Structure and Function of the Cell Envelope of Gram-Negative Bacteria

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INTRODUCTION

The cell envelope of the gram-negative bacterial cell is unique in several respects, and the structure which contributes most to this uniqueness is the lipopolysaccharide (LPS)-containing outer membrane of the cell wall. This layer delimits a zone outside the cytoplasmic membrane and controls the passage of molecules into and out of this “periplasmic space” (Fig. 1) (133). The function of the outer membrane is, in turn, modified by other cell wall components which influence both its penetrability and its vulnerability to attack by external agents. The complex, multilayered cell wall is made up of a variety of structural macromolecules which can trap free molecules and ions so that each structural “layer” defines a zone of particular physicochemical attributes. Perhaps the most interesting aspect of this elaborate cell wall is its continual synthesis, which is necessitated by cellular growth, because the structural macromolecules must either be produced at the cytoplasmic membrane and “programmed” to fit into the correct layer or produced at the specific layer by enzymes that have experienced this same odyssey.

Our conclusions concerning cell wall structure have a profound effect on our concept of the physiological consequences of this organization. Enzymes may be sequestered in the cell wall, if their activities are dangerous to cytoplasmic constituents, and their products are still available to the cell. While these enzymes are “extra-cellular” they are held within the cell wall and thus are not lost to the aqueous environment. Because of the zonal concentrations of molecules and ions, which are produced by attraction to structural macromolecules within the multilayered cell envelope, the “milieu” of a cell envelope-bound enzyme differs from that of a free enzyme and depends on the zone within which it is located. This zonal phenomenon, with the simple barrier function of the outer membrane, affords a measure of protection to envelope-bound enzymes and to the cytoplasmic membrane, and goes some distance towards an explanation of the wide range of environments in which gram-negative bacteria can grow.

STRUCTURAL ORGANIZATION OF THE GRAM-NEGATIVE CELL ENVELOPE

The gram-negative cell envelope consists of a number of layers whose chemical composition and physical nature differ very markedly. This does not mean that each of these “layers” is continuous and discrete and, in fact, some components are clearly discontinuously distributed (Fig. 1). Some layers are intimately associated with neighboring structures and it is difficult to separate them. Because the function of each of these layers is a consequence of its peculiar composition and molecular architecture, an examination of these features is useful.

Cytoplasmic Membrane

The production of protoplasts of a gram-negative bacterium (41) has allowed the isolation of
FIG. 1. Schematic diagram of gram-negative cell envelope. +, Free cation; -, free anion; Ω bound cation; Ω bound anion; § adhesion point produced by ionic bonding; ← hydrophobic zone; → covalent bond; → cross-linking polypeptide in the peptidoglycan; + polysaccharide portion of peptidoglycan; Ω enzymatically active protein; + phospholipid; lipopolysaccharide; lipopolysaccharide (schematic); bp, binding protein; e, enzymes associated with the cytoplasmic membrane whose function is directed to the cytoplasm; em, enzymes associated with the cytoplasmic membrane which synthesize macromolecular components of the cell wall; ep, enzymes localized in the periplasmic zone; es, enzymes localized at the cell surface; lp, lipid portion of Braun's lipoprotein; p, structural and enzymatic proteins of the outer membrane; pl, protein portion of Braun's lipoprotein; ps, permease; s, structural protein of cytoplasmic membrane.
pure preparations of the cytoplasmic membrane of this species, and the chemical composition of this membrane has proved to be similar to that of other biological membranes (123). Purified cytoplasmic membrane fractions obtained from disrupted cells of other species have also been shown to have a chemical composition similar to that of other biological membranes (148, 149, 181, 212). Freeze-etching studies have shown that the frozen cytoplasmic membrane cleaves along a median hydrophobic zone (10, 142) which is traversed by protein "studs" (52, 198; Fig. 2) similar to those seen in red blood cell membranes (74). Fox has suggested (74) that these protein "studs" which traverse the membrane may be permease molecules, and Tourtelotte and Zupnik (198) have also suggested that they are involved in substrate transport. Fox's model proposes, furthermore, that structural membrane proteins are built into the continuous phospholipid bilayer and that other proteins may be associated by hydrophobic interactions with either the inner (cytoplasmic) or the outer aspects of the membrane (Fig. 1).

Certain enzymes are located at the outer surface of the cytoplasmic membrane, such as the peptidoglycan-synthesizing enzymes in gram-positive cells (73) and the adenosine-hydrolyzing enzyme of Escherichia coli (97). Physiological evidence suggests that binding proteins are associated with the cytoplasmic membrane (152), and direct histochemical techniques have shown that the leucine-binding protein of E. coli is on the outside of this structure (Fig. 1) (141). The histidine-binding protein of Salmonella typhimurium binds one molecule of substrate (166), and the galactose-binding protein of E. coli undergoes a substrate-mediated conformational change (15). These are functionally important because the transport of a variety of substrates is greatly reduced if specific binding proteins are released from the cells by osmotic shock (12, 166, 208). Certain binding proteins are induced by the same agent as the specific permease (208).

The cytoplasmic membrane is a vital interface for the cell envelope because it is in this layer that the structural components of the cell wall are synthesized and assembled. The basic chemical units of peptidoglycan (161, 162), LPS (96, 136, 148), and phospholipid (11, 96, 149, 211) are synthesized by enzymes within the cytoplasmic membrane, and present evidence suggests that cell wall proteins are synthesized and transported by this structure (84).

**Peptidoglycan-Lipoprotein Complex**

Peptidoglycan is present in the gram-negative cell envelope in highly variable amounts. It comprises 2.4% of the cell weight in Spirillum serpens (106), and forms an easily resolved electron-dense layer in the cell walls of this organism and of E. coli (139), whereas in the marine pseudomonad B16 it comprises only 1.2% of the cell weight and is so thin that it is resolved only with some difficulty (Fig. 3 and 4) (72). Although other cell wall layers may contribute to cellular rigidity (30, 46), or maintain cellular shape (92), Forsberg et al. (70) have shown that cells bounded only by their peptido-
Fig. 3. Electron micrograph of a section of a cell of the marine pseudomonad (B-16) showing the cytoplasmic membrane (c) and the outer membrane of the cell wall (o). The bar in this and subsequent micrographs indicates 0.1 μm.
glycan layer maintain their shape (Fig. 4). Isolated peptidoglycan structures (murin sacculi) also maintain the shape of the cell from which they are derived (72, 99, 207), and even the apparently incomplete peptidoglycan layer of Myxococcus xanthus maintains a rod shape when isolated and purified (210).

Although electron micrographs of fixed and sectioned cells often show an electron transparent zone between the cytoplasmic membrane and the peptidoglycan layer (43, 139, 155), we have no proof that this “space” exists in the living cell. Rogers (161) has noted that the peptidoglycan layer is assembled from components produced in the cytoplasmic membrane and has suggested that the two structures may be joined by nascent peptidoglycan. And we must bear in mind that the turgor pressure of the living cell would force the cytoplasmic membrane outward against the inelastic peptidoglycan layer (162). The relation of the plasmalemma and the cell wall of plant cells is radically altered by fixation (44), and the relation of the cytoplasmic membrane and the peptidoglycan layer of bacteria may be similarly disturbed by this procedure.

Thus the peptidoglycan layer exerts morphological control over the cytoplasmic elements of the cell, and Braun and his coworkers (20, 22, 23) have shown that a specific lipoprotein, which is 12 to 14 nm long (21) and composed of 57 amino acids (19), is covalently linked to the peptidoglycan of several enteric bacteria in such a way that it extends outward towards the outer membrane (Fig. 1). This layer of protein molecules may correspond to the “G” layer of dePetris (155). Schnaitman (182) has proposed that the covalently linked lipid component of this molecule (19) serves to anchor the outer membrane by hydrophobic interactions with the phospholipids of this membrane-like layer. Inouye et al. (103) have shown that the lipoprotein of E. coli is free in the periplasm when it is first synthesized and that it is subsequently covalently bonded to the peptidoglycan. The oft-repeated observation that proteolytic digestion causes a separation of the outer membrane from the peptidoglycan layer (22, 155, 195) is explicable on the basis of a layer of linking protein between these structures. The reader is referred to Braun and Bosch (19) for the chemical details of this important lipoprotein.

The covalently linked peptidoglycan-lipoprotein complex of the gram-negative cell wall can be thought of as the inelastic foundation of the whole-cell envelope (29, 106) and is, therefore, somewhat analogous to the gram-positive cell wall, the plant cell wall, and the animal cell’s glycosylax. The peptidoglycan of gram-negative bacteria has a relatively open molecular structure due to a paucity of cross-linking (161). Because it contains no lipid it does not provide a cleavage plane in frozen cells (100), or produce a double-track artifact on fixation. Because its polysaccharide chains are approximately 1.4 nm apart, and because peptide cross-linkages are relatively infrequent, we would not expect the peptidoglycan to comprise an effective barrier to the passage of small molecules. DeVoe et al. (53) have shown that its removal from murineoplasts of a gram-negative organism does not affect the uptake of alpha-amino isobutyric acid, but Burman et al. (29) has shown that cholate is excluded by the intact peptidoglycan layer of E. coli. We would expect the peptidoglycan layer to bind specific enzymes, in the same way that penicillinase is bound by the cell walls of Bacillus licheniformis (174) and alkaline phosphatase (APase) is bound by certain gram-positive bacterial (80) and plant (120) cell walls, and to concentrate certain ions, much as gram-positive walls concentrate Al³⁺ (18) and plant cell walls concentrate Ca²⁺ (49). Most eukaryotic exoenzymes are glycoproteins (110), and many are bound to the cell surface by hydrogen bonding or covalent linkages. In Lactobacillus, 75% of the cell’s vitamin B12 is bound to polypeptides in the cell wall (175), and the presence of the adsorbed vitamin molecules protects the polypeptides from the action of proteolytic enzymes. The specific binding of certain molecules within the peptidoglycan layer, and the concentration of certain ions by the Donnan effect (41), in response to the weak negative charge of this zone of the cell envelope (161), would effectively condition the immediate external environment of the cytoplasmic membrane to a marked extent.

Because specific alterations of the peptidogly-

**Fig. 4.** Electron micrograph of a section of a cell of the marine pseudomonad (B-16) from which the outer cell wall layers had been removed (a murineoplast). Where plasmolysis has occurred the peptidoglycan layer (p) is clearly seen to confine both the protoplast and a series of vesicles derived from the cytoplasmic membrane (c).

**Fig. 5.** Electron micrograph of a section of a preparation of isolated outer membrane fragments from cells of the marine pseudomonad (B-16).

**Fig. 6.** Electron micrograph of a section of a cell of Pseudomonas aeruginosa which had reacted specifically for alkaline phosphatase by reaction produce deposition. Note that the reaction product is present exclusively in the periplasmic space between the outer membrane (o) and the cytoplasmic membrane (c).
can layer have been shown to cause pronounced changes in the penetrability of the barrier constituted by the outer layers of the cell wall (29, 90), and in the structural stability of these layers (106), it is clear that the peptidoglycan provides a foundation for the other components of the cell wall. Even specific functions of the outer cell wall layers, such as the ability to assemble flagella, are disturbed if the peptidoglycan layer is disturbed (129). Because of this interdependence it is clearly unwise to draw conclusions from studies in which specific cell wall layers are destroyed by digestion.

Studies of plasmolysed cells have shown that there are regular zones of adhesion between the cytoplasmic membrane and the peptidoglycan complex (8, 9) and that these points of adhesion correspond to phage adsorption sites. These adhesions could be accommodated in the model presented above (Fig. 1) by an ionic association of a structural membrane protein with the peptidoglycan.

**Periplasmic Zone**

The original definition of the “periplasmic space” was made by Mitchell (133), and that definition is valid because he dealt in physiological concepts; his concept of an enzyme-containing compartment bounded on the inside by the cytoplasmic membrane and on the outside by a “molecular sieve” layer is both accurate and useful. The equivalence of the “molecular sieve” and the outer membrane of the gram-negative cell wall has been established (52, 67, 68, 69), so the periplasmic area is that between the cytoplasmic membrane and the outer membrane of the cell wall (Fig. 1 and 3). This means, of course, that the periplasmic area and the peptidoglycan-lipoprotein complex occupy the same zone of the cell wall, and one accommodates periplasmic components whereas the other supports and reinforces the cell envelope.

When the continuity of the outer limiting barrier of the periplasmic space is interrupted by changes in the outer membrane or in the LPS, periplasmic enzymes (41), binding proteins (5, 94, 141, 152), and pigments (77, 78) are released into the menstrum. Treatments which cause this release have been reviewed by Costerton (41) and by Heppel (94), and subsequent work has shown that exposure of cells of *Pseudomonas aeruginosa* to 0.2 M Mg$^{2+}$ (37) or 20% sucrose (39) also modifies the cell wall to allow the inward passage of lysozyme and the release of periplasmic enzymes. The barrier layer of the outer cell wall is also disturbed when cells are grown without an essential amino acid (105), at an elevated pH (37), or in the presence of polymyxin (32), and when either protein (125) or peptidoglycan (106) synthesis is inhibited. Defects in the barrier layer of the outer cell wall may also be genetically determined, and “periplasmic leaky” strains have been described in *E. coli* (116, 117, 122, 173) and in *P. aeruginosa* (36), which release periplasmic enzymes into the medium during growth. In two of these strains (101, 116) the periplasmic enzyme in question (APase) is released as an enzyme-LPS complex.

Physiological studies have shown that guanosine 5'-triphosphate (GTP) is hydrolyzed by phosphatases in the periplasmic space without the molecule being present at any time in the cytoplasm (24), and direct localization of enzymes in the periplasmic area has been effected by reaction product deposition (34, 38, 39, 55, 58, 107, 116, 119, 209) (Fig. 6) and by the use of ferritin-coupled antibodies (119). These enzymes are not exclusively periplasmic, in most cases, but a large proportion of their molecules are found in this zone of the cell wall.

The distribution of enzymes within the periplasmic zone of the cell wall has been examined (58, 119, 209) and Heppel has suggested that these enzymes are not firmly bound to structural wall components (93), but that they are concentrated in “polar caps” where the periplasmic space is enlarged (209). On the other hand, MacAlister et al. (119) have used both reaction product deposition and ferritin-coupled antibodies to show that APase is evenly distributed throughout the periplasmic space of *E. coli*. Garrard (78) has proposed that periplasmic proteins are bound to a variety of structural components by many different types of bonding, and Cheng et al. (39) have concluded that APase is bound to a structural component within the periplasm, since they can disturb the outer layer of the cell wall so as to allow the inward passage of large molecules without any release of the enzyme. Cheng et al. (39) have suggested that the enzyme is associated with LPS (Fig. 1) which has been shown to occur in the periplasmic area (186), and LPS-enzyme complexes have been recovered from growing cells (116) and from sucrose-washed cells (101). The release of this complex has been studied (J. M. Ingram, K.-J. Cheng, and J. W. Costerton, unpublished data), and we have found that both components are released or retained by the cells in an unvaried proportion under a great variety of conditions. Thus, the available evidence suggests that periplasmic enzymes are associated with LPS and with other structural...
components of the cell wall and that they are distributed throughout the periplasmic zone, as here defined, without notable local concentration (Fig. 6).

The precise level at which these proteins are located within the periplasmic space is not yet defined, except in the cases of binding proteins, which are functionally associated with the outside of the cytoplasmic membrane, and of the APase of the marine pseudomonad B16, which is bound to the components of the outer area of the periplasm (42). Although some periplasmic enzymes are completely removed from cells without degradation of the peptidoglycan layer (37, 39), those of other cells appear to depend on alteration of this structure for their release (93, 94). Because the periplasmic space is a functional continuum, the location of enzymes within this area of the cell wall appears to depend on binding to specific structural components rather than on limitation by physical barriers.

Forsberg et al. (71) examined the chemical nature of the structural components of the periplasmic zone of marine pseudomonad B16 and found that this material, known as the "underlying soluble layer," contained both protein and polysaccharide. A small molecular species (<4 × 10^4 daltons) was largely proteinaceous, whereas larger aggregates (>4 × 10^6 daltons) were composed of both protein and polysaccharide and were shown to be morphologically heterogeneous (70).

When mureinoplasts (70) are prepared from cells of the marine pseudomonad all of the structural components of the periplasmic zone are removed except the peptidoglycan (71), but the cells retain their ability to respire and to transport amino acids (53). We assume that the complex structural framework of the periplasmic zone binds enzymes, smaller molecules and ions, and provides a distinctly conditioned and enriched environment at the outer aspect of the cytoplasmic membrane. But it is not strictly essential to membrane function in an osmotically protective and metabolically favorable milieu.

**Outer Membrane Layer**

The profile of this layer, as seen in electron micrographs of embedded and sectioned cells (Fig. 3, 6, 7, and 9), is identical with those of other biological membranes, which suggests that the phospholipids and proteins of this layer form a bilayer which is denatured by fixation to produce a double track (56). Recent studies of the isolated outer membrane (Fig. 5) have shown that it does contain phospholipids and proteins (70, 71, 148, 149, 180, 181, 212) as well as variable amounts of lipopolysaccharide (51, 147–149, 168, 182). Biophysical studies (67, 69) have indicated that the basic continuum is formed by proteins and phospholipids, whereas the oligosaccharide portion of the LPS appears to be associated with the inner (39) and outer (182) surfaces.

Schnaitman (182) and Burman et al. (29) have suggested that the periplasmic lipoprotein of enteric gram-negative bacteria is also associated with the inner aspect of this layer by a hydrophobic interaction (Fig. 1). This would have the effect of linking the outer membrane to the peptidoglycan layer via the lipoprotein so that this relatively rigid inner layer would serve to support the outer membrane. Observations indicating that damage to the peptidoglycan of several gram-negative bacteria changes the penetrability of the outer membrane have led Burman et al. (29) to suggest that this rigid component serves as a foundation for the outer cell wall layers.

Earlier suggestions (28, 169) that the phospholipids of the outer membrane are arranged in an hexagonally close-packed bilayer with a d-spacing of 0.44 nm have been supported by subsequent work (67, 69), which has shown that this structure has the same basic molecular architecture as a typical membrane (65). The phospholipids of the outer membrane are quantitatively similar to (212), but qualitatively different from (149, 212), those of the cytoplasmic membrane in E. coli. This layer is also affected by extraction and digestion of its components (67, 68) in precisely the same way in which membranes are affected, which further indicates a basic similarity in the molecular architecture of these structures. Freeze-etching has shown that there is a cleavage plane in the outer membrane of the cell wall (52, 58, 69, 81), which indicates that the hydrophobic parts of the phospholipid and LPS molecules form a zone (Fig. 1) in the center of this layer which is similar to that found in membranes. Freeze-etching studies of the outer membrane of the cell wall of Pseudomonas aeruginosa (81, 114) have shown that a granular, predominantly proteinaceous, subunit is present in this structure. Exposure to tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate (Tris-EDTA) removes these granules, and their reintroduction, following the removal of these agents, allows the spontaneous reaggregation of the granules within the outer membrane (81). Examination of the outer membrane has shown...
that it contains protein, that these proteins differ from those of the cytoplasmic membrane (181), and that one type of protein may have a structural function in this layer of the cell wall (181), whereas another exhibits phospholipase activity (149). Taken together with the fact that this layer shows a membrane-like plasticity (45), the evidence presented above shows that the outer membrane layer forms a membrane-like hydrophobic barrier which contains integrated and complex synthetic systems.

Physiological studies of cells from which the outer membrane had been removed (53) showed that the ability of these cells to transport a specific amino acid analogue was unimpaired, which suggests that this structure does not carry out active transport. Transport activity in active membranes has been tentatively linked to protein “studs” (74, 198), and the absence of particles in the cleavage plane of the outer membranes of all species studied (52, 64, 142), except in that of P. aeruginosa (81), also suggests that this layer is not a site of active transport. The absence of these protein structures has also been noted in eukaryotic membranes which do not carry out active transport (134). Thus, the outer membrane of the gram-negative cell wall constitutes a barrier which corresponds well with Mitchell’s original concept (133) of a “molecular sieve,” and it is of interest to determine the effective size of the pores in this structure.

By working with E. coli, Payne and Gilvarg (154) showed that the barrier layer of the cell wall allows the passage of tetralysine while restricting that of pentalysine, which indicates that the effective hydrodynamic radius (Kd) of the pore is 0.190. Studies of the penetration of actinomycin D (molecular weight 1,250) through the barriers of the cell envelope have shown that the antibiotic is excluded by intact cell walls of the marine pseudomonad (B16) (118), P. aeruginosa (35), and wild-type E. coli (189). In the latter two organisms the cytoplasmic membrane also acts as a barrier to the penetration of this antibiotic. Damage to the barrier layer of the cell wall, whether caused by specific treatments (35, 77, 118) or by mutations (111, 184, 189, 194), allows the inward penetration of actinomycin D and other antibiotics. The undisturbed cell walls of a variety of gram-negative bacteria have been shown to exclude puromycin (185), benzylpenicillin (89), streptomycin (14), and adenosine 5’-triphosphate (ATP) (127). Similarly, lysozyme (molecular weight 13,930) is excluded from gram-negative cells, while the barrier layer of the cell wall is intact, and can only penetrate to its substrate (the peptidoglycan) when the barrier has been breached by EDTA (13), elevated Mg2+ (36), ion manipulation (43), and a variety of other agents (41).

When the permeability of the outer membrane is increased by the substitution of unsaturated fatty acids for saturated fatty acids, the cells quickly become penetrable by lysozyme and other agents, and the release of periplasmic enzymes after osmotic shock is facilitated (165). This change of permeability when the packing of hydrophobic molecules is disturbed by the introduction of unsaturated fatty acid side chains has also been noted in other membranes (128), and it shows that the outer membrane provides the basis for the barrier layer of the gram-negative cell wall. Although the fatty acids of the phospholipids of the outer membrane are readily interchangeable (3), the proteins of this layer do not, of course, vary with changing nutrient supply (180).

Genetically induced defects in the LPS of the barrier layer also make it permeable by the agents listed above (116, 117, 173). Thus, it is clear that the outer membrane contributes to a barrier within the outer cell wall which excludes a wide variety of molecules and that this barrier is basically a membrane-like arrangement of phospholipids, LPS, and proteins and is susceptible to damage by a variety of agents and by genetic changes. Just as this barrier, or “molecular sieve,” is effective against the inward penetration of extraneous molecules, it also retains enzymes and structural components within the periplasmic space (Fig. 6) (38). However, cells which have genetically determined defects in their barrier layer often release periplasmic enzymes into the medium during growth (36, 37, 116, 117, 189).

Although the basic continuity required to produce a barrier layer in the cell wall is provided by the tendency of the phospholipids of the outer membrane to form a bilayer, LPS is clearly a very important part of this barrier (148, 149, 182). This is illustrated by a comparison of the penetrability of members of isogenic pairs of strains of Salmonella typhimurium which differ only in that the rough strain has a specific and well-understood defect in its LPS (116, 173). This single defect, which shortens the chain of sugars distal to lipid A, produces a marked increase in the inward penetration of antibiotics (173, 176) and lysozyme (173) and a marked increase in the release of periplasmic enzymes during growth (116).

These data show that LPS is an important
component of the barrier layer, and several authors have suggested (39, 116, 147, 182) that the hydrophobic lipid A portion of this molecule associates with the hydrophobic zone of the outer membrane while the polysaccharide portion of the molecule protrudes from the inner and outer surfaces (Fig. 1). Osborn has suggested (147) that the association of the lipid A portion of LPS with the hydrophobic zone of the outer membrane may facilitate the transfer of newly synthesized LPS from the inner to the outer aspect of this layer after its outward movement from the cytoplasmic membrane where its synthesis is initiated (146). LPS has been visualized, by the use of ferritin-coupled antibodies, at the outer aspect of the cytoplasmic membrane (186) and both inside (186) and outside (130, 186) the outer membrane.

The definitive work on the intercalation of LPS into the outer membrane has been done by Rothfield’s group, who have shown that LPS is incorporated into artificial phospholipid monolayers (163, 164). Transferase enzymes involved in LPS synthesis are also intercalated into these artificial systems (96), where they have been shown to be in an active configuration which depends on activation by phosphatidylyethanolamine (136). Two of the three major proteins of the cell envelope have been found in the outer membrane (181, 192), and these workers have suggested that these proteins, which are both glycoproteins, may be either structural molecules or enzymes involved in LPS assembly. The presence of protein inserts into these membrane-like layers would not affect the X-ray diffraction pattern unless they were in a highly ordered pattern (69).

The dissection of the cell wall of a gram-negative pseudomonad by ion manipulation (70) has yielded less information than one would expect concerning the localization of LPS because the LPS of this salt-dependent species requires salts for its molecular integrity and breaks down during the cell wall stripping procedure (145). For this reason the isolated and washed double-track layer of this organism contains only 12.4% of carbohydrate by dry weight (71).

Because ions are important in the stability (59, 145, 214) and in the retention (113) of LPS in this area of the cell wall, we can assume that ionic linkages occur between the polar portions of adjacent molecules and, in salt-dependent species, between parts of the same molecule. Because the polar parts of the LPS protrude from the surfaces of the outer membrane, this arrangement would produce an ordered and stabilized “picket fence” on both sides of the double-track layer (Fig. 1). We can readily understand how the deletion of a distal part of the polysaccharide chain would shorten these polar projections and alter both the thickness of the effective barrier and the extent to which it is cross-linked.

An intact “picket fence” of polysaccharide chains, which carry the “O” antigen at their distal tips, would protect smooth strains of gram-negative bacteria by reacting with antibodies and with complement at a defined distance from the outer membrane (160). This action would protect the outer membrane from the extensive damage wrought by the combination of specific antibody and complement (216) and also from that caused by complement alone (62). Smooth strains of gram-negative bacteria are protected from damage by complement and antibodies, but rough strains, whose polysaccharide chains are abbreviated, are susceptible to cell wall damage by the action of these agents and longer exposure leads to cytoplasmic membrane damage and cell death (63). Smooth strains are also sensitive to immune sera after their protective LPS structure has been deranged by Tris or EDTA (160), or both, and after the protective LPS layer has been disturbed by polymyxin (156).

The specificity at the phage receptor sites of gram-negative bacteria is a function of LPS structure (115), and this specificity resides in the terminal sugar portions of the molecule. Because of this the cell surface must consist partially of the distal ends of the oligosaccharide portions of the LPS, and immunological studies suggest that this is the case (6). Thus, we propose that a part of the surface of gram-negative bacterial cells consists of an ordered and cross-linked mass of the polysaccharide chains (Fig. 1) of the amphipatic LPS molecules which are oriented by the affinity of their hydrophobic lipid A portions for the hydrophobic zone of the double-track layer. Local variations in structure would result from the production of a “patch” of specialized LPS, and it has been noted that whereas phage receptor sites are composed of both lipoprotein and LPS (172, 205), colicin receptors are simply a single exposed glycoprotein (87, 171). Glaueut and Thornley (83) have referred to a “general consensus” that the cell surface is a mosaic of LPS and phospholipid, and it is entirely possible that phospholipids are exposed in specific areas to produce specific local structures. The presence of colicin K on the colicin receptors of tolerant cells of E. coli prevented the access of polymyxin B to the phospholipids of the outer
membrane (124), which indicates that LPS and phospholipids do indeed form a mosaic at the cell surface. And the observation that the presence of colicin K on its specific receptors interferes with phosphate uptake (193) indicates that the receptors may be associated with permeability sites on the outer membrane.

Rothfield and Romeo (168) have postulated that there are local concentrations of specific phospholipids in membranes. If these patches of specific phospholipids also occur in the outer membrane they would exert an allosteric effect on wall-associated enzymes and could specifically associate with LPS to produce a wide variety of specific "sites" (phage receptors, colicin receptors, Bdellovibrio receptors, etc.) at the cell surface. The "L" ring of the basal complex of the flagella have been shown to be associated with the outer membrane (51, 200), and recent studies of the formation of flagella in cells from which these structures had been removed by growth at supraoptimal temperature have led McGroarty et al. (129) to conclude that the cell wall must develop the local capacity to bear a flagellum and that disturbed cells require approximately one generation time to acquire this capacity.

Thus, the available evidence suggests that the surface of a gram-negative cell consists of a protruding pattern of LPS oligosaccharides between which the proteins and phospholipids that constitute the basic outer membrane are exposed to a greater or lesser degree (Fig. 1). This layer constitutes a hydrophobic barrier in the outer cell wall which is structurally strengthened by the interaction of the protruding oligosaccharides, and by the presence of structural proteins, and which is supported and maintained at a predetermined distance from the cytoplasmic membrane by lipoprotein molecules of a definite length.

**External Layers**

There are a number of species, notably aquatic organisms (66, 140, 204) and rumen bacteria (34), which have cell wall layers external to the LPS zone, and some halophilic organisms have regularly arranged protein layers at the outside of their thin and peculiar cell walls (104). In a large proportion of marine organisms (204), and in some rumen bacteria (Fig. 7 and 8) (34), the cell surface is covered by a paracrystalline arrangement of spheres which form multiple layers in some species (204) and which are believed to be proteinaceous. Murray has described an elaborate and highly ordered arrangement of protein subunits on the surface of *Spirillum serpens* (26, 27, 137) which is very readily removed and its loss, by this procedure or by genetic means, has little effect on the survival of the cell in artificial laboratory culture (138).

Although some cells possess proteinaceous outer coats, others have a thick carbohydrate layer outside the LPS zone of the cell wall (66). Staining with ruthenium red (Fig. 9) and freeze-etching (Fig. 10) have shown that many rumen and aquatic bacteria appear to have thick carbohydrate layers at their surfaces (34), and Pate and Ordal (153) have described a similar layer in a myxobacterium. Fletcher and Floodgate (66) have shown that these thick polysaccharide capsules are important in the adhesion of aquatic cells onto solid surfaces and many soil bacteria are surrounded by thick capsules (7). The polysaccharide capsule of *Aerobacter aerogenes* has been chemically defined (217), and it has been shown that a specific phage-induced hydrolase digests this material in certain circumstances (218). Wilkinson's laboratory (88) has reported that a large number of species of the Enterobacteriaceae produce an extracellular carbohydrate slime composed largely of colanic acid. Bacteria may also have extracellular capsules, and it is of great interest that cells of *Salmonella typhimurium* in the tissues of an infected mouse are surrounded by a thick (150 nm) capsule composed of LPS (186). A number of gram-negative bacteria produce other elaborate cell wall structures outside the outer membrane (121), and some of these complex layers function in cell aggregate formation (33, 151).

Thus the surface of a gram-negative bacterial cell may play an important role in the protection of the cell in hostile environments. The simplest cell walls have a "picket fence" of the polar portions of LPS molecules at their surface, and this structure serves to keep antibodies and complement at a distance from the susceptible outer membrane. This array of polysaccharides may also affect the susceptibility of the cell to phagocytosis and account for the pathogenic success of smooth strains of gram-negative bacteria (160), which have complete LPS molecules at their surface. The barrier function of the outer membrane, and its associated LPS, protects the cell from many antibiotics (173, 176) and other potentially harmful molecules, and the ion exchange effect of the multiple layers of the cell wall also protects the cell by binding various ions and molecules. The elaborate external layers of protein and carbohydrate which surround cells in certain crowded and highly
competitive environments (e.g., bovine rumen) would extend this protection by increasing effective cell wall thickness and by providing extra barrier layers to exclude large molecules. These cells would, therefore, be especially well protected from the antibiotics and enzymes produced by competing species by a complex multilayered cell wall, and in the case of *Spirillum serpens* the outer protein layer may protect the cell from attack by *Bdellovibrio* (25, 191).

Studies of highly adapted cells in pure laboratory culture are difficult to relate to mixed populations in natural environments since the cells in pure cultures are not challenged by the same hostile factors found in nature. Thus the loss of a protective layer (cf. the protein “overcoat” of *Spirillum*) may have little effect in a pure culture (138), whereas it would be a critical loss to a cell in its natural environment.

**SYNTHESIS OF THE GRAM-NEGATIVE CELL WALL**

The synthesis and structural assembly of the components of the cell walls of gram-negative bacteria is of interest because this protective structure must grow with the cells and because its highly complex layers and zones are assembled and maintained at a considerable distance from the cell itself (161). With the exception of the peptidoglycan layer, which is immediately adjacent to the cytoplasmic membrane, each of the layers and zones of the cell wall is assembled at a predetermined and definite distance outside the cytoplasmic membrane, and therefore without the direct use of nucleic acids or the machinery of protein synthesis. We will not attempt a comprehensive treatment of the synthesis of cell wall components, because excellent reviews are available for peptidoglycan (146, 147, 161), phospholipids (146, 147, 167, 168), and LPS (146, 147, 167, 168), but we will look at cell wall synthesis in the light of the structural organization we have attributed to this structure.

The components of the cell wall layers are synthesized at the level of the cytoplasmic membrane (11, 136, 146–149, 168, 211) and released into the cell wall where they move outward until they “find” their appropriate layer or zone and are intercalated into it by physical attraction and held there by ionic, hydrophobic, or covalent bonding. Synthesis of these components often involves lipid carriers (4), and the newly synthesized molecules are often transported into the cell wall on a lipid carrier (147, 199). It has been suggested (148) that outer cell wall components may leave the cytoplasm as a protein-phospholipid-LPS complex and be intercalated into these layers in this complex form.

Covalent bonding of components into their specific structural layers requires both synthetic and autolytic enzymes, so that they can be intercalated into existing structures, and these enzymes have been found in the peptidoglycan layer of both gram-positive (16, 61, 161, 196) and gram-negative (16, 91, 206) bacteria. Forsberg and Ward (73) have shown that the amylase which is necessary for cell wall growth in *Bacillus licheniformis* is “bound to the cytoplasmic membrane in such a way that it is accessible to the cell wall”. Similarly, phospholipases have been found in the outer membrane of gram-negative cells (149), and Rothfield (170) has shown that glucosyl and galactosyl transferases are intercalated into a model system which simulates the outer membrane, and that they depend on the resultant hydrophobic associations for their enzymatic activity.

The hydrophobic zone (52, 69) of the outer membrane is most important because it attracts the lipid portion of lipoproteins as well as the phospholipids, LPS, and the hydrophobic proteins of which it is composed (Fig. 1). This layer is expanded to keep pace with cell growth by the intercalation of complex macromolecules which are synthesized and released at the level of the cytoplasmic membrane. The synthesis of phospholipids and proteins is precisely coordinated, because the inhibition of protein synthesis in *E. coli* leads to the release of fatty acids into the medium (47) and inhibition of phospholipid synthesis stops protein production (82). Both the synthesis and intercalation of these components are well understood due to the elegant work of Osborn and of Rothfield, and the reader is referred to their recent reviews and publications on the subject (96, 136, 146–149, 167, 168).

Thus, the cell walls of gram-negative bacteria act on molecules which are released at the cytoplasmic membrane in a variety of ways. Peptidoglycan components and lipoprotein molecules are built into the preexisting peptidoglycan-lipoprotein complex by the action of transpeptidases and glycosidases within the complex (161). In this way the molecules released from the protoplast either reach a predetermined locus in a zone or layer of the cell wall or they diffuse through the multilayered cell wall and are released into the menstruum in which the cell lives.

The enzymes which produce cell wall components such as those of peptidoglycan (4, 161), LPS (96, 136, 147, 148) and phospholipid (11,
211) are located in the cytoplasmic membrane. They must be built into the membrane in such a manner as to allow them to use cytoplasmic substrates, but they must also be able to release their products at the outer aspect of this structure.

The mechanism of the transport of these extracellular proteins out of the bacterial protoplast is much less clear than that of the similar process in eukaryotic cells. In these latter cells exportable proteins are synthesized on the outer surface of the membranes of the rough endoplasmic reticulum (76), passed into the lumen of these membrane systems, and discharged at the cell surface from vesicles derived from this organelle (157–159, 219). Thus, newly synthesized protein is effectively outside the cell when it enters the lumen of the rough endoplasmic reticulum. Glick and Warren (85) have noted that some protein is also synthesized in association with the surface membrane of eukaryotic cells. Redman has shown (157–159) that “exportable” nascent proteins traverse the membrane of the rough endoplasmic reticulum as linear polypeptides and that they undergo tertiary folding within the lumen to form the finished protein molecule (Fig. 2). This conclusion is supported by the observation that incomplete polypeptides are found in the lumen of microsomes derived from the rough endoplasmic reticulum when protein synthesis is interrupted by puromycin treatment (158, 159, 188), and by the observation that nascent protein actually binds ribosomes to the membranes of this organelle (1). This proposed mechanism requires that the initial nascent peptide chain be attracted to the membrane and suggests that hydrophobic amino acid residues may play a part in determining whether a given protein traverses the membranes of the rough endoplasmic reticulum to become an extracellular molecule (Fig. 2) or remains in the cytoplasm of the cell.

The location of protein synthesis in the prokaryotic cell has not yet been established, but many workers (48, 177, 202) have suggested that this process is concentrated at the inner aspect of the cytoplasmic membrane. This would appear to be opposed to the evidence of Miller’s group (131, 132) which indicates that polyribosomes form on nascent messenger ribonucleic acid and that protein synthesis, therefore, occurs in immediate association with the chromatinn of the cell. But Glew and Heath (84) have suggested that protein synthesis may, in fact, occur in both locations with exportable proteins being produced at the inner aspect of the cytoplasmic membrane while intracellular proteins are produced in the cytoplasm. By working with a gram-positive prokaryote these workers established that there was one site of transcription in the cell, but differential sensitivity to puromycin showed that there were multiple sites of translation, with that for extracellular enzymes being the most sensitive, perhaps because of its peripheral location. Schwarz and Landau (183) have noted that protein synthesis is inhibited by high hydrostatic pressure, and they suggest that this indicates that this process is associated with the cytoplasmic membrane in prokaryotic cells.

In this case Redman’s hypothesis would obtain in prokaryotic cells, as suggested by May and Elliot (126), and proteins destined for a location in the cell wall would be produced on polyribosomes on the inner aspect of the cytoplasmic membrane, attracted to the membrane, pushed through the membrane by peptide elongation (84), and folded in their tertiary pattern as they emerge at the membrane surface (Fig. 2). The polypeptide portion of periplasmic cytochrome c is localized in this inner zone of the cell wall of Spirillum thermophilum within a few seconds of its synthesis (78), and this lack of a lag time, coupled with the demonstrated absence of cytoplasmic pools, has led Garrard to speculate that the molecule is extruded from the cell by the above mechanism. The crucial attraction of the nascent polypeptide to the membrane may be basically hydrophobic as has been proposed for the eukaryotic system. Morris and Schlesinger (135) have shown that the
mechanism of extrusion of proteins is non-specific, because APase containing proline analogues was both synthesized and extruded by cells of *E. coli*. In *Micrococcus sodonensis* (84) no intracellular APase is detectable, and the secretion of the enzyme across the cytoplasmic membrane is non-specific and shows a high activation energy (230 kJ/mol) which indicates that the rate-limiting step is not enzymatic and may involve forcing a partially polar linear enzyme molecule through the basically hydrophobic structure of the membrane (Fig. 2).

When the proteins in question emerge and fold at the membrane surface their surface charges would determine whether they would remain in association with the membrane (152), associate with the peptidoglycan-lipoprotein complex, bind to structural components within the periplasm (39), be intercalated into the outer membrane track layer (164), or be released into the menstruum (116). Even though all cell envelope proteins must be associated with the cytoplasmic membrane during secretion, the proteins of this membrane are clearly different from those of the outer membrane (149, 181), and we must presume that the migration of the latter molecules is controlled by their physical properties. Sargent and Lampen (174) have proposed that the penicillinase of *Bacillus licheniformis* (a gram-positive bacterium) is produced on the ribosomes in an extended hydrophobic configuration which causes it to associate with the cytoplasmic membrane. As long as the enzyme retains its hydrophobic nature it remains cell associated, but when it becomes hydrophilic it is released from the cell. Thus, the basic genetic code would contain the specific determinants to cause a specific protein to be exported from the cell and to dictate the precise location of that protein in the complex cell wall of the gram-negative bacterium. The extracellular location of these proteins does not, of course, prevent their being inducible, and APase has been found to be inducible in some strains of *E. coli* (119) and in *Pseudomonas aeruginosa* (37), whereas it is constitutive in some strains of *E. coli* (215) and in certain rumen bacteria (34). Wilkins (213) has shown that low cytoplasmic levels of specific nucleotides are the inducing factor for APase and that phosphate levels per se are unimportant in some cells.

Vesicular exocytosis of fully formed protein molecules, like that seen in many eukaryotic cells (150) and in *Bacillus licheniformis* (174), appears to be unlikely in gram-negative bacteria because, although some cells have elaborate mesosomes with associated vesicles, many cells contain neither mesosomes nor cytoplasmic vesicles and thus could not accomplish vesicular exocytosis. Although it is clear that membrane indentations and vesicles are often lost in fixation, freeze-etching fails to show a regular occurrence of vesicles in most prokaryotic cells (10, 52, 64, 81, 100, 142, 201). Schlesinger's thesis (179), that subunits of a cell wall-associated enzyme (APase) are synthesized in the cytoplasm of *E. coli* and stored therein until they are released into the periplasmic space, would require the outward transport of fully folded protein molecules and it is not clear at this time how this secretion would be accomplished.

Thus, the gram-negative cell wall presents a unique and fascinating case in which both structural subunits and the enzymes responsible for their final structural binding are released at the cytoplasmic membrane and both are attracted to a layer or zone, which may be as many as 15 nm outside the membrane, where the intercalation required to permit cell growth is accomplished.

ASSOCIATION OF ENZYMES WITH SPECIFIC CELL WALL COMPONENTS

In addition to the enzymes involved in synthetic processes, the cell wall also contains a variety of degradative enzymes, and these molecules have been localized both within the periplasmic space and at the cell surface. Wall-associated degradative enzymes include (for reviews: 41, 94) deoxyribonuclease, ribonuclease, 3'-nucleotidase, 5'-nucleotidase, uridine 5'-diphosphatase, adenosine 5'-diphosphate (ADP)-glucose pyrophosphatase, acid hexose phosphatase, cyclic phosphodiesterase (24), ADP-sugar pyrophosphatase (203), asparaginase (31), glucose-6-phosphatase, APase, penicillinase (17), phosphoglucose isomerase (75), and a nonspecific phosphatase.

Both 3' nucleotidase and 5' nucleotidase have been localized by reaction product deposition in the outer cell wall layers of *E. coli* (144), and APase has been localized in the periplasmic space (38, 55, 107) and at the cell surface (39) by the same method. APase has also been localized in the periplasmic space of *E. coli* by using ferritin-coupled antibodies (119). Thus, it is clear that a wall-associated enzyme may occur in more than one zone of the gram-negative cell wall and that we do not know the precise localization of the majority of these enzymes. Studies in our laboratory (101, 116) have suggested that APase is closely associated with
LPS both in the periplasm and at the cell surface, and we may expect that other wall-associated enzymes also form complexes with structural components.

This association of enzymes with cell wall components has at least two effects on the enzyme itself. Firstly, such basic properties as the dimerization of subunits depend on the retention of the enzyme in an association with the cell wall, and cells of E. coli in which the cell wall has been disrupted by EDTA treatment release inactive subunits of APase into the menstruum (178). Cheng et al. (39) have suggested that LPS affords the subunits of APase a locus within the periplasmic space where dimerization is favored by the provision of a favorable physicochemical environment and, perhaps, by a concentration of the Zn2+ or Co2+ ions (Fig. 1) which are essential to this process (197). Secondly, the environment of the active enzyme is conditioned by the ions attracted to that zone of the cell wall and by the exclusion of certain large molecules by the barrier activity of the outer membrane. This is supported by the observation (36) that cell-free APase is denatured when growth reduces the pH of the medium to 4.7 while the wall-associated APase retains its activity. Higgins and Shockman (95) have noted a similar protection of the enzymes associated with the cell wall of a gram-positive cell. Thus, the association of an enzyme with a structural component within a certain zone of the cell wall provides this enzyme with a microenvironment which may differ from that of the menstruum in which the cell lives. The cell wall, therefore, not only protects the protoplasm of the gram-negative cell by “screening” and selecting the ions and molecules which can reach the cytoplasmic membrane, but also provides a variety of microenvironments (Fig. 1) in which essential wall-associated enzymes are protected from damage by physical and chemical agents. This organization clearly makes the gram-negative bacteria capable of growth in a phenomenal range of environments, many of which would kill less well protected organisms. The external coats of protein or carbohydrate, or both, which are found on many aquatic (140, 204) and rumen bacteria (34), constitute an extra layer of ion exchange material which would materially increase the protection of cell wall-associated enzymes from damage by specific ions or molecules.

The observation that several degradative enzymes are cell wall-associated in gram-positive bacteria (40, 79, 84, 108, 109, 174) and in fungi (150) and plants (120) leads us to anticipate that some enzymes will be found to be associated with the peptidoglycan-lipoprotein complex in the gram-negative cell wall. We have found no association of APase with either the peptidoglycan layer or the cytoplasmic membrane (42), but this study involved only one organism and only one enzyme.

The association of APase with LPS (101, 116) provides this enzyme with a structural locus on the inner aspect of the outer membrane, because the LPS is hydrophobically bonded to this layer (182). In smooth strains, in which the barrier function of the outer membrane complex is intact, most of the APase remains in the periplasmic space (116), even though the LPS migrates to the outer surface of the cell (147). In rough strains, in which the barrier function is disturbed, the enzyme is partially located at the cell surface (116) where it is associated with LPS (101), and an LPS-APase complex is shed into the medium during growth (116). Thus certain enzymes in certain cells may form a complex with a structural component which migrates as a unit within the cell wall and may even be shed into the medium. The degradative enzymes associated with gram-positive cell walls have also been shown to be associated with polysaccharide moieties (84).

**FUNCTION OF CELL WALL-ASSOCIATED ENZYMES**

Several authors (143, 168) have noted that the cell wall-associated enzymes of gram-negative bacteria are very similar to the degradative enzymes which are sequestered in the membrane-bound lysosomes of eukaryotic cells (50). Clearly the wall-associated enzymes of gram-negative bacteria are sequestered in an area in which they are separated from the cytoplasmic constituents of the cell, but a complete parallelism with the lysosomal enzymes of the eukaryote would require that they digest certain cytoplasmic constituents of intact cells. In eukaryotic cells the lysosomes can take up and digest both functional (190) and nonfunctional (60) cellular components. Although there is currently no evidence to show that elements of the prokaryotic cell can be extruded into the periplasmic area for digestion, Goldberg (86) has shown that defective proteins are quickly destroyed in both growing and resting cells of E. coli, and periplasmic enzymes may be involved in their degradation.

The degradative cell wall-associated enzymes of gram-negative cells have a digestive function, as have eukaryotic lysosomal enzymes, but their location in a multilayered surface structure...
makes their digestive role somewhat unique. Enzymes located at the cell surface have access to substrates in the medium, and the products of their activity are available for diffusion through the cell wall to binding proteins and permeases at the outer aspect of the cytoplasmic membrane. This arrangement is especially interesting in cells which attack large nondiffusible food substances as in the case of a myxobacter species that feeds on blue-green algae (187) and which must maintain direct physical contact with the algal cell it is digesting because their degradative enzymes are wall-associated. The products of digestion are readily diffusible through the cell wall to the cytoplasmic membrane and are thus available for transport into the cell.

Degradative enzymes which are located in the periplasmic area act on substrates which diffuse into this zone of the cell wall, and their products are immediately available to the binding proteins and permeases at the outer aspect of the cytoplasmic membrane. Thus, periplasmic enzymes act on the wide variety of substrates which a gram-negative bacterium encounters in nature to convert them to molecules which are transportable into the cell. As an example, many of the sugars encountered by bacteria in the rumen are phosphorylated (94) and therefore not immediately transportable through the cytoplasmic membrane. The constitutive phosphatases within the periplasm of these cells cleave the phosphate groups from the sugars to make them transportable by specific permeases (34, 54). In certain strains of E. coli a permease can be induced by glucose-6-phosphate which transports both glucose-6-phosphate and glucose-1-phosphate (54).

Cell wall-associated enzymes provide the gram-negative bacterial cell with a digestive facility unique among unicellular organisms in that complex food molecules are broken down into their component monomers in a zone immediately surrounding the cell, and these small molecules are thus readily available to the cell’s transport mechanisms. The permeases of these cells can therefore be specific for a limited number of essential small molecules, and therefore the cell does not require a mechanism for the uptake of the huge variety of macromolecular substrates which serve as its food. The number of permeases required is further reduced by the activity of isomerases, such as phosphoglucose isomerase (75), in the periplasmic space so that some sugars are isomerized into molecules for which the cell already has a permease.

Although the loss of the capacity to produce a particular degradative periplasmic enzyme may not adversely affect a cell growing in a rich medium in which specific transportable monomeric substrates are supplied, the loss of such an enzyme would be critical to a cell in a natural environment with a varied and changing content of food materials. Although some laboratory-adapted gram-negative bacteria release degradative enzymes (116, 117, 122, 189) most wild-type strains hold these enzymes in a close association with the cell wall (34, 39, 116). This is in contrast with the gram-positive bacteria which release a large proportion of their extracellular enzymes into the medium (94, 108, 126, 161). The gram-positive pattern is clearly advantageous to an organism growing in conditions of high population density and high substrate concentration, because the products of digestion by extracellular enzymes are available to all of the cells in the immediate vicinity, but the extracellular enzymes are not protected by association with the cell wall.

On the other hand, gram-negative cells are well adapted for life in a dilute aqueous environment because, unlike gram-positive bacteria, they retain their degradative enzymes in a highly protective association with the cell wall (161), and because the products of digestion by these enzymes are immediately available to the transport system of the cell so that they are not lost into the water. For this reason the majority of bacteria found in the sea and in river water are of the gram-negative type (98).

Gram-negative cell walls contain other enzymes, in addition to the degradative enzymes described above, and Lundgren et al. (2) have suggested that potentially toxic autotrophic reactions are also localized within this structure. An iron-binding LPS-phospholipid-protein complex has been isolated from cell walls of a gram-negative autotroph (57), and Lundgren has suggested (2) that inorganic substrates such as iron may be oxidized while in a complexed form in the cell wall. This sequestration of autotrophic processes in the cell wall would serve to separate toxic intermediates and products from the cytoplasm while making nontoxic products available for transport into the cell.

**EPILOGUE**

This consideration of the gram-negative cell envelope leads towards an explanation of the ability of these bacteria to live in an extraordinarily wide variety of environments. This facility depends on a multilayered cell wall of
considerable complexity which confers rigidity, excludes certain toxic molecules, and prevents the access of antibodies to the gram-negative cell. Furthermore, the cell wall acts as an ion exchange material and specifically regulates the passage of ions into the immediate vicinity of the cytoplasmic membrane. Some of the constituent molecules of the cell wall are especially well suited to their many functions as in the case of LPS which contributes to the hydrophobic barrier layer, binds specific enzymes, attracts certain ions, and holds antibodies away from the outer membrane.

A variety of enzymes are associated with specific layers and zones within the cell wall, where their ionic and molecular environment is conditioned and maintained. These enzymes are synthesized within the cell and extruded through the cytoplasmic membrane into the cell wall where they associate with specific structural components of these layers and zones. Enzyme activities which are essential to the cell, but potentially damaging to cytoplasmic constituents, are sequestered in this area outside the cytoplasmic membrane.

Because these enzymes are retained in the areas just outside the cytoplasmic membrane their products may be readily transported into the cell or allowed to diffuse into the medium. Thus, the cell wall-associated enzymes of the gram-negative bacterial cell are capable of digesting a very wide variety of "food" molecules so that simple monomeric molecules can be selectively transported into the cell by specific binding proteins and permeases. In this way the cell wall constitutes a metabolically important functional organelle of the gram-negative bacterial cell and a unique biological design.

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