells was constant regardless of cell size confirming the results of Donachie and Masters (13).

In contrast the specific activity of carboxypeptidase II showed a threefold fluctuation, being highest in recently divided or about-to-divide cells, and lowest in cells of intermediate size (Fig. 5C). This demonstrates that the specific activity of carboxypeptidase II is highest around the time of septum formation, indicating a temporal correlation between carboxypeptidase II activity and cell division in D11-1.

Relationship between specific activity of carboxypeptidase II and cell division in BUG 6. Since the previous experiment indicated a correlation between the activity of carboxypeptidase II and cell division in D11-1, the relationship between carboxypeptidase II activity and cell division was studied in BUG 6, a temperature-sensitive mutant that grows normally at 32 C and grows, but does not divide at 42 C (51). Upon shift from 42 to 32 C, cell division resumes after 15 min, indicating that the temperature-sensitive lesion is in one of the later stages in the cell division process.

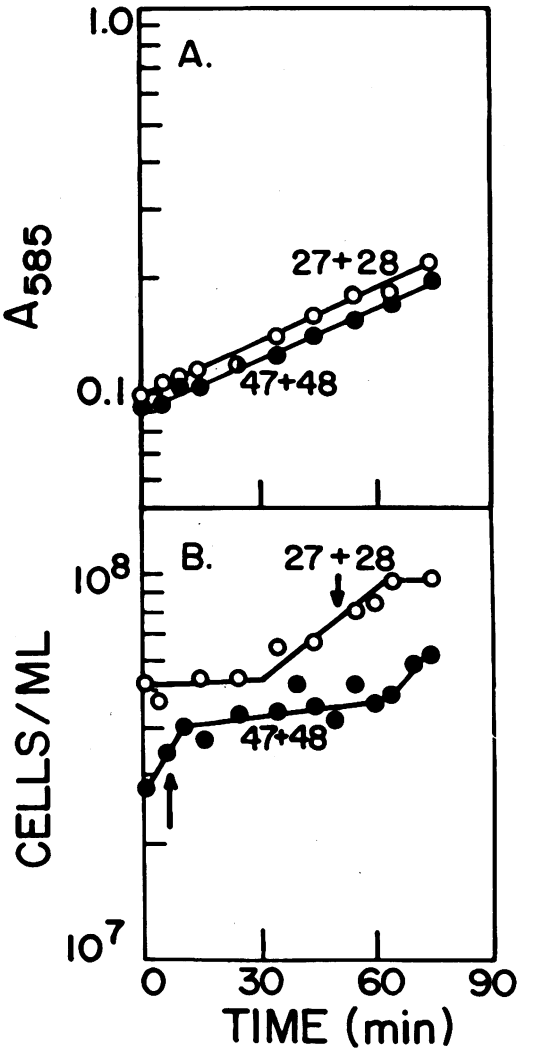

FIG. 4. *Synchronous growth of cells of D11-1 sized by sucrose gradient centrifugation in the MSE zonal rotor. (A) Cells from fractions 27 + 28 and 47 + 48 in the experiment described in Fig. 3 were diluted 1:10 in prewarmed E medium plus lactose and grown at 37 C. Samples were taken for absorbance readings. (B) Cell numbers were determined.*

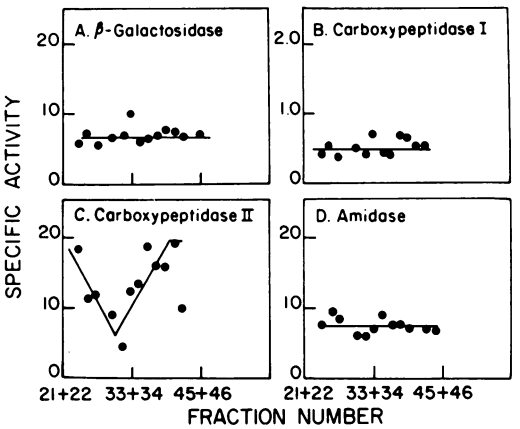

FIG. 5. *Relationship between specific activity in murein hydrolases and cell division in D11-1. Cells fractionated on the MSE zonal rotor were toluene treated and assayed for murein hydrolase activities and β-galactosidase activity.*

A definite correlation between cell division and carboxypeptidase II specific activity was found in toluene-treated cells of BUG 6 (Fig. 6). The basal activity at 32 C, when cells were dividing, was three- to fourfold higher than the basal activity at 42 C, when cells were not dividing. Even more dramatic was the 10-fold increase in specific activity of carboxypeptidase II 15 min after a shift from 42 to 32 C. At this time, there was also a resumption of cell division. Carboxypeptidase II itself was not the temperature-sensitive component of BUG 6. Enzyme from either nondividing or dividing cells showed no temperature sensitivity when assayed at 32, 37, or 42 C (results not shown).

DISCUSSION

Septum formation in *E. coli* is a precisely controlled process involving the synthesis of several complex surface components. The ingrowth of the rigid murein layer, dividing one cell into two daughter cells, is an important feature of septation. The specific involvement of a murein hydrolase(s) in septation has often been postulated (15, 16, 22, 62), but no firm experimental proof for the involvement of a specific enzyme has yet been furnished. To find out if any of three *E. coli* murein hydrolases, carboxypeptidase I, carboxypeptidase II, or amidase, was specifically involved in septum formation, we measured the specific activities of these enzymes as a function of cell size. Since cell size is related to cell age (38), such an approach could reveal which murein hydrolase was most active at the time of division.

We separated cells of D11-1 by centrifugation through a sucrose gradient in a zonal rotor. The relative rate of sedimentation was a function of cell mass as determined by measurements of the absorbance per cell across the gradient (Fig. 3B). In addition, the cells at the top of the gradient were younger than the cells further down the gradient, since, upon dilution into prewarmed medium, the cells from the top were the last to divide (Fig. 4B). Toluene-treated cells of different size had the same specific activities of both carboxypeptidase I and amidase (Fig. 5B, D). This suggests that carboxypeptidase I and amidase exert no special function in septation. The specific activity of carboxypeptidase II, on the other hand, varied threefold across the gradient, being highest in recently divided or about-to-divide cells and lowest in cells of intermediate size (Fig. 5C). The division-associated increase in carboxypeptidase II activity in toluene-treated cells does not reflect changes in total enzyme protein, because com-

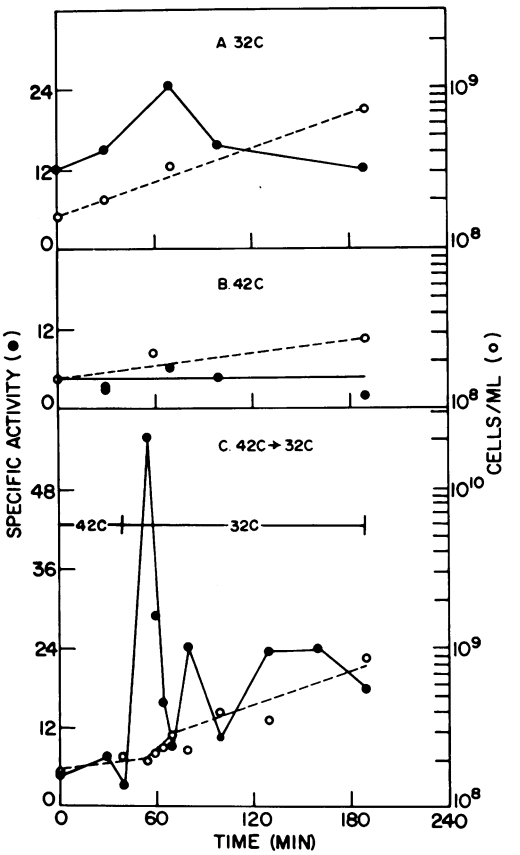

FIG. 6. *Relationship between carboxypeptidase II activity and cell division in BUG 6. BUG 6 was grown in E medium plus glucose for 110 min at 32 C to A_{585} = 0.35. The culture was shifted to 42 C for 60 min and then back to 32 C (C). Control cultures were maintained at 32 C (A) and 42 C (B). Samples were taken for cell counting and toluene treatment.*

plete disruption of cells by sonic oscillation liberated equal amounts of enzyme from nondividing and dividing cells. There appear to be multiple causes for the fluctuations in carboxypeptidase II activity. These causes may include increased substrate uptake under dividing conditions, greater release of carboxypeptidase II from dividing cells, and enzyme inhibition under nondividing conditions (to be published).

Further evidence for a correlation between carboxypeptidase II activity and cell division came from studies using the temperature-sensitive division mutant, BUG 6 (49–51). BUG 6 grows and divides normally at 32 C. At 42 C BUG 6 grows, but does not divide, resulting in the formation of filamentous cells. Upon a shift from 42 to 32 C, cells begin to divide after about 15 min. The specific activity of carboxypeptidase II in cells grown at 42 C was about one-third the specific activity of carboxypeptidase II in cells grown in 32 C (Fig. 6A, B). In addition, 15 min after a shift from 42 to 32 C, there was a 10-fold increase in the specific activity of carboxypeptidase II. At this time, there was also a rapid resumption of cell division.

Thus we have shown that the detectable activity of carboxypeptidase II in toluene-treated cells is temporally correlated with cell division in two situations: in cells of D11-1 sized by sucrose gradient centrifugation and in cells of BUG 6 after a shift from 42 to 32 C. The observed fluctuation in carboxypeptidase II activity during the cell cycle will be discussed in a future paper.

Another interesting finding of this work is the observation that carboxypeptidase II is released from L broth-grown cells of D11-1 by two methods which are known to release periplasmic enzymes (Table 2). These methods were osmotic shock (55.4% release) and Tris-EDTA treatment (100% release). No carboxypeptidase II was released during lysozyme-EDTA-induced spheroplast formation or polymyxin B treatment. Penicillinase, a periplasmic enzyme (21, 56), was released by both of these methods. The release of carboxypeptidase II may be correlated with the release of lipopolysaccharide (LPS). Thirty to 50% of total LPS is released during Tris-EDTA treatment (30). LPS is also released during stage 1 of osmotic shock (1). On the other hand, no LPS is released during the Osborn method of lysozyme-EDTA-induced spheroplast formation (44) or during polymyxin B treatment (11). A future paper (manuscript in preparation) will show that the release of carboxypeptidase II by Tris-EDTA is a division-associated phenomenon, and this release partially explains the fluctuations in activity reported in this paper.

Since polymerization and cross-linking of murein occur on the membrane (2, 27), the periplasmic location of carboxypeptidase II suggests that it may act on murein at a step subsequent to polymerization and cross-linking. Since none of the murein repeating units in the cytoplasmic membrane is linked to lipoprotein (6), it is possible that the covalent association between murein and lipoprotein occurs after polymerization and cross-linking. By analogy to the transpeptidase reaction in which the terminal D-alanine residue is cleaved, providing energy for linking the subterminal D-alanine to an adjacent diaminopimelic acid, the cleavage of the subterminal D-alanine by carboxypeptidase II may provide the energy for linkage of diaminopimelic acid to the lipoprotein (25). Alternatively, carboxypeptidase II could prevent linking between murein and lipoprotein by removal of the subterminal D-alanine.

This work represents an advance in the method of studying the relation of murein hydrolases to septation and cell separation in that the activities of individual hydrolases were examined, whereas earlier studies have usually measured gross destruction of the murein (54, 55). A fuller evaluation of the role of murein hydrolases in cell division would require measurement of other murein hydrolases, namely muramidase (45) and a second more active muramidase that produces a nonreducing disaccharide-tetrapeptide (19, 24, 58).

ACKNOWLEDGMENTS

This work was supported by Public Health Service Research grant no. AI05090 from the National Institute of Allergy and Infectious Diseases. Barbara D. Beck was a predoctoral trainee of Public Health Service grant no. GM01136 from the National Institute of General Medical Sciences. The enthusiastic assistance of Cynthia Neumann is gratefully acknowledged.

LITERATURE CITED

1. **Anraku, Y., and L. Heppel.** 1967. On the nature of the changes induced in *Escherichia coli* by osmotic shock. J. Biol. Chem. 242:2561–2569.
2. **Araki, Y., A. Shimada, and E. Ito.** 1966. Effect of penicillin on cell wall mucopeptide synthesis in *Escherichia coli* particulate system. Biochem. Biophys. Res. Commun. 23:518–525.
3. **Bachman, B. J.** 1972. Pedigree of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36:525–557.
4. **Boman, H. G., K. G. Eriksson-Grennberg, S. Normark, and E. Mattson.** 1968. Resistance of *Escherichia coli* to penicillins. IV. Genetic study of mutants resistant to D,L-ampicillin concentrations of 100 μg/ml. Genet. Res. 12:169–185.
5. **Braun, V., and V. Bosch.** 1973. *In vivo* biosynthesis of murein-lipoprotein of the outer membrane of *E. coli*. FEBS Lett. 34:302–306.
6. **Braun, V., and V. Bosch.** 1972. Repetitive sequences in the murein-lipoprotein of the cell wall of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 69:970–974.

7. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. 10:426–438.
8. **Braun, V., and V. Sieglin.** 1970. The covalent murein-lipoprotein structure of the *E. coli* cell wall. The attachment site of the lipoprotein on the murein. Eur. J. Biochem. 13:336–346.
9. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Septum formation in *Escherichia coli:* characterization of septal structure and the effects of antibiotics on cell division. J. Bacteriol. 119:303–324.
10. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Electron microscope study of septum formation in *Escherichia coli* strains B and B/r during synchronous growth. J. Bacteriol. 119:1039–1056.
11. **Cerny, G., and M. Teuber.** 1971. Differential release of periplasmic versus cytoplasmic enzymes from *Escherichia coli* B by polymyxin B. Arch. Mikrobiol. 78:166–179.
12. **Cerny, G., and M. Teuber.** 1972. Comparative polyacrylamide electrophoresis of periplasmic proteins released from gram-negative bacteria by polymyxin B. Arch. Mikrobiol. 82:361–370.
13. **Donachie, W. D., and M. Masters.** 1969. Temporal control of gene expression, p. 37–76. *In* G. M. Padilla, G. L. Whitson, and I. L. Cameron (ed.), The cell cycle. Academic Press Inc., New York.
14. **Eshdat, Y., and D. Mirelman.** 1972. An improved method for the recovery of compounds from paper chromatograms. J. Chromatogr. 65:458–459.
15. **Ghuysen, J. M.** 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. 32:425–464.
16. **Ghuysen, J. M., and G. D. Shockman.** 1973. Biosynthesis of peptidoglycan, p. 37–130. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, Inc., New York.
17. **Ghuysen, J. M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls, p. 685–699. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
18. **Hall, T. C., and E. C. Cocking.** 1965. High-efficiency liquid-scintillation counting of ^{14}C-labeled material in aqueous solution and determination of specific activity of labeled proteins. Biochem. J. 96:626–633.
19. **Hartmann, R., J. V. Holtje, and U. Schwarz.** 1972. Targets of penicillin action in *E. coli*. Nature (London) 235:426–428.
20. **Heppel, L. A.** 1967. Selective release of enzymes from bacteria. Science 156:1451–1455.
21. **Heppel, L. A.** 1971. The concept of periplasmic enzymes, p. 224–247. *In* L. I. Rothfield (ed.), Structure and function of biological membranes. Academic Press Inc., New York.
22. **Higgins, M. L., and G. D. Shockman.** 1971. Procaryotic cell division with respect to wall and membranes. CRC Crit. Rev. Microbiol. 1:29–71.
23. **Hoffman, B., W. Messer, and U. Schwarz.** 1972. Regulation of polar cap formation in the life cycle of *E. coli*. J. Supramol. Struct. 1:29–37.
24. **Holtje, J. V., D. Mirelman, N. Sharon, and U. Schwarz.** 1975. Novel type of murein transglycosylase in *Escherichia coli*. J. Bacteriol. 124:1067–1076.
25. **Inouye, M., J. Shaw, and C. Shen.** 1972. The assembly of a structural lipoprotein in the envelope of *E. coli*. J. Biol. Chem. 247:8154–8159.
26. **Ito, E., S. G. Nathanson, D. N. Dietzler, J. S. Anderson, and J. L. Strominger.** 1966. Formation of UDP-acetylmuramyl peptides. p. 324–337. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
27. **Izaki, K., M. Matsuhashi, and J. L. Strominger.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin sensitive enzymatic reactions in strains of *Escherichia coli*. J. Biol. Chem. 243:3180–3192.
28. **Izaki, K., and J. L. Strominger.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIV. Purification and properties of two D-alanine carboxypeptidases from *E. coli*. J. Biol. Chem. 243:3193–3204.
29. **Lawrence, P. J., and J. L. Strominger.** 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XVI. The reversible fixation of radioactive penicillin G to the D-alanine carboxypeptidase of *Bacillus subtilis*. J. Biol. Chem. 245:3660–3666.
30. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. 21:290–296.
31. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology 1:190–206.
32. **Leutgeb, W., and W. Weidel.** 1963. Oligo-mucopeptide aus der stutzmembranen von *E. coli*. Z. Naturforsch. 18:1065–1069.
33. **Levy, S. B., and L. Leive.** 1970. Release from *Escherichia coli* of a galactosyltransferase complex active in lipopolysaccharide synthesis. J. Biol. Chem. 245:585–594.
34. **Lindström, E. B., and K. Nordström.** 1972. Automated method for determination of penicillins, cephalosporins, and penicillinase. Antimicrob. Agents Chemother. 1:100–106.
35. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagents. J. Biol. Chem. 193:265–275.
36. **Martin, H. H., and H. Frank.** 1962. Quantitative bausteinanalyse der stutzmembranen in der zellwand von *Escherichia coli* B. Z. Naturforsch. 17b:190–196.
37. **Mitchell, P.** 1961. Approaches to the analysis of specific membrane transport, p. 581–603. *In* T. W. Goodwin and O. Lindberg (ed.), Biological structure and function, vol. 2. Academic Press Inc., New York.
38. **Mitchison, J. M., and W. S. Vincent.** 1965. Preparation of synchronous cell cultures by sedimentation. Nature (London) 205:987–989.
39. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. 240:3685–3692.
40. **Neuhaus, F. C., and W. G. Struve.** 1965. Enzymatic synthesis of analogs of the cell-wall precursor. I. Kinetics and specificity of uridine diphospho-N-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine-D-alanyl-D-alanine ligase (Adenosine diphosphate) from *Streptococcus faecalis* R. Biochemistry 4:120–131.
41. **Nguyen-Distèche, M., J. M. Ghuysen, J. J. Pollock, P. Reynolds, H. R. Perkins, J. Coyette, and M. R. J. Salton.** 1974. Enzymes involved in wall peptide cross-linking in *Escherichia coli* K-12, strain 44. Eur. J. Biochem. 41:447–455.
42. **Nguyen-Distèche, M., J. J. Pollock, J. M. Ghuysen, J. Puig, P. Reynolds, H. R. Perkins, J. Coyette, and M. R. J. Salton.** 1974. Sensitivity to ampicillin and cephalothin of enzymes involved in wall peptide crosslinking in *Escherichia coli* K-12, strain 44. Eur. J. Biochem. 41:457–463.
43. **Nikaido, H.** 1973. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of gram-negative cell wall, p. 131–208. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, Inc., New York.
44. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characteri-

zation of cytoplasmic and outer membrane. J. Biol. Chem. 247:3962-3972.
45. **Pelzer, H.** 1963. Mucopeptidhydrolasen in *Escherichia coli* B. I. Nachweis und wirkungsspezifitat. Z. Naturforsch. 18:950-956.
46. **Pelzer, H., D. Maass, and W. Weidel.** 1963. Determination of the role played by an identified mucopeptide during the synthesis of the supporting membrane of *Escherichia coli* B. Naturweiss 50:722-723.
47. **Platt, T., B. Muller-Hill, and J. H. Miller.** Unit VII, p. 351-376. *In* J. H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor, N.Y.
48. **Pollock, J. J., M. Nguyen-Disteche, J. M. Ghuysen, J. Coyette, R. Linder, M. R. J. Salton, K. S. Kim, H. R. Perkins, and P. Reynolds.** 1974. Fractionation of the DD-carboxypeptidase-transpeptidase activities solubilized from membranes of *Escherichia coli* K-12, Strain 44. Eur. J. Biochem. 41:439-446.
49. **Reeve, J. N., and D. J. Clark.** 1972. Cell division of *Escherichia coli* BUG-6: effect of varying the length of growth at the nonpermissive temperature. J. Bacteriol. 110:117-121.
50. **Reeve, J. N., and D. J. Clark.** 1972. Cell division of *Escherichia coli* BUG-6: effect of varying the temperature used at the nonpermissive growth condition. J. Bacteriol. 110:122-125.
51. **Reeve, J. N., D. J. Groves, and D. J. Clark.** 1970. Regulation of cell division in *Escherichia coli:* characterization of temperature-sensitive mutants. J. Bacteriol. 104:1052-1064.
52. **Rogers, H. J.** 1970. Bacterial growth and the cell envelope. Bacteriol. Rev. 34:194-214.
53. **Rosen, B. P., and L. A. Heppel.** 1973. Present status of binding proteins that are released from gram-negative bacteria by osmotic shock, p. 209-239. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, Inc., New York.
54. **Schwarz, U., A. Asmus, and M. Frank.** 1969. Autolytic enzymes and cell division of *Escherichia coli*. J. Mol. Biol. 41:419-429.
55. **Schwarz, U., and W. Weidel.** 1965. Zum wirkungsmechanismus von penicillin I. Isolierung and charakterisierung 2,6-diaminopimelinsaure-enthaltender niedermolekularer peptide aus penicillin-spharoplasten von *Escherichia coli* B. Z. Naturforsch. 20:147-153.
56. **Smith, J. T., and J. M. Wyatt.** 1974. Relation of R-factor and chromosomal β-lactamase with the periplasmic space. J. Bacteriol. 117:931-939.
57. **Takebe, I.** 1965. Extent of cross-linkage in the murein sacculus of *Escherichia coli* B cell wall. Biochim. Biophys. Acta 101:124-126.
58. **Taylor, A., B., C. Das, and J. van Heijenoort.** 1975. Bacterial cell-wall peptidoglycan fragments produced by phage λ or Vi II endolysin and containing 1,6-anhydro-*N*-acetylmuramic acid. Eur. J. Biochem. 53:47-54.
59. **van Heijenoort, Y., and J. van Heijenoort.** 1971. Study of the NAcMur-*L*-amidase activity in *E. coli*. FEBS Lett. 15:137-141.
60. **van Heijenoort, Y., and J. van Heijenoort.** 1975. Envelope-bound N-acetylmuramyl-L-alanine amidase of *Escherichia coli*. Eur. J. Biochem. 58:611-619.
61. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli:* partial purification and some properties. J. Biol. Chem. 218:97-106.
62. **Weidel, W. H., H. Frank, and W. Leutgeb.** 1963. Autolytic enzymes as a source of error in the preparation and study of gram-negative cell walls. J. Gen. Microbiol. 30:127-130.
63. **Weidel, W., and H. Pelzer.** 1964. Bag shaped macromolecules. Adv. Enzymol. 26:193-232.