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Synthesis of the Cell Surface during the Division Cycle of Rod-Shaped, Gram-Negative Bacteria

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INTRODUCTION

When, where, how, and how much cell surface is made during the division cycle? What is the rate of synthesis of the wall components during the division cycle? Where is the wall inserted? How are the rates and locations of wall synthesis determined? And perhaps most important, how are these biosynthetic rates related to the observed regularity of division during the cell cycle? This last question arises from the observation that, during normal growth, there is neither too much nor too little synthesis of peptidoglycan.

Most of the work on the biosynthesis of the cell surface is related to peptidoglycan synthesis, as peptidoglycan appears to be the most stable portion of the cell surface. The peptidoglycan of the rod-shaped cell can be isolated from the other cell constituents and retains the original shape of the cell. This observation may imply that peptidoglycan is involved in the determination of cell shape. Another interpretation suggests that once cell shape is determined, the peptidoglycan in an isolated state merely retains the original shape.

STRUCTURE OF THE CELL SURFACE OF GRAM-NEGATIVE BACTERIA

The gram-negative bacterial cell is covered with a three-layered coat consisting of an inner membrane adjacent to the cell cytoplasm, a peptidoglycan or murein layer encompassing the inner membrane, and an outer membrane layer enclosing the cell (Fig. 1). Weidel and Pelzer (152) coined the word murein in 1964 to describe the wall-like properties of the bacterial peptidoglycan. The word murein is analogous to protein and nuclein and does not have any biochemical connotations, but rather refers to the functional aspects of the cell wall. Currently, the word peptidoglycan dominates the literature.

The surface of a rod-shaped bacterial cell is described as a cylindrical side wall capped by two hemispherical poles. During the early part of the division cycle, when cells are not invaginating, the cells elongate and grow only in the cylindrical wall area. In the later part of the division cycle, cells invaginate and produce two new poles in the middle of the parental cell.

Peptidoglycan Structure

Composition and cross-linking of the peptidoglycan subunits. The cross-linked peptidoglycan network of the gram-negative cell wall is made up of chains of subunits composed of two sugars and four amino acids (126, 127). The sugars are arranged in chains, and the amino acids are engaged in the cross-linking between glycan chains. After insertion of subunits, the linear strand is made up of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid subunits. (Muramic acid is *N*-acetylglucosamine with a D-lactic acid ether substituted at C-3). The tetrapeptide side chain is attached to the muramic acid at the lactic acid substituent and may be either free or involved in cross-linking between chains.

The amino acids of the side chain are L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanine. If a cross-link is formed between strands, there is a peptide bond between the carboxyl group of the D-alanine in one chain and the ε-amino group of the diaminopimelic acid in an adjacent strand. By a combination of cross-linking and strand extension, a large network is produced that completely encloses the cell. All of the peptidoglycan material is covalently attached to other peptidoglycan material, so the peptidoglycan sacculus may be visualized as a single macromolecule (Fig. 1). The cell must retain this macromolecular structure while also being able to grow as new material is inserted into the peptidoglycan.

Because it is a single macromolecule, the peptidoglycan does well at holding the cell together against the large internal turgor pressures found within a bacterial cell. (The internal pressure has been estimated to be 75 to 90 lb/in^2, approximately the pressure found inside a high-pressure bicycle tire.) This pressure would cause the cells to burst if it were not for the presence of the strong peptidoglycan layer. This is supported by the classic observation that enzymatic digestion of the peptidoglycan leads to cell bursting unless the cells are suspended in a hypertonic medium (high concentrations of sucrose or glycerol). The fact that peptidoglycan is a single, covalently linked network leads to the central question of wall growth: how does the cell wall grow without weakening its essential structural features?

Amount of peptidoglycan per cell. What is the thickness of

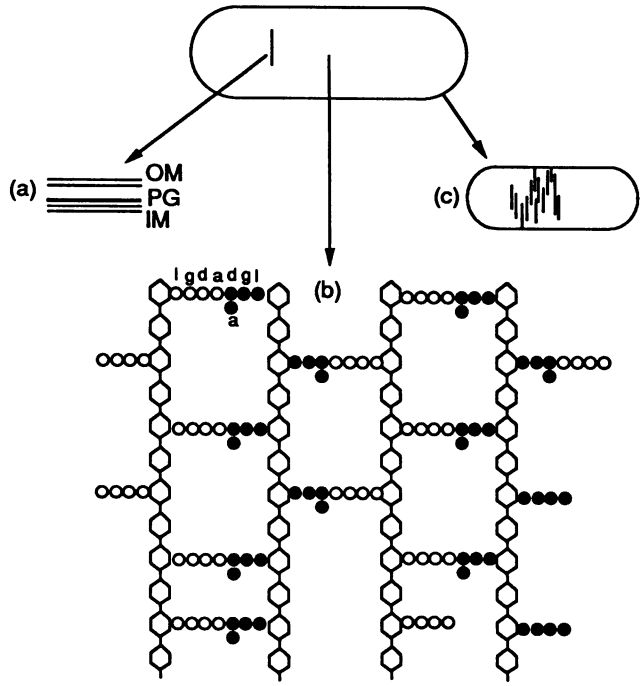

FIG. 1. Cellular and molecular structure of the gram-negative bacterial cell wall. The rod-shaped, gram-negative cell wall is composed (a) of a peptidoglycan (PG) layer sandwiched between an outer membrane (OM) and an inner membrane (IM). At a molecular level (b), the peptidoglycan consists of chains of sugar residues cross-linked by amino acid chains. The amino acids are labeled as follows: l, L-alanine; g, D-glutamic acid; d, *meso*-diaminopimelic acid; a, D-alanine. The sugar chains are preferentially arranged perpendicular to the long axis of the cell (c). No information is available on the orientation of strands in the spherical polar regions. As the cross-links are drawn, the open circles are the donors to the closed circles. Reprinted from reference 33 with permission.

the peptidoglycan, and how many layers of it are on a cell? Early work involving chemical determinations of the amount of diaminopimelic acid per cell gave a value of 2.7×10^6 diaminopimelic acid residues per cell (13). The cell surface had an average area of 30 to 40 μm^2 per sacculus. This yields a calculated surface area per diaminopimelic acid residue—the diaminopimelic acid moiety indicating the unit tetrapeptide of the peptidoglycan—of 12 nm^2 per residue. This area of a residue is too small to cover the cell surface; there must be more residues in a cell, or the cell must be smaller than measured.

More refined chemical measurements of the diaminopimelic acid content of cells by using amino acid analysis revealed that there were 3.1×10^6 diaminopimelic acid residues per cell for cells growing slowly in minimal medium and 5.6×10^6 residues per cell for cells growing rapidly in rich medium. The surface area of rapidly growing cells is larger than that of slowly growing cells (140); these measurements give a more acceptable amount of diaminopimelic acid per cell surface area. More refined measurements of the amount of diaminopimelic acid per cell, combined with surface measurements on the same cells, gave a value of 4 to 5×10^6 subunits per cell, with a value of 7 nm^2 per diaminopimelic acid residue. Interpretation of these values requires consideration of the dimensions of a pentapeptide subunit. Model-building and X-ray-scattering data reveal that the length of a repeating disaccharide subunit is 0.98 nm and that the average separation of glycan chains is about 1.9 nm. Other measurements give 2.5 nm between chains. With these data, the area per disaccharide unit is 2.0 to 2.5 nm^2. With 4×10^6 units per cell, this is enough peptidoglycan to cover about 9 to 12 μm^2. As the average surface area of these smaller cells is 7 μm^2, this is too much peptidoglycan for a monolayer and too little for a trilayer, but there is enough for a bilayer.

Recent chemical measurements of the amount of diaminopimelic acid per cell, combined with electron-microscopic determinations of the surface area of a cell, have suggested that there is less peptidoglycan per cell (154). Direct chemical determinations indicated that there were 3.6×10^6 molecules per *Escherichia coli* sacculus while the surface area was 8.9 μm^2. This indicated that the surface area per diaminopimelic acid molecule was 2.4 nm^2. This is consistent with a monolayer but excludes a complete bilayer or trilayer.

A view of the peptidoglycan layer. Given the uncertainty about the amount of peptidoglycan on the bacterial cell surface, one can only attempt to fit the data to a reasonable model of surface structure. One possible way of looking at surface structure is presented in Fig. 2. In this three-dimensional representation of the cell, there is a single load-bearing monolayer of peptidoglycan with additional, non-load-bearing layers beneath. The strands below the surface are only loosely connected by cross-links between the glycan strands. This view of the load-bearing structure of the cell incorporates the idea of "make before break." The taut bonds of the peptidoglycan are eventually going to be severed, allowing new material to be inserted. When the new material is in place before the bond cutting, new surface growth can take place with no loss of the structural integrity

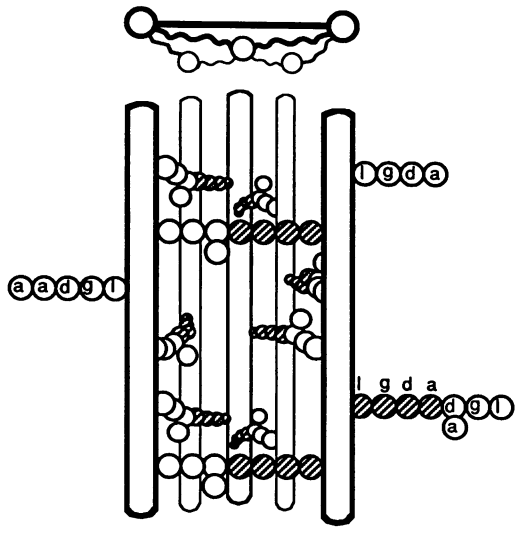

FIG. 2. Three-dimensional representation of peptidoglycan structure. This is an idealized representation of the peptidoglycan structure as seen from the outer surface. The thick bars represent chains of sugars at the outside of the peptidoglycan layer. The thinner bars represent chains below the outer layer. The stretched chains of circles represent amino acids cross-linking the glycan chains. The chains below the stretched surface of the cell rise to the outer layer when the taut layers of the peptidoglycan are hydrolyzed. Above is a cross-sectional view through the glycan chains illustrating the taut outer layer and the more loosely inserted inner material. Reprinted from reference 33 with permission.

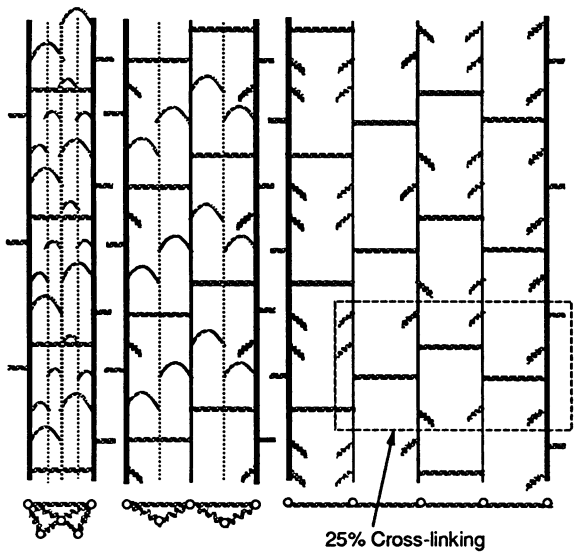

FIG. 3. Growth of the peptidoglycan area by cutting of stretched bonds. A taut peptidoglycan layer is shown at the left, with two layers of inserted peptidoglycan below. As the stretched bands are cut, the diagrams to the right illustrate the growth in surface area. When the fully stretched peptidoglycan is produced, the free amino acid chains can be acceptors for further new chain insertion. A unit area of peptidoglycan illustrating the degree of cross-linking is shown in the dotted box. One-quarter of the possible bonds are involved in cross-linking; thus, the degree of cross-linking is 25%. This calculation is made by noting that each side chain contains the material for one complete cross-link, a donor and an acceptor. There are a total of 16 side chains within the box, and there are four complete cross-links, so 25% of the possible cross-links are made. Reprinted from reference 33 with permission.

of the peptidoglycan layer. As shown in Fig. 3, there may be more than one layer of strands below the load-bearing layer. When the cross-links in the load-bearing layer are cut, a large increase in the cell surface can be produced without any break in the structural continuity of the cell surface. Koch (98) discussed the enzymological requirements for a "smart" autolysin that would not cut a stressed murein bond unless there were strands in place to connect the separating glycan strands.

This view of the cell surface allows a variable amount of cell peptidoglycan, depending on how many strands are located below the load-bearing layer. It is not necessary, as depicted in Fig. 2, to have every interstrand region filled with non-load-bearing strands. It may be that only a fraction of the load-bearing strands have these sublayer strands in place. Depending on growth conditions or the rate of growth, the density of peptidoglycan per unit of surface area could vary, with the single restriction that there must be at least a minimal load-bearing layer of peptidoglycan. If some, but not all, interstrand regions have loosely connected strands below, the cell must have a mechanism for ascertaining that a strand is in place before a load-bearing cross-link is severed (98). If a cell had such a protective mechanism, no severing of cross-links would occur in the rightmost diagram of Fig. 3 until new non-load-bearing strands and cross-links were inserted between the load-bearing glycan strands. There is evidence that whatever the actual peptidoglycan density on the cell surface, this density is constant and independent of growth rate (165).

Multilayered peptidoglycan and inside-to-outside growth. An alternative view of peptidoglycan growth has been supported by a number of experiments. This alternative view postulates an inside-to-outside pattern of peptidoglycan growth in the gram-negative cell, in a manner similar to that for rod-shaped, gram-positive cells. New peptidoglycan would be laid down adjacent to the cytoplasmic membrane. With successive new layers forming below this peptidoglycan, the peptidoglycan would age, would be successively externalized, and would eventually slough off into the medium. The initial evidence for this type of model is that in certain strains of *E. coli* there is an observable turnover of peptidoglycan; peptidoglycan fragments are secreted into the medium (63). The actual degree of turnover is thought to be greater than the observed release of material because there is some reutilization of released material (59, 61). This turnover has been measured primarily by direct pulse-labeling methods (59).

The postulation of turnover has been questioned on the basis of membrane elution experiments demonstrating that in both *Salmonella typhimurium* (29) and *E. coli* (36), peptidoglycan is as stable as protein and is not released from the cell. Protein in a growing cell is stable, so these membrane elution experiments imply that there is no turnover of peptidoglycan in gram-negative cells, at least in the sense of release of peptidoglycan from the cell. One may surmise that there is turnover in these cells with an immediate and complete reutilization of the peptidoglycan fragments. In the description of peptidoglycan growth presented here, it will be seen that there is no theoretical reason to require peptidoglycan to turn over at all. Although turnover is possible, growth with turnover represents an energetic loss to the cell if it could grow without turnover. Why make wall and then replace it with new wall if the original wall suffices? In a more detailed discussion, Koch (98) points out that a patchwork, localized turnover system leads to instability of cell wall growth and loss of the rod shape.

Direct evidence for a multilayered structure for peptidoglycan comes from small-angle neutron scattering. In this technique, isolated sacculi are suspended in D_2O and placed in a neutron beam. The angular scattering of neutrons is measured. By Fourier analysis, the thickness of lamellalike objects can be determined (109). Although the predominant conclusion is that there is a multilayered peptidoglycan, there are other valid interpretations of the data (109). For example, if only part of the cell were multilayered, such as the pole areas, one would have a model compatible with the data. It is difficult to eliminate interactions between the sacculi in solution or a collapse of the sacculi upon themselves (as an empty balloon appears double layered). These interactions may give the multilayered result, and they have been considered by Labischinski et al. (109). The introduction of small-angle neutron scattering is an exciting development, but it is difficult to reconcile this approach with the quantitative determinations of peptidoglycan per cell.

A more conventional experimental support for multilayered, inside-to-outside growth comes from determinations of the percentage of radioactivity in the donor portion for different fragments as they mature following a pulse-label (55). Immediately after labeling with diaminopimelic acid, the tetra-tetra fragment (a dimer formed with both peptide side chains containing four amino acids) was only 79% labeled in the donor portion, and 90 min later it was 30% labeled in the donor peptide. (A peptide chain is a donor if the diaminopimelic acid in that chain is not cross-linked to an adjacent D-alanine, whereas a peptide chain is an acceptor if the diaminopimelic acid is cross-linked with an adjacent

D-alanine.) In contrast, the tetra-tri fragment started out with 94% donor labeling, which decreased to 2% after 90 min. This was interpreted (55) as compatible with the conclusion that tetra-tri dimers are connections between an innermost layer and a middle layer above and that the tetra-tetra dimers are connections within a layer of peptidoglycan. This result is subject to numerous experimental difficulties: labeling took place at high cell concentrations in Penassay broth, the tetra-tri fragments are present at very low concentrations, and there is a problematic pool of diaminopimelic acid in the strain used. It should be noted that the total acceptor-donor pattern, primarily as a result of the abundant tetra-tetra fragments, is not consistent with the very low acceptor measurements in *S. typhimurium* (37). Assuming that the tetra-tri fragment exclusively interlinks murein layers of different planes (in a triplanar model, there would be an inner, a middle, and an outer plane of peptidoglycan), one would get the observed donor-acceptor pattern because the initial tetra-tri fragments would be donors from the inside layer to the middle layer. The final tetra-tri fragments would be acceptors from the middle layer to the outside layer. In contrast, the tetra-tetra fragments are involved only in intraplane linkage (55). There might be some maturation by intercalation, but there would be less loss of donors in the tetra-tetra fragments than in the tetra-tri fragments. Although the reasoning of Glauner and Höltje (55) is impeccable, and in many ways ingenious, it is based on the assumption of a multilayered structure of peptidoglycan, on the assumption that tetra-tri fragments are involved in interplane linking, and on the assumption that tetra-tetra fragments are involved in intraplane linking. If different fragments reacted differentially to intercalation, e.g., if the tetra-tetra fragments were more resistant to intercalation of new strands, one would also get the observed donor pattern during maturation.

Glauner and Höltje (55) have suggested abandoning the acceptor-donor radioactivity ratio (ADRR) to describe results. They propose that the percentage of the donor alone should be given. Since the ADRR is a ratio of two related components, the calculation of the ratio may be misleading. I support this change. In future work in which acceptors and donors are determined, the results should be reported in terms of the percentage of donor (or, if preferred, the percentage of acceptor) rather than as a ratio of acceptor to donor. When further analysis of the ADRR is presented here, it will be discussed in the terms currently used in the literature, rather in this recommended form.

Heterogeneity of peptidoglycan structure. This simplified picture of peptidoglycan structure has been made more complicated, but more interesting, by the description of a large variety of fragments in peptidoglycan digests. By using high-performance liquid chromatography (HPLC) it is possible to observe up to 80 different fragments from the cell wall peptidoglycan (53–57, 78, 79, 142). The major components of a peptidoglycan digest are tetrapeptide-containing monomers (a single, un-cross-linked subunit containing two sugar residues) and dimers (two cross-linked monomers with four sugars) and even trimers and tetramers. In addition, the heterogeneity is due to fragments formed by permutations involving different numbers of amino acids (tripeptides, tetrapeptides, and pentapeptides), different cross-links (diaminopimelic acid–diaminopimelic acid in addition to the D-Ala–diaminopimelic acid link), and different sugar structures (depending on whether the sugars were located within or at the end of a glycan chain). Other minor modifications are observed. The covalent attachment of lipoprotein to a significant fraction of the peptidoglycan produces additional variability. In addition, growth in rich medium leads to the incorporation of glycine in place of a D-alanine (55). All of these variations may combine to form all the permutations and combinations possible to give a large variety of fragments after enzyme digestion of peptidoglycan.

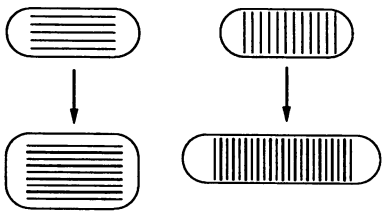

FIG. 4. Cell growth with either hoop or lengthwise arrangement of peptidoglycan. With an intercalation mechanism, if chains were arranged parallel to the long axis the cell would increase in width. Chains arranged perpendicular to the long axis would allow lengthwise extension of the cell, which is the observed mode of cell growth.

Do any of these minor fragments have a function within the cell? For example, it could be imagined that these minor components of the peptidoglycan are regulatory signals that allow the cell to start constriction at a particular time and at a particular place. At present, however, there is no evidence regarding the function of these minor subunits. A nonfunctional origin of these subunits would exist if the subunits were produced because of errors during the normal biosynthetic reactions; the cell may survive without having an elaborate proofreading mechanism for correcting minor errors in cell wall structure. Another argument against the notion that these minor fragments have a role in cell wall structure or morphogenesis is that a component present at 0.1% of the total peptidoglycan would be present at 3,000 subunits per cell; this is enough for one complete circumference of the cell. It is difficult to imagine how such a small amount of material in the total cell peptidoglycan could influence the regular morphogenesis of the cell.

Arrangement of Peptidoglycan Strands on the Cell Surface

Electron-microscopic analysis of partially digested cell walls (151) and the results of controlled-sonication studies (149) suggest that the strands of the cylindrical side wall are arranged primarily perpendicular to the long axis of the cell; i.e., the strands encircle the cylindrical side wall as hoops encircle a barrel. When walls were observed after fragmentation, the strands tended to go preferentially in one direction, perpendicular to the long axis of the cell. Although there may not be a perfect alignment, as has been suggested by Koch (95, 96), a hoop arrangement is theoretically the best way to place peptidoglycan strands to allow lengthwise extension of a growing cell (Fig. 4). New strands are inserted between preexisting glycan chains, and the cell grows primarily in the lengthwise direction between divisions. If the glycan chains were placed in the axial direction, i.e., in the lengthwise direction of the cell, with the cross-links in the hoop direction, then insertion of new strands between preexisting glycan chains would lead to an increase in the cell circumference. It is clear from even the earliest observations of cells in the light microscope (1, 5, 141) that rod-shaped cells grow primarily lengthwise. This gives strong a priori support to the experimental evidence that the strands are arranged primarily as hoops around the cell circumference.

Biochemical analysis indicates that glycan strands are relatively short and cannot extend around the entire circumference of the cell (55, 71). The recent introduction of amidase digestion with HPLC separation of individual glycan chains indicated that there was a broad distribution of chain lengths, from 2 to 23 subunits. This means that the peptidoglycan in the hoop orientation is actually made up of short strands that collectively encircle the cell. Presumably the short strands overlap to make an effective encircling hoop. The absence of long-range order in the arrangement of peptidoglycan strands around the circumference of the cell cylinder should not obscure the conclusion that the insertion of new glycan strands between resident glycan strands leads to the growth of the cell in the axial or lengthwise direction. The cell surface should be viewed with the strands going primarily in the hoop direction. As with combed hair, the strands go primarily in a common direction, although there may be many strands out of place.

The absence of long-range order around the circumference of the bacterial cell makes the homeostasis of cell width or circumference a problem. If single hoops or long helical structures (15) encircled the cell, then new hoops made in a one-to-one correspondence with the resident hoops could keep the cell circumference invariant. The data on strand length make such a mechanism unlikely.

Strand arrangement can be reanalyzed by considering a cell with its wall removed and allowed to grow so that the peptidoglycan is synthesized de novo. If spheroplasts are placed on an agar medium, some cells will recover and produce colonies. The new peptidoglycan may grow in all directions, with no proper cell wall formed in most cells. If an occasional cell has strands going primarily in one direction, then future strands would be constrained to grow in the same direction as the new strands are laid down adjacent to existing strands. A successful cell has daughters with strands going perpendicular to the long axis of the cell. Thus the arrangement of strands in the cell is determined by the preexisting strands. The cells we observe are those that successfully arranged their peptidoglycan around the hoop direction; daughter cells preserve this arrangement.

The Peptidoglycan Macromolecule and the Problem of Cell Growth

Because each of the peptidoglycan subunits is covalently linked to another subunit in the bacterial cell wall, the murein layer must be considered a single macromolecule. The concept of the bag-shaped macromolecule leads to the main question of bacterial cell growth. How does a macromolecule grow while retaining its topological configuration and enclosing all of the cell cytoplasm? The high turgor pressure on the cell wall requires that the peptidoglycan remain intact during growth so that the cell does not explode. The cell has developed a unique set of biosynthetic steps and biochemical safeguards to allow cell growth while maintaining the structural integrity of the peptidoglycan.

BIOCHEMISTRY OF PEPTIDOGLYCAN SYNTHESIS

Synthesis of the Pentapeptide Precursor

Although the subunit of the peptidoglycan was described above as a tetrapeptide, the unit building block for peptidoglycan is actually a pentapeptide. The biosynthesis of the pentapeptide precursor in the cytoplasm has been described by Park (126). The final product of a series of biosynthetic steps—a disaccharide with an attached pentapeptide—is transferred to a membrane-bound lipid carrier that transports the pentapeptide subunit out of the cytoplasm for insertion into the extant peptidoglycan. The pentapeptide precursor is made in an amount that reflects the rate of synthesis of cell wall at any time during the division cycle. Evidence supporting this comes from measurements of the uptake of diaminopimelic acid and *N*-acetylglucosamine during the division cycle (29, 36). Small deviations from exponential uptake indicate that incorporation of radioactive precursors reflects the synthesis of peptidoglycan during the division cycle. Whatever pool variations may be postulated, these are the result of changes in the rate of peptidoglycan synthesis and not the cause of these changes.

Transfer of the Pentapeptide to the Growing Chain

There are a number of transglycosylases that may be involved in the transfer of the pentapeptide to the peptidoglycan (116, 126). Chains are extended by the transfer of the disaccharide-pentapeptide of the end of a chain. No information is available on how new chains are started. New chains presumably start in some random manner and extend in a progressive manner until chain extension randomly stops. These stops occur frequently—at least compared with the progressive synthesis of protein, DNA, or RNA—producing short chains 6 to 50 subunits long. End group determinations (56) indicate that the average chain length is short, compared with the circumference of the cell or the products of other biological polymerases. The end group determinations give an average chain length of 33 disaccharide units. Recently, the distribution of chain lengths has been determined by using amidase digestion followed by HPLC separation of the peptidoglycan chains according to length (71), and the results were similar but differed in detail. The length of peptidoglycan chains was disperse, with a wide range from 2 to 23 subunits, with some chains extending up to 30 disaccharide units. The predominant lengths were between 5 and 10 disaccharide units. (The HPLC results are slightly different from the end group results; it is very likely that the HPLC assay is a more accurate measure of the strand length in peptidoglycan.) This supports the earlier reports that peptidoglycan chains are not extremely long and certainly are not long enough to encircle a cell about its circumference. For a cell with a width of 0.6 μm, the glycan strands are about 1/300 of the cell circumference.

Cross-Linking of Peptidoglycan Chains

After, or perhaps simultaneous with, glycan chain extension, there is a cross-linking of the newly synthesized chain to an adjacent resident chain. This occurs by the formation of a peptide link between the penultimate D-alanine of the donor pentapeptide and an ε-amino group of an adjacent acceptor diaminopimelic acid residue. (A donor is the peptide chain with the D-alanine participating in the cross-linking, while the acceptor is the chain that has the diaminopimelic acid participating in the cross-linking.) The energy for the cross-linking appears to be located in the peptide bond between the fourth and fifth amino acids of the donor chain. At the time of cross-linking, the D-Ala–D-Ala bond in the donor pentapeptide is broken and the donor chain is converted to a tetrapeptide.

There are carboxypeptidases in the cell that remove the final D-alanine from the pentapeptide, whether or not cross-linking has occurred (45). Shortly after a pentapeptide is

inserted into the surface layer, the pentapeptide is shortened either by cross-linking or by carboxypeptidases to produce a tetrapeptide (55). In either case, the ability to serve as a donor in cross-linking is essentially lost. Recent work has revealed many additional, albeit minor, cross-links (such as between two diaminopimelic acid molecules), which may be brought about by the energy from the bonds between the third and fourth amino acids (54–56). The tetrapeptide can still serve as an acceptor in cross-linking when new strands are inserted adjacent to the tetrapeptide.

RELATIONSHIP OF MASS SYNTHESIS TO PEPTIDOGLYCAN SYNTHESIS

We must now consider the regulation of cell surface synthesis. It appears that the cell makes neither too much nor too little peptidoglycan. There is a uniform peptidoglycan layer with no buckling due to excess and no cell lysis due to deficiency.

Figure 2 is a schematic diagram of the murein of a cell. The stretched linkers are at the outside of the cell. Before the stretched cross-links are broken, new cross-links are in place, connecting the glycan strands destined to separate. Koch (92–94, 97) proposed that the stretching of peptidoglycan leads to bends in the bond angles in the cross-links, lowering the energy of activation of the cutting reaction. The energy of activation for the hydrolysis of a peptide bond is approximately 10 to 20 kcal/mol (approximately 42 to 84 kJ/mol). If a stress is applied to this bond—by stretching it, for example—the calculated decrease in the energy of activation is approximately 4 kcal/mol (approximately 17 kJ/mol). This leads to an increase in the rate of hydrolysis by as much as 10^5- to 10^{10}-fold when comparing stressed with unstressed peptidoglycan (92). Over a short time, there is a small increase in the mass of the cytoplasm leading to a small increase in the turgor pressure over the entire cell surface. The pressure is due to growth of the cell mass by incorporating nutrients, synthesizing macromolecules, and performing other activities, while the cell surface remains constant. This increase in turgor pressure, whether produced by the actual presence of more high-molecular-weight cytoplasmic material or by the influx of ions due to active or passive transport, leads to an increased stress on the load-bearing bonds all over the surface. As the bonds stretch, there is a steady lowering of the energy of activation for cutting the stretched cross-links. Somewhere on the cell surface, the energy of activation is low enough to allow an enzyme to hydrolyze an existing load-bearing cross-link. Cutting of load-bearing bonds leads to a separation of the strands previously held together by the cross-link. Assume that the cut does not remain localized but continues down the strands in a zipperlike fashion. This allows the insertion of the strand that was below the surface into the load-bearing layer. The zipperlike movement of the hydrolytic activity may come about because a single cut leads to a large increase in the stress on the adjacent bonds between two strands; thus the cutting action would progress between two adjacent glycan strands. As the strands separate, there is a slight increase in the total volume of the cell due to the increase in cell surface (Fig. 3). This increase in volume relieves the stress throughout the cell surface. Bonds all over the cell are slightly relaxed. However, this respite does not continue for long. The cell mass increases continuously and exponentially during the division cycle (28). Again, there is an increase in mass or internal cytoplasm and in turgor pressure; further bond stretching, bond cutting, and strand separation; and finally, an increase in cell volume. As the cutting of the bonds is distributed randomly over the surface, there is a diffuse intercalation of new strands between the old strands. The volume of the cell just accommodates the volume of the mass through this mass increase and surface growth. If mass synthesis is inhibited, there is a cessation of cell surface growth. In the cell, the stress on the cell surface is constant, the internal pressure is constant, and as the volume expands to just enclose the cell mass, the cell density is constant. This view of the growth of the cell surface has been called the surface stress model by Koch (92).

The surface stress model was originally developed to explain the shape of the poles of gram-positive streptococci (now called enterococci). A zonal growth pattern at a leading edge produces the streptococcal cell shape. The surface stress model was then extended to explain the growth of gram-positive, rod-shaped bacilli. Layers of completed but unstressed peptidoglycan are first synthesized adjacent to the cytoplasmic membrane to form the innermost layer of peptidoglycan. Then cell growth leads to a continuous series of externalizations of this interior material, with a final sloughing off of the peptidoglycan. Considering that the poles are more stable than the side wall, the mathematical analysis of the rate of turnover of cell wall material fits the observations. The surface stress model is a general explanation of bacterial surface growth, and its application to gram-negative cell walls is a special case of a more general theory.

In gram-negative cells, the load-bearing layer of peptidoglycan is essentially a monolayer of material surrounding the cell. When the cell grows, there must be a cleavage of the load-bearing layer. If the layer were merely cut, with no prior preparation, then the cell would lyse and the cell cytoplasm would burst out of the cell. According to the postulates of the surface stress model, the cell does not cut a preexisting load-bearing bond before there is another bond in place; there exists, according to Koch, a "smart autolysin" (98). As an analogy, consider a high-pressure bicycle tire. The pressure inside this rubber tube is of the same magnitude as that inside a bacterial cell. One way to make the tube longer is to cut it perpendicular to the circular axis, place a new piece of rubber between the exposed ends, and seal the new edges to the old rubber. As soon as the tire is cut, the air would leak out and the tire would be deflated. An alternative approach is to go inside the inflated tube, seal in place a rubber patch approximately the length the tube is to lengthen, then go outside the tire and cut the outer rubber. The tire will grow, the patch will expand, and the air will be retained in the tire. This elongation is successful because the patch was inserted before cutting the tire. The surface stress model postulates that there must always be an intact or structurally continuous cell wall with new strands in place before any part of the cell wall under stress is cut. Synthesis occurs by a make-before-break method; the strands to be inserted are in place before any cutting of load-bearing bonds. This view of the internal pressure of the cell, approximately 5 to 7 atm (approximately 507 to 709 kPa) means that the internal pressure not only is a problematic force that the cell must contend with, but is actually a positive force that allows the cell to grow. It is the turgor pressure, producing the stress on the cell surface, that leads to the continuous increase in cell surface and cell volume during the division cycle.

Any variation in the rate of peptidoglycan synthesis is ultimately related to mass synthesis. What may change is the relationship of how much surface is made per unit increase in

cell mass, but the ultimate cause of cell wall growth is cell mass increase. The surface stress model implies that there is no timing mechanism, or regulatory system, that affects the rate of surface synthesis other than the increase in cell mass. If there were such a timing mechanism, one could alter the rate of peptidoglycan synthesis independently of the rate of mass synthesis; such a regulation has not been observed.

With this view of the regulation of peptidoglycan synthesis, we can now look at the pattern of cell surface synthesis during the division cycle of rod-shaped, gram-negative bacteria. However, before describing this pattern, it is important to review the historical context from which our understanding has emerged.

EARLY STUDIES ON THE PATTERN OF CELL SURFACE SYNTHESIS DURING THE DIVISION CYCLE

The history of the analysis of cell surface synthesis during the division cycle is different from the history of DNA synthesis during the division cycle. The pattern of DNA synthesis was discovered relatively early (in 1967 to 1968), and the large amount of work on DNA synthesis that followed was placed within a single framework. In contrast, for many years there was no consensus on the synthesis of cell surface during the division cycle. There were many different views, many interpretations, many experimental results, and no common thread to the analysis. Only within the last few years has there emerged a single foundation for understanding cell wall synthesis during the division cycle. Thus, some of the early work on cell surface synthesis is obsolete. Nevertheless, it is important to review these early studies if only to see what was correct and what must be reinterpreted in the light of later results.

Early Studies on the Location of Cell Surface Synthesis

The first two decades of the study of gram-negative bacterial cell wall growth were dominated by the notion of growth zones—a growth zone is a localized area of cell surface growth. Changing the number of these zones could explain alterations in the rate of surface synthesis. There are three sources for the idea of zones. One is the early recognition that in *Streptococcus* spp. the cell wall is a rigid structure that grows at one edge (74). In *Streptococcus* (now *Enterococcus*) spp., old cell wall is not metabolized and new material is not inserted within the old wall material. This zonal growth pattern served as a model for zonal growth in gram-negative bacteria. The second source for the notion of growth zones is the analogy to DNA synthesis. DNA replication is regulated by the insertion, at appropriate times, of new replication points at the origins of DNA (35, 72). This idea, applied directly to cell wall synthesis, suggests that there are a particular number of growth zones and that, at some time or times during the division cycle, new growth zones are inserted. At the time of insertion or activation of these zones, there would be a change in the rate of cell wall synthesis. At the simplest level, if a cell had one zone and another zone were activated, there would be a doubling in the rate of surface synthesis. This is directly analogous to the situation of chromosome replication, in which the termination of one round of replication can occur at the same time as the initiation of two new rounds of replication; in this case there is a doubling in the rate of DNA synthesis at the time of termination and initiation. The third element leading to the idea of zonal growth was the proposal of the replicon model to explain DNA segregation. Because there is no visible mitotic apparatus, the regular segregation of DNA at division was explained by the binding of DNA strands to the cell surface with cell wall growth taking place between the bound DNA strands (82). The wall growth between the surface-bound DNA strands could lead to their separation and sequestration in the two new daughter cells. It appeared that zonal growth, particularly in the center of the cell, was an important requirement for DNA segregation. Thus we see the emergence of growth zones from a theoretical point of view. At the time of the proposal of the replicon model, however, there was definitive evidence that cell surface growth was diffuse and not zonal (148).

Zonal growth of the cell wall may be contrasted with diffuse growth, in which the entire surface of the cell is available as sites of cell wall growth. Different types of zonal growth have been postulated, from the proposal of a conserved unit cell to the growth of one or a few zones over the cell surface.

Experimental support for zones of wall synthesis. When cells were labeled for a short time with diaminopimelic acid and analyzed for the location of grains by autoradiography under the electron microscope, cells of all sizes had a preferential location of zones of incorporation in a relatively narrow band at the center of the cell (138). This result was interpreted as indicating a preferential zone of growth in the center of the cell. New zones would appear in the new daughter cells at some later time. Immunofluorescence analysis also supported the insertion of discrete zones of synthesis in the side wall of rod-shaped cells (21). When pulse-chase experiments were performed, the evidence for zones was ambiguous (143), and it was concluded that there was a randomization of the material in the initial zone. At the same time that autoradiography results were suggesting a zonal growth mechanism, kinetic measurements of cell wall synthesis during the division cycle indicated that in the middle of the cycle there was a sudden doubling in the rate of peptidoglycan synthesis (75, 125). This result was compatible with the idea of zonal growth.

The main controversy to emerge from these studies was not whether there were zones, but when zones were activated. One model proposed that growth zones were produced at particular times during the cell cycle and that the zones grew at rates proportional to the growth rate (66, 67, 134, 135, 164). Pierucci (128) suggested that new growth zones, with a finite life span, were activated at the initiation of new rounds of chromosome replication. Pritchard (131) proposed that cell wall synthesis was determined by an unregulated gene located near the terminus of the chromosome. A doubling in the rate of surface synthesis was predicted to occur when this gene replicated; the rate of envelope synthesis at each of these zones was assumed to be constant. Another model was proposed (46, 47) wherein cells abruptly increased their rate of elongation at a critical length; this length was proposed to be twice the minimal cell length.

Unit cell model. The unit cell model has been proposed as a particular zonal growth pattern (46). It proposes that cells grow only from one pole, producing one daughter cell with completely new peptidoglycan in the side wall and one daughter with completely old peptidoglycan in the side wall. One might imagine an analogy with the growth of *Saccharomyces cerevisiae*, in which a new bud appears on a conserved mother cell. The experiments demonstrating the unit cell were microscopic observations of cells growing in only one direction; i.e., one pole appeared fixed and the other pole moved when the cells grew in length. In retrospect, the preferential attachment of one cell pole to the

substrate and the free movement of the other pole could not be eliminated (although it was considered); this preferential attachment could produce the appearance of growth in one direction. This proposal was supported by additional data (9, 10) indicating that phage attachment sites are inserted asymmetrically on the cell surface. The unit cell model (at least for peptidoglycan) was decisively eliminated by the analysis of the distribution of radioactive diaminopimelic acid on each of the two cell halves of dividing cells (150). Statistical considerations alone suggest that one of the two halves would show more grains than the other. Verwer and Nanninga (150) determined whether the smaller number of grains in one half of the cell was attributable solely to statistical variation or whether there was actually a bias to fit an asymmetrical (unit cell) pattern of peptidoglycan synthesis. Their results showed that synthesis of cell wall in the two cell halves was the same; this result decisively eliminated the unit cell model.

Evidence against zonal growth. Quantitative analysis of the dispersion of label indicated that there were no conserved portions of the bacterial cell (148), although the presence of numerous but small conserved regions could not be eliminated. The earliest experiments on cell wall growth, in which fluorescent antibodies were used to label the cell surface, indicated that the wall grew diffusely with no conserved areas (6, 23, 117). Additional evidence against zonal growth was based on autoradiographic evidence that new cell wall material can be inserted over the surface, i.e., side wall, of the cell (16, 17). Similar results were obtained for the matrix protein attached to the peptidoglycan (8). More refined autoradiographic evidence was then presented, which showed that there were no apparent zones of synthesis before invagination (158). Biochemical support came from studies of the ADRR, a technique that measures the pattern of strand insertion into the cell wall. These studies indicated that there were essentially no conserved areas of the cell wall and that new material was inserted between any two strands (15, 45).

One of the strongest results supporting diffuse growth of side wall peptidoglycan comes from membrane elution experiments. If there were few zones of insertion, then one would expect that after a time there would be a sudden drop in the elution of radioactive diaminopimelic acid from cells bound to the membrane. Such a drop is not observed, indicating that growth of the side wall is diffuse (29). Not only did these membrane elution experiments support diffuse growth, but also they supported the conclusion that there was no release or turnover of peptidoglycan.

To keep the history straight, one must note that both of these results—the stability of peptidoglycan (i.e., no release or turnover of peptidoglycan) and the diffuse growth of the surface—were originally demonstrated by the classic experiments of Van Tubergen and Setlow (148), who used simple quantitative autoradiography and poisson distribution analysis.

Early Studies on the Rate of Peptidoglycan Synthesis during the Division Cycle

Early measurements of the rate of peptidoglycan synthesis during the division cycle supported the zonal growth model. One study reported that the rate of diaminopimelic acid incorporation increased shortly before the end of the division cycle (75). Other studies indicated that the rate of peptidoglycan synthesis accelerated (i.e., there was a step function when the rate of incorporation of diaminopimelic acid was measured) toward the end of the division cycle (75, 102, 125, 138, 143).

A different approach to the problem took advantage of the measurement of cell growth during the division cycle. The cell surface of the rod-shaped cell is relatively rigid, so one can measure the lengths and widths of cells during the division cycle and derive the pattern of cell surface growth. When this was done for synchronized cells, two different results were obtained for two different strains (118). Later analyses suggested that the original data were compatible with an exponential increase in length during the division cycle (101). Observations of growing single cells suggested that growth was also close to exponential, or at least was continuous (141).

The original finding of two different growth patterns in different strains of what is normally called *E. coli* raises interesting philosophical questions. Is it expected that strain differences within the same species would produce two different patterns of growth, an aspect of the cell that one might consider fundamental to its nature? I take the position that there is going to be only one answer to the question of how *E. coli* grows. There are certain aspects of cell growth that are so fundamental as to override strain differences. For example, two strains may differ in the sequence of a particular enzyme because the exact sequence is not believed to be essential to the growth or existence of the cell. In contrast, I suggest that there are some aspects of the cell—the pattern of cell growth and cell surface extension—that are so fundamental as to be found in all cells described as *E. coli*. Just as human beings come in many colors, sizes, and shapes, certain aspects of the structure of human beings—bilateral symmetry and the placement of hands and feet—are constant. In the same way, I postulate that there will be only one pattern of surface growth. This postulate of a unity of cell growth pattern means that we can compare results in different strains and try to find the best experiments to understand the mode of cell surface growth in gram-negative bacteria.

Another approach to determining the pattern of cell growth during the division cycle is the Collins-Richmond method (24). Length is the easiest cell size variable to measure, and most work has centered on the determination of length as a function of cell age. This method is not as useful as was originally believed (30). One set of analyses, on very good data, was unable to distinguish among a number of different models of cell growth during the division cycle (65, 103). Nevertheless, the results of the Collins-Richmond analysis are consistent with an exponential growth pattern during the division cycle. As we shall see, the actual pattern of growth is extremely close, but not quite, exponential. The Collins-Richmond method could not have distinguished the actual pattern from exponential.

Summary of Early Work on the Rate of Cell Surface Synthesis

Two fundamental ideas emerged from the early work on surface growth during the division cycle. The most important idea on the regulation of cell surface growth was that triggers and discrete mechanisms regulated the synthesis of peptidoglycan independently of other synthetic processes occurring in the cell (139). As we shall see, this idea has not survived. The second idea to emerge from these early studies was that the methodology used to measure cell growth could not solve the cell surface regulation problem. For each question there were at least two answers; con-

flicting data abounded, and most results were interpreted without considering the growth of the entire cell.

RATE AND TOPOGRAPHY OF PEPTIDOGLYCAN SYNTHESIS DURING THE DIVISION CYCLE: NEW MODEL BASED ON CONSTANT CELL DENSITY

A proposal that considers cell wall synthesis within the context of total cell growth can accommodate and explain almost all of the data on cell surface growth. This proposed pattern is interesting because it is derived from a priori considerations as well as strong experimental results.

According to the surface stress model, the relationship of mass synthesis to the growth of the cell surface implies that the cell surface is made to perfectly enclose, without excess or deficit, the cytoplasm synthesized by the cell. The amount of cell cytoplasm increases continuously and exponentially during the division cycle (28, 43, 44). Therefore, cell surface is made continuously. We now come to the question of the actual rate of surface synthesis during the division cycle. What is the precise pattern of synthesis for cell wall, or peptidoglycan in particular, during the division cycle? As will be seen, although wall is made continuously and is dependent upon an increase in the amount of cytoplasm, it is not made exponentially during the division cycle.

Consider an imaginary cell in which the cytoplasm is enclosed in a tube that is open at each end. Assume that the cytoplasm remains within the bounds of the tube. The cytoplasm in the newborn cell is encased in the cylinder of cell surface made up of membrane and peptidoglycan. As the amount of cytoplasm increases exponentially, the tube length increases to exactly enclose the newly synthesized cytoplasm. The cell surface increases exponentially in the same manner as the cytoplasm. The cell length is directly proportional to the amount of cytoplasm present, and thus cell surface in this particular (and imaginary) cell increases exponentially. When the amount of cell cytoplasm doubles, the tube divides into two new cells and the cycle repeats. In this imaginary open-ended cell, the amount of cytoplasm increases exponentially, the internal volume of the cell increases exponentially, and the surface area increases exponentially as well. The density of the cell, i.e., the total cell weight per cell volume, is constant during the division cycle. Furthermore, because both the surface and the cytoplasm increase exponentially, the ratio of the rate of cytoplasm synthesis to the rate of cell surface synthesis is constant throughout the division cycle. However, a real rod-shaped cell does have ends, and therefore the pattern of cell surface synthesis during the division cycle is not exponential. If the cell surface were synthesized exponentially, the cell volume could not increase exponentially, and there would have to be a change in cell density. Cell density, however, is constant during the division cycle (105).

An illustration of a proposal for cell surface synthesis that allows an exponential increase in cell volume, and therefore a constant cell density (29), is presented in Fig. 5. Before invagination, the cell grows only in the cylindrical side wall. After invagination, the cell grows in the pole area and the side wall. Any volume increase required by cell cytoplasm increase that is not accommodated by pole growth is accommodated by an increase in the side wall. The cell is considered a pressure vessel (92), and when the pressure in the cell increases, there is a corresponding increase in cell surface area. The pole is assumed to be preferentially synthesized (when invagination is taking place) by mechanisms that are not yet known. Because a continuously changing volume is being accommodated in the growing poles, there is a varying amount of growth in the cylindrical wall which accommodates the exponentially increasing cell mass (29, 36). Whatever volume is not accommodated by increases in pole growth, after the start of invagination, is accommodated by side wall growth. The sum of the volume increase in the pole

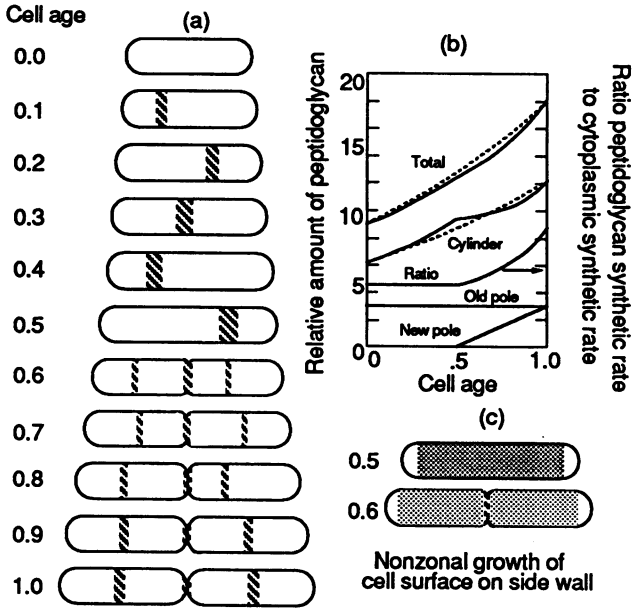

FIG. 5. Rate and topography of peptidoglycan synthesis during the division cycle. The newborn cell at the left (a) is drawn with a cylinder length of 2.0 and a radius of 0.5. Before invagination, the cell grows only by cylinder extension. The cells in panel a are drawn to scale, with the volume of the cells increasing exponentially during the division cycle. The shaded regions of the cell indicate the amount and location of wall growth (whether in the pole or the side wall), during 10% of a division cycle. The width of the shaded area is drawn to scale. Cell surface growth actually occurs throughout the side wall (c), and not in a narrow continuous zone. Before invagination the ratio of the rate of surface increase to the rate of volume increase is constant. When pole synthesis starts, at age 0.5 in this example, there is an increase in this ratio. Any volume not accommodated by pole growth is accommodated by cylinder growth. At the start of pole growth there is a reduction in the rate of surface growth in the cylinder. As the pole continues to grow, there is a decrease in the volume accommodated by the pole and an increase in the rate of growth in the side wall. This is schematically illustrated by the thinner sector in the expanding side wall immediately after the start of constriction. As the new pole increases in increments of equal area between the indicated ages, the volume accommodated by the new poles continuously decreases. Therefore, the growth rate in the cylindrical portion increases continuously during the constriction period. At the end of the division cycle, the rate of synthesis in the cylinder is the same as the rate for a newborn cell. There is no sharp change in the rate of cylinder elongation at the instant of division. At the upper right (b) is a plot of the expected pattern of accumulation of peptidoglycan or cell surface during the division cycle. The total accumulation of peptidoglycan is the sum of the individual accumulations of new pole, old pole, and cylindrical side wall. The dotted line represents the expected pattern for exponential synthesis. The ratio of the rate of surface synthesis to the rate of cytoplasm synthesis is indicated. At the lower right (c) an explicit illustration of the dispersive, nonzonal growth of the side wall is illustrated for cells of ages 0.5 and 0.6. There is a decrease in the density of incorporation of new cell wall material after invagination starts, as indicated by the side wall shading. Reprinted from reference 33 with permission.

and the side wall precisely accommodates the exponential increase in cytoplasm. Thus, at the start of pole growth, in the example given in Fig. 5, a large volume is accommodated in the pole and there is a small amount of side wall extension. As the pole volume made by pole growth decreases, the amount of growth in the cylindrical side wall increases. At the very last instant of pole growth, at the end of the division cycle, when the volume accommodated by the growing pole is infinitesimally small, essentially all of the growth of the cell is in the side wall. The rates of side wall growth before and after the act of pole completion are almost identical, and at division there is a smooth transition with no abrupt changes in the rate of side wall synthesis.

The resulting pattern of synthesis is approximately exponential (Fig. 5b). The formula describing surface synthesis during the division cycle is complex and includes terms for the shape of the newborn cell, the cell age at which invagination starts, the pattern of pole synthesis, and the age of the cell. It is simpler to understand the pattern of surface synthesis by considering and measuring the ratio of the rate of surface synthesis to the rate of cytoplasm synthesis. Before invagination, cell surface growth occurs only by cylindrical extension. As the width of an individual cell is constant during the division cycle (see the discussion below), the rate of surface synthesis before invagination is directly proportional to the rate of cytoplasm synthesis. This can be understood by noting that side wall synthesis occurs as a narrow coin-shaped disc, indicated in Fig. 5a by the shaded areas on the cells between ages 0.0 and 0.5. (As noted in Fig. 5c, side wall synthesis is actually diffuse, but can be represented by a single coin-shaped area as drawn in Fig. 5a.) Each of these coins has an edge and a volume. The volume of two coins is twice that of one coin, and the edge area of two coins is also twice that of one coin. The thickness of the shaded area is a measure of the rate of both area and volume increase in the growing cell, so the ratio of the rate of surface synthesis to the rate of volume or mass increase is constant before invagination. After invagination starts, there is an increase in the rate of surface synthesis relative to the rate of cytoplasm synthesis. This is due to a higher ratio of surface to volume in a sphere than in the side wall of a cylinder. This can be seen in Fig. 5a, which shows that as the pole nears completion, equal areas of pole increase are associated with decreasing cell volumes. In Fig. 5a, the surface of the pole is assumed to increase with equal surface areas during the period of invagination. The volume accommodated by the pole decreases as the edge of the growing pole goes out from the cylinder. Thus, the ratio of surface to volume must increase. When the combined increases in volume and area in the poles and side walls are considered, one sees an increase in the ratio of the rate of surface synthesis to cytoplasm synthesis after invagination starts.

When invagination starts, the growth of the pole accommodates some of the volume increase required by the increase in cell mass or cytoplasm. Whatever mass is not accommodated by pole growth is accommodated by side wall growth. This model predicts that when invagination or pole growth starts, there will be a relative decrease in the rate of side wall synthesis. By having this pressure relief system, the volume of the cell increases perfectly exponentially to accommodate the exponential increase in cell mass. This view of cell surface synthesis leads to the powerful "ratio-of-rates method," in which the ratio of the rate of surface synthesis to the rate of cytoplasm synthesis is measured, rather than the rate of surface synthesis alone. This ratio is predicted to be constant during the first part of the division cycle, when there is only side wall growth, and to increase during invagination (Fig. 5b). This pattern of cell surface synthesis has been observed in *S. typhimurium* (29) and *E. coli* (36). One consequence of this model of surface synthesis is that at no time is surface synthesis exponential, because the rate of surface synthesis is not proportional to the amount of surface present over any time interval during the division cycle. Even though the incorporation of radioactivity into wall and protein is parallel prior to invagination, this does not mean that wall synthesis during this period is exponential, even though protein synthesis is exponential.

Quantitative Analysis of Wall Growth during the Division Cycle

What is the formula for the pattern of surface synthesis during the division cycle? Historically, much of the effort expended on analyzing the synthesis of the bacterial cell surface has been spent searching for a particular formula—either linear, bilinear, stepwise, or exponential—and performing experiments that would prove one or another formula correct. Some of these formulas were of interest because they predicted density variations during the division cycle. These variations would then have been clues to the control of cell cycle events such as the initiation of DNA synthesis. A qualitative description for cell wall synthesis was presented in the previous section with a central assumption that the cell density during the division cycle is constant; i.e., the volume of the cell increases exponentially with the cytoplasm. Let us look at this model in a more rigorous and quantitative way.

Analysis of the simple cylindrical wall, hemispherical pole model of the bacterial cell—special theory. A formula for the amount of surface synthesis present on a cell, for a special case, can be derived for the proposal illustrated in Fig. 5. The assumptions made for the calculation are that (i) the rate of mass and volume increase is exponential during the division cycle; (ii) cell density is constant during the division cycle (which means that cell volume increases exponentially); (iii) constriction starts at a particular age during the division cycle; (iv) the cell can be approximated by a cylinder capped with two hemispheres; (v) the cell grows with a constant width (diameter) during the division cycle; (vi) any volume increase in the cell not accommodated by the increase in new pole volume is accommodated by an increase in the cylindrical wall of the cell; and (vii) the new pole grows at a constant rate of area increase after the start of constriction. The last assumption is more of a mathematical convenience, and it, as well as the assumption that the pole is a hemisphere, can be relaxed to obtain a more general description of the pattern of wall growth.

Given these assumptions, the surface area at any time during the division cycle (where the age is is given by α) is (29)

$$A_{\alpha_{tot}} = \frac{8}{3}(2^{\alpha})\pi r^2 + 2(2^{\alpha})\pi r L_{\alpha_{cy}} + \frac{4}{3}\pi r^2 + \frac{4}{3}\pi\frac{h_{\alpha}{}^3}{r}$$

In this formula, r is the radius of the cell, $L_{\alpha_{cy}}$ is the length of the cylindrical portion of the cell, and h is the height of the growing pole measured from the end of the cylindrical portion of the cell. Inspection of the equation of surface growth during the division cycle clearly indicates that surface synthesis is not exponential. The precise pattern is very close to exponential (Fig. 5b), and by direct, standard methods (synchronization, single labels, size measurements,

etc.), this pattern would be very difficult to distinguish from exponential.

If we consider how cell wall is synthesized in comparison with cell mass, which is synthesized exponentially (28, 43, 44), we can derive an equation for the relative rate of surface (A) compared with mass (M) synthesis:

$$\lambda\left(\frac{dA}{dM}\right) = 1 + \kappa\left(\frac{h_\alpha{}^2}{2^\alpha}\right)$$

(The λ and the κ are constants inserted to make the formula dimensionally correct.) Before invagination, when h, the height of the new pole, is zero, the ratio of the rate of surface synthesis to the rate of mass synthesis is constant. After invagination, this ratio increases, because the second term has a positive value. The exact pattern of increase depends on the relationship of the height of the new pole at particular ages during the division cycle.

Analysis of the simple cylindrical wall, hemispherical pole model of the bacterial cell—general theory. A general formula, independent of the pattern of pole synthesis or cell density, has been derived by Keasling (89) and is given by

$$\frac{dA}{dM} = \left(\frac{1}{\rho} + \frac{1}{\gamma}\cdot\frac{h_\alpha{}^2}{2^\alpha}\cdot\frac{dh_\alpha}{d\alpha}\right)\cdot\frac{1}{\beta}$$

where β is the ratio of the cylinder surface area to volume, ρ is the density of the cytoplasm, γ is the adjusted initial cell mass, and h_α is the height of the new pole as a function of the cell age, α. When pole synthesis is linear, the rate of pole synthesis is constant (i.e., $dh_\alpha/d\alpha = k$) and the general formula reduces to the specific formula given above for a constant rate of pole surface increase during invagination.

There are many other generalizations of this formula. For example, one could relax the restriction that the cell pole be approximated by a sphere. Consider two extremes, with the pole being either pointier or flatter than a sphere. If the pole were flatter, during invagination more pole surface would be synthesized per volume of cytoplasm extended in the pole. If other aspects are unchanged, the final ratio in the rate of surface to mass synthesis would be higher. In contrast, a pointer pole would have less surface per volume in the pole region and hence a lower ratio would be found. In addition to variations in the shape of the pole, one can consider other patterns of pole synthesis. If the rate of pole synthesis was proportional to the radius of the growing pole at the leading edge of pole growth, the increase in the pole would be rapid at the start of invagination and would slow as pole synthesis neared completion. Other combinations of rates of synthesis and pole shape may be considered, and future work will involve theoretical, biochemical, and biophysical analysis of the pattern of pole growth. It does not appear feasible to distinguish different models by using current methodology.

It is interesting to speculate that the relationship between cell surface increase and cell mass increase fits the classic allometric growth relationships developed at the beginning of this century. The exciting aspect of this observation is that this simple system obeys the classical allometric growth laws. That simplicity allows it to serve as a example for understanding more complicated allometric relationships.

Partitioning of surface synthesis between side wall and pole. It is generally believed that the best time to measure the synthesis of pole material is toward the end of the division cycle, when the pole is being completed. For this reason, experiments with synchronized cells have usually compared the peptidoglycan synthesized in newborn cells with the peptidoglycan synthesized in cells late in the division cycle (41). Inspection of Fig. 5 reveals that this initial assumption is not necessarily correct. Rather, it may be predicted that cells immediately after the initiation of pole formation are synthesizing the greatest proportion of pole as a fraction of the total new surface area. This relationship may be simply derived from the equations above, giving the proportion of material in either the pole or the side wall. Before invagination, all of the newly synthesized material is in the side wall. However, at the start of invagination, there is a maximum in the percentage of peptidoglycan devoted to pole synthesis which continuously decreases during the remainder of the division cycle.

This conclusion indicates that the idea of a switch from side wall to pole synthesis at some time during the division cycle (for example, see the discussion in reference 11) may not be the way to think about or describe cell surface growth. Instead of a switch, there is a partitioning of surface synthesis between the pole and side wall in such a manner as to allow the volume of the cell to increase exponentially during the division cycle. The partitioning is not constant, but varies continuously during pole formation. Regulation of cell surface is not an all-or-none decision between side wall and pole synthesis.

Implications of the pressure model of wall growth. The precise rate of surface synthesis and its measurement are less important than the unanticipated conclusion, presented here, that there is no description of the rate of peptidoglycan synthesis in terms of a simple mathematical formulation. Peptidoglycan synthesis is neither exponential nor linear, but is a complex pattern that is easy to describe. Before the start of constriction, when cylindrical extension is the only means of cell growth, the rate of peptidoglycan synthesis appears exponential because the differential rate of wall increase is similar to the differential rate of mass and volume increase. It is incorrect to say that synthesis is exponential. Exponential synthesis means that the rate of synthesis is exponential and that the total amount of material increases exponentially. For no period during the division cycle is this the case for cell surface synthesis.

Woldringh and colleagues have analyzed the pattern of cell surface growth during the division cycle by using electron-microscopic autoradiography. They noted that when the cell grows before invagination, there is an increase in the amount of mass per cell surface compared with that present at the start of the division cycle. However, the ratio of mass to surface at the end of the division cycle must return to be precisely that of the newborn cells. Therefore, during invagination there must be a preferential accumulation of cell surface compared with mass, in order to have the correct ratio of surface to mass at division (159, 163). This concept, which stimulated the proposal of the jump-invagination model (158), is rigorously explained by the equations presented here. If we take the ratio of the amount of surface to the amount of mass at any time during the division cycle, rather than the ratio of the rates of synthesis, there is an initial decrease in the ratio which is followed by an increase during the latter portion of the division cycle. This is because there is an increase in the ratio of the rate of surface to cytoplasm synthesis after invagination starts. The pattern of wall synthesis proposed here accounts for the ratio of surface to mass during the division cycle, as well as the ratio of the rates of synthesis of surface and mass during the division cycle.

Experimental Support for the Pressure Model of Cell Surface Synthesis

Given the constant density of the cell and the regulation of surface synthesis by mass synthesis, the general proposal made here is a priori correct. In addition, there are experiments that support the proposal.

Ratio of peptidoglycan synthesis to protein synthesis during the division cycle by using the membrane elution method. A simple proof of the proposed pattern of wall and pole synthesis could come from measurements of the rate of peptidoglycan synthesis during the division cycle. The problem, however, is that the predicted rate of synthesis is so similar to exponential that it is difficult to perform the requisite measurements and demonstrate the precise nonexponential pattern. The approach that was successful analyzed the prediction that the ratio of the rates of mass and surface synthesis would vary in a predictable way during the division cycle. The ratio-of-rates method uses two radioactive labels, one for cell surface (diaminopimelic acid) and one for cytoplasm (e.g., leucine), and determines the ratio of the rates of incorporation during the division cycle. The experiment is simple. Exponentially growing cells are pulse-labeled with differentially labeled diaminopimelic acid and leucine. The labeled cells are placed on the membrane elution apparatus, the newborn cells are eluted with fresh medium, and the radioactivity in the eluted cells is determined for the two labels. The ratio of the rates expected according to the proposed model is shown in Fig. 5b. The predictions of this model were confirmed by using diaminopimelic acid for *S. typhimurium* (29) and *N*-acetylglucosamine for *E. coli* (36). The initial results were obtained with *S. typhimurium* because it is 30 to 50 times more efficient at incorporating diaminopimelic acid than is *E. coli* when short pulses of label are used (38). Once the concept of the double-label experiment was understood, *N*-acetylglucosamine was used to demonstrate that the pattern of wall synthesis in *E. coli* was similar to that in *S. typhimurium*. The data, however, are not precise enough to prove that pole growth is linear. The important point regarding the ratio-of-rates method is that even slight deviations from exponentiality are experimentally detectable.

Cell density. A number of models of cell growth have used variations in cell density to regulate cell division (131). In contrast to those theoretical proposals, experiments indicate that the density of *E. coli* (and, by extension to a homologous organism, the density of *S. typhimurium*) is invariant during the division cycle (99, 105, 106, 107, 115). The relationship of constant density to the pattern of wall synthesis during the division cycle can be examined in two complementary ways. The proposed pattern of the synthesis of cell surface to cell mass means that the cell density is invariant during the division cycle. Thus, the stress model proposed here is consistent with constant cell density. Alternatively, for the assumptions made in the quantitative analysis, any model that is not equivalent to the proposal made here must predict a variation in cell density during the division cycle. Given a constant density and the rod-shaped organism, it is necessary that the organism grow as described in the model in Fig. 5. Only the pole shape or the precise pattern and timing of pole growth can be varied.

Segregation of peptidoglycan. Van Tubergen and Setlow (148) were the first to look at the segregation of peptidoglycan. They noted that there was a uniform segregation pattern indicating a large number of randomly segregated subunits. This was supported by the theoretical arguments of Koch (92) and by other experiments demonstrating a random dispersion of peptidoglycan (16, 17, 110). They also observed that peptidoglycan was stable and was not released from the cell.

The membrane elution apparatus gives a very simple and quantitative approach to the segregation problem. The results from long-term elution of diaminopimelic acid and leucine from the membrane elution apparatus indicate that cell wall material is subdivided randomly for at least six generations (29). The results are consistent with a halving of the lateral wall at each division and a random distribution of material at cell division. Any unit cell or zonal-growth model is incompatible with the experimental results.

Rate of cell length growth. From measurements of the cell length distribution of exponentially growing cells, it was concluded that cell growth is exponential (104, 108). The length of the cell, according to the model proposed here, increases almost exponentially; it increases in the same manner as the total surface area (Fig. 5b). Given the uncertainties in length measurements of cells and, in particular, measurements of living cells growing synchronously, the results on the length or surface increase during the division cycle are consistent with the proposal that cells grow so that cell volume increases exponentially.

Location of newly synthesized peptidoglycan. Woldringh et al. (158) noted that the incorporation of diaminopimelic acid into the lateral walls of cells with constrictions was significantly lower than incorporation into the lateral walls of cells without constrictions. This unexpected observation is predicted by the model proposed here (Fig. 5a and b). At the start of invagination, lateral-wall synthesis decreases. Woldringh et al. suggest that this increase in synthesis at the pole occurs " . . . at the expense of the activity [i.e., synthetic or growth activity] in the lateral wall." What is the mechanism of this redistribution of synthesis between the pole and the side wall? If there were a limited supply of enzymatic machinery or wall precursors available for peptidoglycan synthesis, then when pole synthesis started there would be a reduction, by competition, in the rate of lateral-wall synthesis. The alternative view presented here (Fig. 5a and b) suggests that there is no limitation in the amount of precursor or the enzymatic machinery for cell wall synthesis. When pole synthesis starts, by mechanisms not known, the increase in cell volume as a result of pole growth relieves the stress in the cylinder, and the rate of insertion of peptidoglycan in the lateral wall is reduced. The reduced stress means that there is less bond breakage and thus less insertion of new strands. Within the terms of the surface stress model, no proposal is made for any specific mechanism for changing the rate of wall synthesis other than the passive one of altering the amount of stress that a particular part of the cell receives during the division cycle.

Autoradiographic analysis of the rate of peptidoglycan synthesis. An alternative proposal for the rate of peptidoglycan synthesis during the division cycle was made by Woldringh et al. (158). Regarding the pattern of pole synthesis, they assumed an exponentially increasing pole area during constriction. The model proposed that the synthesis of pole area starts slowly and increases in rate while the pole area yet to be synthesized continuously decreases. More significant, this model proposes a jump in the rate of peptidoglycan synthesis when invagination starts, which is supported by autoradiographic evidence (158). Although the results summarized by Woldringh et al. show that there is a jump in the rate of peptidoglycan synthesis at the start of invagination, the original unpublished data kindly supplied by Woldringh

(155) indicates that a distinction cannot be made between a jump and a smooth increase in the rate of peptidoglycan synthesis. Thus, the autoradiographic analysis is compatible with the proposal made here of a smooth, continuous increase in the rate of peptidoglycan synthesis during the division cycle.

Leading-edge model of pole growth. Wientjes and Nanninga (153) have looked at the incorporation of radioactive diaminopimelic acid into cells with slight, medium, and deep constrictions. They found that the amount of radioactivity in the pole area appeared constant, as did the width of the peak of incorporation. They concluded that the rate of pole synthesis was constant and that synthesis occurred only in a narrow leading edge at the junction of the two daughter cells. This proposal is equivalent to the case in which the pole is synthesized linearly during invagination (29), as shown in Fig. 5. The additional proposal of a jump in peptidoglycan synthesis at the start of invagination is dealt with below.

Variation or constancy of diameter during the division cycle. One of the assumptions of the pressure model illustrated in Fig. 5 is that cell diameter is constant within a division cycle. The evidence for this proposition is unclear. Some experiments support a constant width, whereas others suggest a decreasing width during the division cycle. A study of the width of *Salmonella* cells as a function of cell length (3) indicated that the diameter did not systematically vary during the division cycle.

An alternative proposal was made by Trueba and Woldringh (145), who measured the lengths and widths of a large number of cells and observed a negative correlation, i.e., as the cell length increased, the diameter decreased. This effect was slight, but definitely within statistical bounds. As longer cells are presumed to be later in the division cycle, it was concluded that cells passing through the division cycle decrease their cell width. An explanation for this experimental result with a constant cell diameter has been proposed (33). If one considers that cells (i) are born with a relatively constant cell size, (ii) divide with a relatively constant cell size, (iii) have a constant cell density during the division cycle, and (iv) have some variability in cell diameter about some mean value, it must follow that thicker cells will be shorter at birth and division and thinner cells will be longer at birth and division. When the predicted results for a population are plotted, there is a negative correlation between length and width, but there is no systematic variation in cell width during the division cycle (33). The data of Trueba and Woldringh (145) are compatible with the postulate of a constant cell diameter.

Variation in cell diameter about a mean value without a systematic variation during the division cycle means that cells are constantly varying their diameter. However, this variation is extremely small within any one division cycle. A cell of mean diameter may produce daughter cells with an imperceptible increase in width; subsequent descendants may also increase in width. Thus, over many generations, cell width may increase to produce wide cells (or, conversely, decrease to produce thin cells), but the sum of these variations produces no systematic variation during the division cycle (33). Given the constant properties of cultures started from single cells, a thin cell must give rise to wider cells and a wide cell must give rise to thinner cells. This proposal accounts for not only the reported inverse correlation between cell length and cell width, but also the reported jump in peptidoglycan synthesis at the start of invagination (33).

Stability and turnover of peptidoglycan. There have been a number of reports of turnover of peptidoglycan in *Escherichia coli*. There is a significant release of labeled cell wall material to the external medium (19, 59, 63). A general review of turnover in a large number of bacteria has been published (48). Cells can take up some of these excreted fragments, so the amount released to the medium is a minimal measure of the turnover of peptidoglycan. It is suggested here that this observed turnover may be a special case, as studies using the membrane elution method reveal that the peptidoglycan is extremely stable.

The standard method for measuring turnover is to label cells, remove them from the label, grow them in unlabeled medium, and measure how much of the label is either excreted into the medium or found in the soluble pool of the cell. How can one measure the turnover of peptidoglycan by using the membrane elution method? Cells are labeled in cell wall and cell protein and filtered onto a membrane. The membrane is inverted, and the bound cells are allowed to grow. Consider that there are 100 cpm in both the wall and the protein. The protein would be released from the membrane elution apparatus, and its amount would decrease by one-half each generation. Thus, 50, 25, 12.5, 6.25, etc., cpm would be released each generation. If there were turnover of peptidoglycan, the first generation would be expected to give 50 cpm by division, with an additional 5 cpm if there were 10% turnover from the bound sister cell. Thus, 55 cpm would be released to the medium in the first generation. In the next generation, half of the remaining 45 cpm would be released, plus 10% from the bound cell, approximately 25 cpm. In the next generation, there would be 11 cpm, and so on. The ratio of diaminopimelic acid to leucine released from the membrane would decrease over time (55:50, 25:25, 11:12.5, etc.). A decrease is not seen in the membrane elution experiments, and the conclusion is that peptidoglycan, in *Salmonella* cells, is at least as stable as protein (29). A similar conclusion can be made for cell wall on the basis of comparison of *N*-acetylglucosamine-labeled peptidoglycan with leucine-labeled protein (36). Goodell and Asmus (60) suggest that there is some turnover in the peptidoglycan of *S. typhimurium*. This experiment was done with a strain that did not take up excreted peptides from the medium. Reanalysis of their Fig. 3 indicates that there is actually no turnover of peptidoglycan. There is no significant decrease in the radioactivity in the labeled cells during a 2-h period of growth; any decrease could be corrected for the loss of some other stable material such as DNA or protein.

The difference between *E. coli* and *S. typhimurium* with regard to turnover may be attributable to the fact that almost all the experiments on turnover in *E. coli* were carried out with a diaminopimelic acid auxotroph, strain W7. *E. coli* is rather impermeable to diaminopimelic acid (38). It is possible that the physiology of the organism is disturbed by a diaminopimelic acid limitation. This is supported by Driehuis and Wouters (49), who noted that limiting diaminopimelic acid led to the production of abnormal peptidoglycan.

It is likely that the last word on turnover of peptidoglycan has not been written, but a distinction must be made between two types of turnover that have been proposed. The first type of turnover is when material is excreted to the medium; this is an experimentally verifiable phenomenon. The second type of turnover is when previously made material is broken down and immediately reused for synthesis without excretion. It has been suggested that the peptidoglycan fragments are retained within the periplasmic space between the inner and outer membranes. This type of turnover is very difficult to measure. Some propose that in

cells with no measurable turnover, there may be turnover of the second type. It is my belief that since turnover with release does not occur in the first place, there is no need to postulate the existence of a second type of unmeasureable and unobservable turnover. If turnover with release of material had not been observed in the first place, no suggestion of turnover without release would have been proposed.

The stability of peptidoglycan postulated here assumes that cells are in balanced, exponential growth. When cells undergo transitions, such as inoculation of a culture or upon entering stationary phase, there may be a breakdown and rearrangement of peptidoglycan. This type of turnover should be distinguished from turnover occurring during normal cell growth.

The degradation of peptidoglycan in *E. coli* has been the center of a general model of peptidoglycan synthesis proposing that a large component of wall biosynthesis is due to an inside-to-outside movement of peptidoglycan, as in *Bacillus subtilis* (56, 78, 142). This proposal is based on the observed recycling of murein, the calculated amount of peptidoglycan per cell, and the metabolism of different dimeric peptidoglycan fragments (e.g., tetra-tetra and tetra-tri fragments). This model is based on a multilayered peptidoglycan structure. The recycling of peptidoglycan may be a strain-specific result, as there is no apparent release or recycling of peptidoglycan in *S. typhimurium* (29) or in *E. coli* B/r (36). Koch (98) has presented the arguments against such an inside-to-outside mechanism of peptidoglycan growth; but at this time the inside-to-outside mode of surface growth cannot be excluded. It has been suggested (77) that there is turnover even in *Salmonella* cells, but the release of degradation products to the medium is prevented by the outer membrane, and the fragments are reutilized by further growth. Thus, the absence of released material is not necessarily an impediment to the inside-to-outside model of growth.

CONTROL MECHANISMS FOR CELL WALL SYNTHESIS

Freeman Dyson, the noted physicist, has described the stance to be taken when confronted with a new scientific idea (51):

> . . . the professional duty of a scientist confronted with a new and exciting theory is to try to prove it wrong. That is the way science works. That is the way science stays honest. Every new theory has to fight for its existence against intense and often bitter criticism. Most new theories turn out to be wrong, and the criticism is absolutely necessary to clear them away and make room for better theories. The rare theory which survives the criticism is strengthened and improved by it, and then becomes gradually incorporated into the growing body of scientific knowledge.

It is in this spirit that I approach a number of theories related to the control of wall synthesis, control of invagination, and control of the division cycle. Since many of these proposals are mutually exclusive, it is clear that they must be reevaluated.

Stringent Regulation of Wall Synthesis

It has been proposed that the synthesis of cell wall is under the control of the stringent-relaxed system for the regulation of RNA synthesis (80, 81, 132). Conditions that lead to increased RNA synthesis (e.g., mutation to relaxed phenotype, addition of chloramphenicol to amino acid-starved cells) also lead to an increase in peptidoglycan synthesis. A simple explanation for the observation that peptidoglycan and RNA syntheses vary in parallel is that the increase in RNA leads to the increase in cell wall synthesis. The apparent regulation of peptidoglycan through the stringent response may be nothing more than the response of the cell surface to an increase in cytoplasm as a result of increased RNA synthesis. To demonstrate a direct stringent control mechanism, it is necessary to distinguish cytoplasm synthesis as the immediate cause of surface increase from direct regulation by the stringent control mechanism.

Regulation of Surface Synthesis by Cytoplasmic Signals

There have been a number of proposals stating that specific signals at particular times during the division cycle affect cell shape and surface synthesis. For example, a sudden change in the cytoplasmic calcium concentration has been proposed as a signal for a number of cell cycle events (76, 123). Cyclic nucleotides have also been suggested as being involved in cell cycle regulation (25). The evidence for division cycle variations in the concentrations or amounts of various cytoplasmic substances have been considered. It was concluded that there is no compelling evidence for variation in the concentration of any substance in the cytoplasm as a function of the division cycle (33). Cytoplasm does not vary in composition during the division cycle, and all cytoplasmic components (ribosomes, tRNAs, proteins, ions, metabolites, etc.) increase exponentially during the division cycle (33). If this general model of cell cytoplasm composition is correct, there is no specific increase in any cytoplasmic component that will trigger invagination.

An alternative to the cytoplasmic triggering model is the ratio model, analogous to the ratio model invoked to explain the initiation of DNA replication during the division cycle. I propose that if (and I stress the "if") a cytoplasmic component is the trigger of invagination, the ratio of the cytoplasmic signal to the cell surface initiates invagination. Consider the ratio of a certain substance to the number of poles on a cell. A newborn cell was two poles, and so during cell growth the ratio of the trigger to the number of cell poles continuously increases as the amount of cytoplasm increases. when the ratio reaches a certain value, invagination could be triggered. At this instant, there are now four poles per cell (two old and two new poles; for the sake of this discussion, we assume that even part of a pole is counted as a pole) and the ratio of trigger to pole is instantly halved. Thus one would observe a cyclic and recurring initiation of invagination at every doubling in cell mass. In this way, the division of the cell is correlated with the growth of the cell mass.

Regulation of Wall Synthesis by Peptidoglycan Structure

The structure of the peptidoglycan in different parts of the cell has been proposed as a trigger of invagination. This triggering may be due to a change in the chemistry of cell wall synthesis. For example, the peptidoglycan structure in the pole may be different from the side-wall structure. Mutant strains that grow as spheres show a rise in the total number of cross-links and a decrease in the average length of the glycan strands (142). However, other work shows that there are no major differences between cells growing as either long rods or spheres; any differences were due to

different growth rates (50, 147). Chemical analysis of poles from minicells compared with whole cells did not reveal any difference in composition between poles and side walls (64). More direct evidence comes from studies of cells labeled during the cell cycle. No changes in the composition of peptidoglycan synthesized during cell elongation or septum formation could be detected (41). Although it is not clear that the decisive experiment has been performed, no available evidence indicates that peptidoglycan composition changes during invagination.

Part of the problem with measuring differences in peptidoglycan structure as a function of the division cycle has been the possibly erroneous notion that the greatest difference would be seen by comparing the peptidoglycan synthesized in the youngest cells (with only side wall growth) with peptidoglycan synthesized in the oldest cells (with pole growth). As seen in Fig. 5, cells with the greatest proportion of pole growth may be those in the middle of the division cycle. In future experiments, this possibility should be taken into consideration.

A precise model for the regulation of wall synthesis based on structure proposes that the differential availability of tripeptide or tetrapeptide chains affects the activation of either side wall synthesis or pole synthesis (11). This model is based on the peptidoglycan composition of cells with mutations in different peptidoglycan-synthesizing functions and filamenting cells. It is not clear how this proposal explains the triggering of invagination at a particular time during the division cycle.

Heat Shock and Cell Division

When cells are exposed to a sudden increase in temperature, there is a period during which a small number of diverse proteins are preferentially synthesized (39). It has been suggested that the heat shock response or the heat shock proteins may be involved in cell division (146). This suggestion comes from the observation of altered patterns of cell division following a temperature shift in cells genetically altered in their heat shock response. Slight changes in temperature, however, affect cell division irrespective of genetic background. It is difficult to make a clear distinction between a defined relationship of heat shock to cell division and a pleiotropic effect of the heat shock response.

Cell Division Related to Specific Cytoplasmic Proteins

It is widely thought that the protein determined by the *ftsZ* gene may play a key role in triggering cell division (85, 112). Evidence has been presented that the protein determined by *ftsZ* is synthesized linearly during the division cycle (133). It is of interest that it was previously thought that the protein might have been synthesized at a particular time during the division cycle. When it was found that the *ftsZ* gene product was synthesized throughout the division cycle, the experimental measurements were plotted on rectangular graph paper to demonstrate that there were periods of linear synthesis of the protein. The method used to synchronize cells, the phosphate-starvation-entrainment method, very probably produces artifacts (33). A large number of starvations may produce abnormal and altered cells. In addition, it is difficult to distinguish exponential synthesis from linear synthesis by total measurement (33), so a rate measurement is necessary to distinguish the two patterns.

Other enzymes related to peptidoglycan synthesis have been reported to vary during the division cycle. For example, a carboxypeptidase has been reported to have its maximal activity at the time of division (7).

Proposals of cyclic variations in protein during the division cycle should be reviewed. Analysis of experimental approaches to determining cell cycle variations in protein synthesis indicates that there are many possible artifacts. If rigorous criteria were used for accepting synchronization experiments, many of the experiments proposing cell-cycle-specific variations in protein synthesis would be subject to reinterpretation (33).

Gearbox Model for Control of Septum Formation

The observation of a constant amount of expression for various genes related to septum formation has led to the proposal that some proteins are made at a constant amount per cell (1a). Since all cells have only two poles per cell, one could imagine that these proteins are present at a constant amount per septum or per pole. Although the data are intriguing, the model is still in a speculative stage.

Control of Cell Cycle by Phospholipid Flip-Out

A general model for the regulation of the division cycle envisions a once-per-cycle flip-out of phospholipids from the inner membrane to the outer membrane (122). It is conjectured that there is an increase in the phospholipid density in the inner monolayer compared with the outer monolayer of the bilayer membrane of the cytoplasmic membrane during the division cycle. At a critical lipid density there is a rapid movement of lipid from the inner monolayer to the outer monolayer. At this time a transient nonbilayer structure is proposed to be formed. When this flip-out occurs, a variety of events are thought to be triggered. The DNA initiation complex seems to be bound to lipid, so a change in membrane fluidity at a particular time in the division cycle may allow an initiation of DNA replication. The phospholipid flip-out may control the cell cycle by affecting the calcium concentration in the cell. The phospholipid flip-out model is speculative. In contrast to the flip-out model, it has been proposed that the surface density of the membrane remains constant as the membrane is synthesized in direct response to the cell surface area; no change in surface phospholipid density would be expected during the division cycle (33).

Control of Cell Cycle by Variation in Calcium Concentration

Variations during the division cycle of the degree of sequestration of calcium in the cell membrane have been proposed as a general mechanism for the regulation of the division cycle. The experiments supporting calcium as a regulatory element in the cell cycle are electron probe microanalyses of the calcium concentrations as a function of the cell cycle (20). By using this sophisticated approach to spatial ion measurements, it was concluded that at the time of cell division there is an increase in the concentration of calcium in the cell. Dividing cells were defined as cells over a certain length and containing a visible invagination. Only a small subset of the dividing cells were considered to be the dividing population. No indication of calcium concentration as a function of cell length for the entire culture is given, so the class of cells defined as dividing cells may be unrepresentative. Furthermore, the calcium concentrations in the cells (from electron probe microanalysis) may not be consistent with measurements of total calcium concentrations. A

pertinent question arises when one asks whether very high or very low concentrations of calcium in the medium affect cell division. It might be expected that if the cell were reacting to different calcium concentrations, the calcium in the medium would affect the pattern of cell division. Cells grow normally whether or not calcium is added to the medium. Thus it appears that calcium may not have any regulatory role in cell division.

Nucleoid Occlusion Model

One possible way in which DNA replication may affect cell division is that the mere presence of DNA in the middle of the cell exerts a negative effect on the synthesis of the septum. This idea was first articulated by Helmstetter and colleagues (73). They proposed that if chromosome replication were not completed, the chromosomes would not segregate and division would not take place. This proposal that DNA interferes with division has been extended by the nucleoid occlusion model, which also proposes that DNA exerts a negative effect on both septum formation and septum completion (119, 161). A nucleoid that has not finished replicating is located in the middle of the cell, thus preventing septum formation from starting. Upon termination of DNA replication, the nucleoids move apart (by mechanisms unrelated to the cell surface); this separation frees the center of the cell for invagination. The mechanism for nucleoid separation involves ribosome assembly compartments that develop around the duplicated gene clusters coding for rRNA and protein. This accumulation of newly synthesized ribosomes causes the gene clusters, together with the interposed origin, to drift apart (162). The central proposition of the nucleoid occlusion model is that there is a negative nucleoid effect ". . . and a positive compensating termination signal" (119). It is proposed that the termination of DNA replication causes a transient change in the pool of deoxyribonucleotides and that this change in concentration serves as a localized trigger that is converted into a diffusible cytoplasmic activator of peptidoglycan synthesis. Both the negative and positive effects originate from the actively transcribed and replicating nucleoid and produce a signal that influences the peptidoglycan-synthesizing system in the plasma membrane. This balance of forces determines when and where invagination will occur. A formal description of the nucleoid occlusion model has been presented previously (160, 161).

There are a number of problems with the nucleoid occlusion model. For example, the proposal that there is a localized increase in the level of deoxyribonucleotides at the instant of termination of DNA replication is difficult to understand when one considers DNA replication over a range of growth rates. If the time for a round of replication is 40 min (35), cells growing with a 41-min doubling time will have a 1 min "gap" in the synthesis of DNA. Although one might not see this gap in a population of cells, one might envision that such a gap exists in individual cells. As the growth rate increases from a 41-min doubling time to a 40-min doubling time, the gap disappears and there is a doubling in the rate of DNA synthesis at the instant of termination. This is because two new rounds of replication start at the instant when one round of replication is ending (32). Thus, rather than an increase in deoxyribonucleotide concentration, one would expect a decrease. According to the nucleoid occlusion model, there would be a disruption in normal cell division as cells increase their growth rate over the period when the gap disappears. No such disruption has been observed (72, 140).

Furthermore, it is difficult to imagine how a localized concentration of nucleotides can be maintained within a cell that has dimensions on the order of microns. Diffusion of the nucleotides will erase any localized variations in concentration. Any proposal of spatial nucleotide concentration variations should include calculations of the effects of diffusion.

At an experimental level, there are many disparities between the nucleoid occlusion model and the extant data. For example, it is put forth that "it will never occur that a constriction is initiated at the site of a nucleoid" (160; subsequent quotes are also from this article). Yet it is reported that constricting cells can have continuous nucleoids (156, 157). The nucleoid occlusion model also predicts that a shift-up leads to a "postponement of division" and a "transient drop in the percentage of constricting cells." The rate of cell division following a shift-up has been determined by analyzing the pattern of elution of cells bound to a membrane. Since the rate of elution of cells from the membrane is a measure of the rate of cell division, if there were a temporary inhibition of cell division one would find a large decrease in the number of cells eluted. No such decrease is observed (27, 90). There is no cessation in the rate of cell division after a shift-up. Data in support of the nucleoid occlusion model are derived from the pattern of nucleoids in filament-forming mutant cells during filament formation and after recovery of division (160). However, any model of cell division can be applied to, and fit, the same experimental data. The data in support of the nucleoid occlusion model fit any model proposed for the regulation of cell division.

Jump in Peptidoglycan Synthesis

One description of peptidoglycan synthesis during the division cycle suggests a jump in peptidoglycan synthesis at the start of invagination (153). Cells of similar length sorted into invaginating and noninvaginating cells appeared to have more incorporation of diaminopimelic acid in the invaginating than the noninvaginating cells. Detailed examination of the experimental results shows that the error bars on these data are large and that they not only overlap each other but also overlap the mean of the other cells. Thus, it can be said that there is no experimental difference between the invaginating and noninvaginating cells. One explanation of the data is that cells of the same length may be of different sizes as a result of differences in width. If cells initiate invagination at a relatively constant size, then for cells of similar length the thinner cells will be noninvaginating and the thicker cells will be invaginating (33; also see discussion above on variations in cell diameter during the division cycle). If the increase in the amount of cytoplasm produces the increase in the cell surface, it follows that the larger cells make more cytoplasm than the smaller cells do. Thus one would expect the invaginating (i.e., thicker, and hence larger) cells to make more surface than the noninvaginating (i.e., thinner, and hence smaller) cells of the same length. Both theoretical and experimental considerations argue against the evidence for a sudden jump in the rate of peptidoglycan synthesis at the start of invagination. The experimental evidence is consistent with the pressure model proposing a smooth increase in the rate of peptidoglycan synthesis during the division cycle.

Variable T Model

The variable T model is not so much a specific model for regulation of peptidoglycan synthesis and invagination as an insightful restatement of the pattern of peptidoglycan synthesis in terms of physical forces. The surface tension (T) of an area or surface is the work required to expand the area by a unit amount. If the surface tension is high, it takes a lot of work or energy to add to that surface. A low surface tension means that it takes less energy to make a unit amount of cell surface. For a given amount of energy, a low-surface-tension material will increase in area more than a high-surface-tension area. This formal concept of surface tension offers a way of restating the pole formation problem and may even present ideas about the mechanism of pole formation.

In the basic pattern of pole synthesis (Fig. 5), when cells are invaginating, the increase in surface area per increase in cell volume is greater in the pole area than in the cylindrical wall area. This can be seen by noting that the amount of volume associated with a unit increase in surface area is lowest during pole formation and highest during side wall formation. This means that the surface tension during pole synthesis is lower than the surface tension during side wall synthesis. No biochemical explanation for this difference in surface tension is available. Koch and Burdett (92, 94, 100) have analyzed the shape of the poles of gram-negative bacteria and have proposed the variable T model for pole growth. They proposed that slight decreases in the surface tension of wall synthesis in the middle of the cell, and a continuous change in this value with continued constriction, can explain the shape of the bacterial pole.

The formulas for surface increase presented above are mathematical restatements of the variable T model. Since one finds a continuously increasing ratio of surface to mass synthesis during the period of invagination, there must be a continuously varying T during invagination. The importance of the variable T model is that it allows one to look for specific mechanisms leading to a lowering of the energy required for pole formation. This change in energy may be due to changes in the mode of peptidoglycan synthesis or in the ionic and chemical environment in different parts of the cell. The variable T model provides a way of connecting theoretical concepts to biochemical principles.

Divisome Model

Nanninga (120) has proposed the idea that there is a particular structure, the divisome, that forms when a cell begins invagination. This macromolecular complex exists at the leading edge of a constriction and is proposed to encompass the cytoplasm, the membrane, and the periplasm of the cell. The composition of the complex varies depending on whether division is in progress. The evidence for the divisome is indirect. The divisome is offered because of the need to take all the different protein and gene products involved in invagination and accommodate them to their locations in the cell. Among the components of the divisome are penicillin-binding protein 3, penicillin-binding protein 1, the product of FtsZ, a proposed penicillin-insensitive, peptidoglycan-synthesizing activity, and an X factor, which is a transmembrane protein that mediates the interaction between the cytoplasm FtsZ and the periplasmic penicillin-insensitive, peptidoglycan-synthesizing activity. Numerous observations ranging from the biochemical (location, hydrophobicity) to the genetic (interactions between different mutants) are brought within the framework of the divisome proposal. The main thrust of this proposal is that invagination is turned on at a particular time by the formation or activation of the divisome. This is a form of dual control for peptidoglycan synthesis, with side wall and pole formation being formed by different peptidoglycan-synthesizing systems.

Dual Control of Peptidoglycan Synthesis

The dual-control model posits that during the cell cycle, two morphogenetic processes alternate. First cell elongation occurs by an increase in the cylindrical portion of the sacculus. Then septation occurs with the formation of two new hemispherical poles in the center of the cell. In addition to the idea of alternation of morphogenetic processes, the dual-control model proposes that different proteins are responsible for the different processes. For example, the proteins responsible for the maintenance of the cylindrical shape during the elongation phase include RodA and penicillin-binding protein 2, whereas other proteins, including penicillin-binding protein 3, carry out septation (for a discussion of the origin of this model, see reference 11).

Another view of the dual-control model was presented by Lleo et al. (111). They proposed the "two-competing-sites model" for peptidoglycan assembly and bacterial cell shape regulation. This model postulates two reaction sites, one responsible for lateral (cylindrical) wall elongation and the other responsible for septum formation. The two-competing-site model accounts not only for gram-negative, rod-shaped cells, but also for different coccus-shaped cells. This model differs from the pressure model proposed here (29, 33) in that the dual-control model has the two reactions competing with each other so that no lateral wall can be formed during septum formation and vice versa. Electron-microscopic autoradiographic measurements of wall synthesis have shown no discrete separation between side wall and pole synthesis. Cells which make pole are also labeled in the side wall, and it is only a statistical relationship that cells are preferentially making pole.

Unitary Pattern of Peptidoglycan Synthesis

An alternative to the dual-control model for peptidoglycan synthesis is the unitary model. The unitary model implies that all peptidoglycan, in all parts of the cell and at all times during the division cycle, is synthesized or dependent upon the same peptidoglycan-synthesizing structures and activities. Differences in peptidoglycan synthesis, or apparent differences in sensitivities to different antibiotics, are due to differences in the rate of synthesis of peptidoglycan rather than to differences in the biochemical elements involved in synthesis.

An example of the application of the unitary model concerns the effect of β-lactams at different times during the division cycle. Cefsulodin is an antibiotic that binds specifically to penicillin-binding-proteins 1a and 1b (42, 121). In a study of the effect of cefsulodin-induced lysis during the first division after a nutritional shift-up or chromosome replication alignment, it was concluded that cefsulodin lysis was linked to cell division (52). Because of the possibility that the production of synchronously dividing cells alters the biochemistry of the cells, a study was done with cells produced by the membrane elution technique. These cells are relatively free of biochemical perturbations. When cefsulodin was added to cell populations at different stages of the division cycle, all cells lysed rapidly (83). There was no cell

cycle specificity of the antibiotic. It was concluded that cell-cycle-dependent events do not determine the sensitivity of lysis to cefsulodin. One must be cautious before accepting synchronization studies as indicators of cell-cycle-specific variations in a physiological process. The results with cefsulodin may be limited at present to only one antibiotic (83); it may be that this will be a general finding for all antibiotics. If one goes back to the original postulation of a dual-control model (144), the more antibiotic added, the more likely a cell was to lyse rather than to form filaments. The unitary model proposes that if penicillins were added at similar "activities," with activity not rigorously defined, the biochemical effects of penicillins will be the same.

Physical Forces and Initiation of Invagination

An explicit statement that physical forces may be the key effector of cell cycle events when it comes to cell morphogenesis has been proposed by Harold (70). Although this analysis dwelt mainly with complex single-celled organisms, the ideas have direct application to gram-negative bacteria. This review (70) should be consulted for the myriad details supporting physical forces as the agent of cell morphogenesis.

Periseptal Annuli

The periseptal annuli are two concentric rings found beside a newly forming septum (26, 114, 136, 137). The annuli are zones of adhesion between the inner membrane and the peptidoglycan. When cells are plasmolyzed by being placed in a hypertonic medium (e.g., a high sucrose concentration), zones of adhesion that delimit the plasmolysis bays are observed. Besides their association with the septum, the periseptal annuli can appear at sites of future septum formation before any septum is visible (113). In the longest cells of a population, periseptal annuli were observed at one-quarter and three-quarters of the cell length, presumably where the septum will be forming in the future daughter cells. Another support for the involvement of periseptal annuli in the invagination process comes from the study of temperature-sensitive mutants. At elevated temperatures, as filaments formed, the annuli were present with normal spacing and the sites were separated by a distance equivalent to a unit cell. When the cells were allowed to defilament by lowering the temperature, the new septa formed at the sites where the annular structures were found at the elevated temperatures.

How does the cell localize the annuli at the precise midpoint of the future daughter cells? It appears that new annuli come from preexisting annuli. They first appear close to the older annuli, then move away from the center of the cell (possibly by membrane synthesis between the septum and the periseptal annulus), and finally come to rest at the midpoint between the pole and the septum.

The periseptal annulus proposal suggests that sites for invagination are chosen at some time earlier than the division cycle that is currently under observation. The formation of periseptal annuli is analogous to the initiation of DNA synthesis prior to the division cycle in which DNA synthesis is terminated.

What is the trigger for the formation of periseptal annuli? One possibility is that the cell titrates something in a manner similar to the formal titration related to the initiation of DNA synthesis. For example, if the cell titrated the amount of surface area per cell pole, then when the area per two poles was above a certain value, a new set of periseptal annuli would be initiated. At the instant when new annuli were initiated, there would be a decrease in the ratio, and thus a stable situation would be present. This model predicts that new periseptal annuli would appear earlier and earlier at faster growth rates (33). This prediction is in accord with the limited data available.

The Problem of Initiation of Invagination—Where Does It Stand?

The mechanism of cytoplasm increase may be complicated in detail, but is understood in principle. Enzymes work on small metabolites to derive energy and synthesize precursors of macromolecules. This leads to the synthesis of new cytoplasm, which makes more cytoplasm. Thus, cytoplasm grows exponentially. Synthesis of DNA, once initiated, is well understood. The problem of initiation of DNA synthesis is currently being examined by using cell-free systems; its ultimate resolution is anticipated within the next few years. In contrast, the invagination problem is a mystery, even though we have some good starts on understanding the biosynthesis of cell wall. There is no consensus on the mechanism of invagination initiation. The field is wide open to new ideas, and it is difficult to state what form the ultimate answer will take.

Among the best clues to understanding the invagination problem are the data on the periseptal annulus from Rothfield and associates (26, 113, 114, 136, 137) and the theoretical insights of the variable T model of Koch (100). These ideas direct our attention to the chemical conditions leading to periodic formation of annuli and the change in the energetics of wall formation during pole synthesis. Whether these ideas continue to be fruitful, or whether newer ideas will arise to replace them, will be the principal challenge in the near future.

MATURING OF PEPTIDOGLYCAN

Change in Acceptors and Donors

If new single strands of peptidoglycan are inserted between older strands, and if cross-links are formed only from pentapeptides present on the newly inserted strands, then all donor links will come from the new strand. This means that the resident strand is always an acceptor. If a cell is labeled for a short time with diaminopimelic acid, all of the radioactive diaminopimelic acid will be in the newly inserted strand. The nonradioactive resident strand will accept the cross-link from the adjacent D-alanine, so the two types of diaminopimelic acids in a cross-link can be chemically distinguished (58, 62). After a short labeling period, there will be no label in the acceptor portion of dimers, and all of the label will be in the donor moiety. The results of measurements of acceptors and donors can be summed up in the ADRR (14, 15). An ADRR of 0.0 means that there are no acceptors and that all of the material is present in the donor form. (As noted above, the ADRR should be replaced by reporting of percentage of donors [or acceptors], as this method is not subject to the problems of a ratio of related components. However, for this discussion I will refer to the ADRR because the original data were couched in these terms.)

Measurements of the ADRR have not always been consistent with this simple model of single-strand insertion. For example, measurements of initial ADRR values and their change during extended growth indicated that the pepti-

doglycan strands entered the cell wall as pairs, with two new strands inserted adjacent to each other; after 8 min the strands encircled the cell and returned to their original point of insertion. This was based on the finding (15) that the initial ADRR was not zero, and the value was constant for 8 min before it began to increase. Other investigators have found lower initial ADRRs (39), indicating that single strands are inserted during peptidoglycan synthesis. When different dimers have been studied, different ADRRs have been found (53, 55). The insertion of single strands is indicated in Fig. 2 and 3. The precise kinetics of the initial ADRR and its increase is a matter for further work, but the final value of the ADRR is an indication of the degree to which strands can be separated and new strands placed between them.

There are apparent variations in the ADRR during the division cycle. If such a variation exists, the numerical results should be consistent with the ADRR in an exponentially growing culture. That is, the average ADRR for a culture should be consistent with the ADRRs for cells of different ages. As will be noted below, this has not always been the case.

Increase in Cross-Linking

The peptidoglycan matures by increasing the cross-linking frequency after synthesis (14). The observation that cross-linking increases after a pulse-label is unexpected and paradoxical. Consider a strand with 1,000 pentapeptide subunits inserted during a labeling period. Assume that the frequency of cross-linking is such that 25% of these inserted peptides will form cross-links with adjacent strands. These 250 cross-links are distributed equally to each side, 125 each to the adjacent left and right strands. At some later time, a new strand is inserted between the radioactive strand and the original unlabeled material. This newer strand acts in the same way as the original labeled strand: it forms 250 cross-links with its adjacent strands. These cross-links are also equally distributed with 125 cross-links to the unlabeled strand and 125 to the labeled strand. Thus, 125 cross-links were broken (from one side of the labeled strand) when the second strand was inserted, but 125 cross-links were reformed. No change in the degree of cross-linking is expected. Although the actual subunits linked may or may not be the same, the total cross-linking fraction of the original material does not change.

How can we explain the observed increase in the total amount of dimers? Consider that the number of cross-links is not precisely the same for each strand but is statistically distributed about some mean value. For two strands inserted in different parts of the cell, one strand may have 300 cross-links formed from 1,000 inserted subunits; the other strand may have 200 cross-links. The average would be the same as described above, 250 cross-links per strand. However, now assume that the next strands inserted preferentially replace the links between the low-cross-linked strands; there is a preference that the 200 cross-links will be replaced by 250 cross-links the next time a new strand is inserted so that strands with 300 cross-links are relatively stable. The strand with 200 cross-links has a below-average cross-linking density, so it is expected that the newly inserted material will have a higher cross-linking density. There will be a steady increase in the cross-linking fraction as the low cross-linked material is replaced by higher cross-linked material. This proposal is illustrated in Fig. 6.

Consider the different stresses on strands connected by either 200 or 300 cross-links. The internal pressure of the cell makes the turgor pressure the same all over the cell surface, so it is expected that the strands with 300 links will have less stress per cross-link than the strands with 200 links between them. For a given length of strand, the higher number of links means that there is less stress per link. The surface stress model predicts that the replacement will preferentially occur between the strands that are poorly cross-linked. The average replacement strand is above the cross-linking value, so there will be a steady drift to higher cross-linking values (Fig. 6). By this mechanism the cell will continue to strengthen the peptidoglycan by a drift to increasing cross-link density.

Cross-Linking and ADRR during the Division Cycle

Differences in the mode of synthesis of peptidoglycan have been suggested as explanations for the synthesis of either poles or side walls. In a study involving elutriation to produce cells in two different parts of the division cycle, it was found that there was a slight increase in the degree of cross-linking in the cells in the latter part of the division cycle (41). In addition to the increase in cross-linking, there was an increase in the ADRR in the oldest cells. A mathematical derivation of the relationship between the ADRR and cross-linking indicated that one must correct the increased cross-linking values for the increased ADRR (41). After making this correction, it was concluded that there was no increase in cross-linking in invaginating cells.

A detailed analysis of the relationship of the acceptor-donor radioactivity measurements and the degree of cross-linking indicates that no correction need be applied to cross-linking data as a result of variations in the ADRR (31). The cross-linking value and the ADRR are independent measures of peptidoglycan structure and synthesis. This result shows that the data on the changes in cross-linking during the division cycle demonstrate a cell-cycle-dependent variation in cross-linking (41). No statistical analysis of the difference is available, so it cannot yet be concluded that there is a difference in cross-linking between the peptidoglycan made at the poles and the side walls.

Distinction between Cross-Linking and Cross-Linkage

Höltje has pointed out (77) that one can consider a distinction between the final product (the cross-linkage value) and the mode of synthesis (the cross-linking value). The distinction between the two values becomes important when one considers whether insertion of strands occurs by insertion of single strands between existing strands or whether new strands are inserted as pairs or higher multiples of preformed units. The difference arises because when a single strand is inserted, each of the previous strands must be broken to have a strand inserted. After two strands are inserted, there will be two complete rounds of strand breakage. If, however, a pair of strands are cross-linked and inserted as a pair, only one round of strand breakage need occur in order to insert these two strands. Thus, it is energetically more efficient (i.e., less costly in terms of energy) to have multiple strands inserted during wall synthesis. Nevertheless, it is not clear that such multistrand insertion occurs. It is difficult to distinguish between multistrand insertion and very rapid insertion of independent single strands as the cause of an elevated ADRR. If, during a short pulse-label, two single strands were inserted adjacent to each other in rapid succession, labeled acceptor would be found. This labeling would be indistinguishable from that of

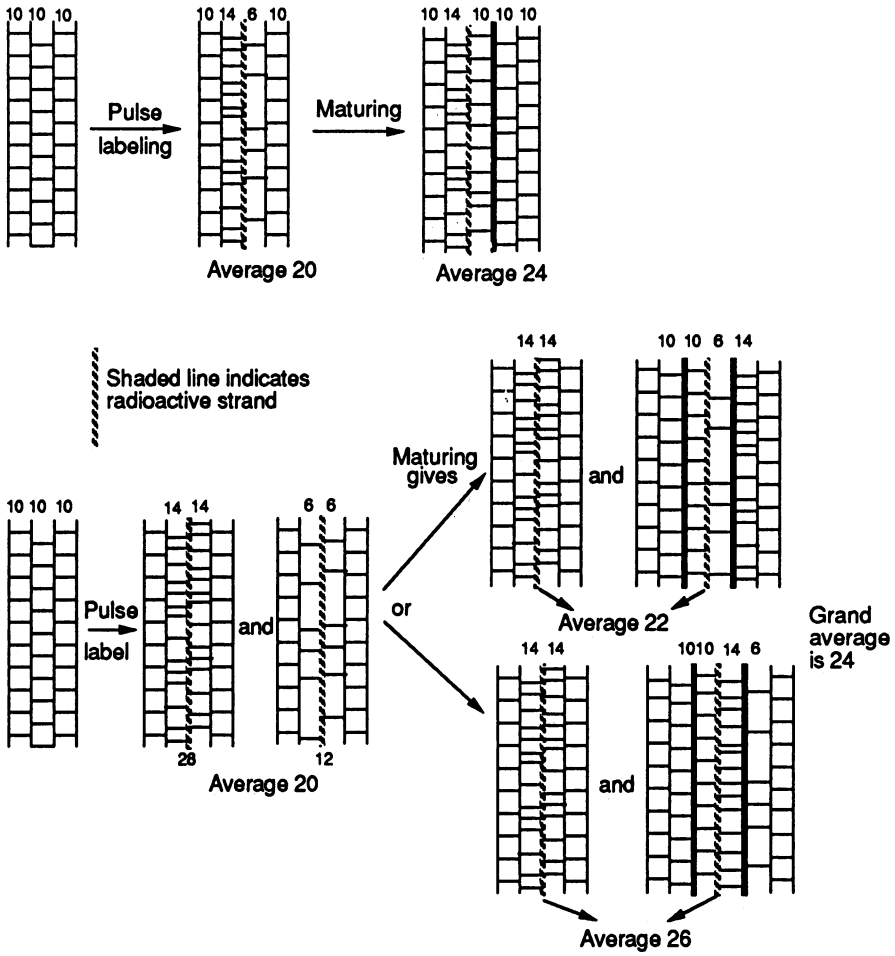

FIG. 6. Increase in cross-linking by natural selection. In the upper panel a single strand is inserted to give an average of 20 cross-links. The cross-links on the left and right are not equal. When another, unlabeled strand is inserted, it will preferentially replace the less dense cross-links (the six cross-links), and there will be an increase in cross-linkage. The lower panel shows that the inequality of strand cross-linkage at the time a labeled strand is inserted does not have to be restricted to one strand. Over the population, different strands may have cross-linking densities above or below the mean. When new strands are inserted during further growth, the lower-density cross-links are preferentially replaced by new strands. Although sometimes the replacement does not change the cross-link values, over the entire population there will be the same total increase in cross-linkage. Reprinted from reference 33 with permission.

a two-strand unit being formed and then inserted. However, it is simpler for the cell to have a unitary model for peptidoglycan synthesis, with all chains inserted as single strands—whether in the pole or the side wall—rather than a dual mode for strand insertion, with some insertion occurring as single strands and some as double strands. This last proposal means that the mode of pole synthesis may be different from the mode of side wall synthesis. Pole synthesis may occur at a single point again and again to form a leading edge, whereas the side wall is made exclusively by random intercalation between existing strands. The biochemical elements involved in this pole synthesis are not necessarily different from those involved in side wall synthesis.

When Does Invagination Occur?

It is generally believed that invagination starts sometime in the middle of the division cycle. Woldringh (156) presented evidence to link the start of invagination with the termination of DNA replication or the start of the D period. This experiment provides evidence for the idea that termination of DNA synthesis is related to, or possibly triggers, invagination. Recently this has been extended into a more specific regulatory mechanism whereby the DNA or nucleoid itself prevents invagination by its very presence in the center of the cell. This nucleoid occlusion model (160, 161) states that the charge on the DNA or some other property of DNA has a negative effect on invagination. When the nucleoids separate, the negative effect is removed and the cell invaginates. However, there are pictures in the literature (156) showing cells invaginating even in the presence of a definite nucleoid stretching along the inside of the cell.

If cells do not invaginate until the middle of the division cycle, newborn cells should not show any signs of invagination. Cells eluted from a membrane elution apparatus have been observed in the electron microscope, and a significant amount of invagination was present (101). The cells studied were slow-growing cells (165-min doubling time), so it would be expected that if DNA replication initiated invagination, no newborn cells would show invagination. It was proposed that the newborn cells were contaminated with a significant number of cells that were randomly eluted from the mem-

brane. By calculating the degree of contamination (assuming that true newborn cells were not invaginated), Koppes et al. (101) proposed that up to 57% of the eluted cells were randomly eluted and not newborn. Of course, if this were the case, one would expect an ever-decreasing number of cells eluted from the membrane at each generation of elution. A decrease in cell number is not usually observed, so it may be valid to conclude that newborn cells can be invaginated. Any models that related the termination of DNA replication and the start of invagination would be eliminated if newborn cells could invaginate.

MEMBRANE SYNTHESIS DURING DIVISION CYCLE

Less is known about membrane synthesis during the division cycle than about peptidoglycan synthesis. The model developed in this review (Fig. 5) predicts that the membrane would increase in the same manner as the peptidoglycan.

Lipid Synthesis during Division Cycle

If the lipid of the bacterial cell is in close association with the peptidoglycan and increases along with the peptidoglycan, we would expect a pattern of synthesis similar to that of peptidoglycan. To observe such a pattern, the ratios must show very slight deviations from exponential. There have been a number of studies on lipid or membrane biosynthesis during the division cycle. Ohki (124) concluded that cytochrome b_1 (localized predominantly in the cytoplasmic membrane) and L-α-glycerol-phosphate transport increased in steps during the division cycle. Furthermore, the turnover of phospholipid was proposed to vary in steps during the division cycle. Carty and Ingram (18) observed abrupt increases in lipid synthesis coincident with the initiation of cross walls. Similar transient increases in glycerol incorporation in synchronized cultures of both *E. coli* and *Bacillus megaterium* were seen (40). James and Gudas described a cell-cycle-specific incorporation of lipoprotein into the outer membrane (84). In contrast, a continuous increase in the amount of membrane components was reported by two groups (4, 22). More recently, Joseleau-Petit et al. (86) proposed a bilinear pattern for membrane synthesis during the division cycle. This has been referred to as a doubling in the rate of phospholipid synthesis, shortened to the acronym DROPS. DROPS have been reported (68, 69, 129, 130). A cessation of phospholipid synthesis during the division cycle has also been reported (118a).

Attempts to find cell-cycle-specific synthesis of a particular part of the membrane, i.e., the zones of adhesion between the membrane and the peptidoglycan (OM_L), showed that there was no cell-cycle-specific formation of these sites (87). Synthesis at specific portions of the cell cycle were predicted on the basis of studies of certain cell division mutants. This experiment was performed by the phosphate starvation method (91), which has been criticized as not being suitable for cell cycle analysis (33).

Although this review of the experimental determination of the rate of surface synthesis during the division cycle has dealt primarily with peptidoglycan, it also applies to membranes and other surface-associated elements. This is because the cell membrane grows in response to the increase in peptidoglycan surface and coats the peptidoglycan without stretching or buckling. The area of the membrane should increase in the same way as peptidoglycan. The observed DROPS during the division cycle (86, 88) may be attributable to artifacts of the synchronization procedure (33). Pierucci's (129) data on phospholipid synthesis, obtained by the membrane elution method, are consistent with the conclusion that the rate of phospholipid synthesis is similar to peptidoglycan synthesis; i.e., there are no DROPS. Experiments performed by the membrane elution method and the ratio-of-rates analysis (used successfully for peptidoglycan synthesis, as described above) support the suggestion that the rate of membrane synthesis during the division cycle is similar to that of peptidoglycan synthesis (34). However, anomalous patterns of incorporation have been observed which may be consistent with the proposal that there are regions of high rates of membrane synthesis at the midpoint of incipient daughter cells (34). This finding is consistent with the proposal that periseptal annuli act as the agents of new pole formation.

Membrane Protein Synthesis during Division Cycle

In addition to lipid, the membranes of the cell contain specific membrane proteins. Measurements of the amount of protein per unit surface area for cells growing at different rates indicate that the density of proteins is constant (2). This means that the protein composition in the membrane does not vary as the cell surface varies over a factor of 2. If membrane proteins were inserted into the membrane in direct proportion to the increase in cell surface area, we would expect that the pattern of membrane protein synthesis would be similar to peptidoglycan synthesis. Measurement of protein synthesis during the division cycle showed that the bulk membrane protein was synthesized at a constant rate throughout the cycle, with an abrupt doubling in rate approximately 10 to 15 minutes before division (12). This result is reminiscent of the reported increase in peptidoglycan synthesis relative to total mass synthesis during invagination.

INTEGRATION OF PEPTIDOGLYCAN SYNTHESIS WITH CELL GROWTH

Primacy of Mass

The primary source of regulation of peptidoglycan synthesis is the increase in cell mass or cell cytoplasm. In accordance with the postulates of the surface stress model, increased mass leads to increased surface. There is no independent synthesis of surface other than that responding to the increase in cell mass. As DNA synthesis is also regulated by cell mass (32), it is seen that the cell is regulated in its entirety by the increasing cell mass.

Does DNA Synthesis Regulate Peptidoglycan Growth or Pattern?

Since 1968, when the temporal constancy of the period between termination of DNA replication and cell division was noted, it has been thought that termination of replication may trigger invagination. This observation is consistent with the alternate proposal that there is only a coincidental relationship between termination and division. It may be that the constant D period is a result of the cell evolving to have DNA replication terminate prior to division and that there is no causal relationship between division and termination.

It is unknown whether cell mass can be replaced by cell area or surface as the regulatory agent of initiation of DNA synthesis. If this were the case, we would see an indirect

initiation of DNA replication by cell mass, with the initiation being caused by an increase in cell surface. The data supporting the mass initiation model are, in general, compatible with the conclusion that surface is the initiator.

Aggregation Theory and Growth Law of the Bacterial Cell

With regard to the division cycle, it has been proposed that only three categories of material need be considered, i.e., the cytoplasm, the genome, and the cell surface (32, 33). All of the cell material fits into one of these categories. The problem of cell growth is to describe how each of these components is synthesized during the division cycle. DNA is synthesized at one or more linear rates during the division cycle, cytoplasm is synthesized exponentially during the division cycle, and cell surface is synthesized almost (but not precisely) exponentially during the division cycle. When all of these components are summed, one can arrive at the general cell growth law, which is that the cell grows approximately exponentially during the division cycle (32). This simple result means that the cell is the sum of its individual biosynthetic patterns and that there is no overriding central law to which all of the components of the cell conform. Just as there is no central timer to the division cycle, there is no central regulator of cell growth other than each of the cell components reacting locally to the needs of the cell. The mass grows as fast as it can in a given environment. Upon occasion, when a certain mass is reached, a new round of DNA synthesis is initiated; and cell surface is made continuously and almost exponentially in response to the increasing cell mass.

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