THE ANATOMY OF THE BACTERIAL SURFACE[1]

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I. Introduction

Despite its minute dimensions, the bacterial cell has been quite amenable to dissection into its component parts and, during the past ten or so years, a great deal has been learned about the chemical and biochemical anatomy of microorganisms. The term "bacterial anatomy" has become firmly entrenched in our literature and its usage no longer mystifies or amuses those biologists who are used to handling the "coarse" structures of higher animal and plant cells. The transition from the older study of bacterial cytology to the modern field of bacterial anatomy has really dated from the introduction of the electron microscope, the shadow-casting technique, and the development of elegant methods for slicing up the bacterial cell into incredibly thin sections. These advances have occurred at the time of a tremendous expansion of our biochemical knowledge and the emergence of a wealth of biochemical and biophysical techniques which have accompanied this period. Thus bacterial anatomy has been concerned not solely with the microscopic appearance of the various morphological entities of the cell, but also with the more exciting and broader problem of integrating both the structural and functional properties of the bacterial cell.

The student of human anatomy is only too well aware that *shape* is a most important anatomical attribute. The general importance of *shape* is amply illustrated by the anatomical and aesthetic qualities of the female form (unfortunately, for technical reasons, the slide illustrating this point in the lecture could not be reproduced here). So with bacterial anatomy, the shape of the cell is of great interest and, as realized long ago by Leeuwenhoek, it is the surface structure which determines the characteristic shape of the cell. The elucidation of the surface structure of bacterial cells has attracted much attention in recent years and for diverse reasons. Thus the immunologist has been interested in the presence of specific antigenic groupings on the cell surface. The biochemist has concerned himself with the transport of substances into and out of the cell across the permeability barriers and those interested in the control of microorganisms by antibiotics have become intrigued with the possibilities of killing bacteria by interfering with the biosynthesis of surface structures. The results of all these diverse investigations have begun to focus attention on the bacterial surface and we can now begin to talk about its detailed anatomy.

[1] The Office of Naval Research Lecture at the Society of American Bacteriologists Annual Meeting at Philadelphia in 1960 with the above title formed the basis for this contribution. I should like to express my sincere thanks to the Office of Naval Research and the Program Committee of the Society for making this lecture possible. This review of a rapidly growing field is not intended to be comprehensive but has been prepared in the hope that it may focus attention on certain aspects and perhaps stimulate some interest, to eliminate the large gaps in our knowledge.

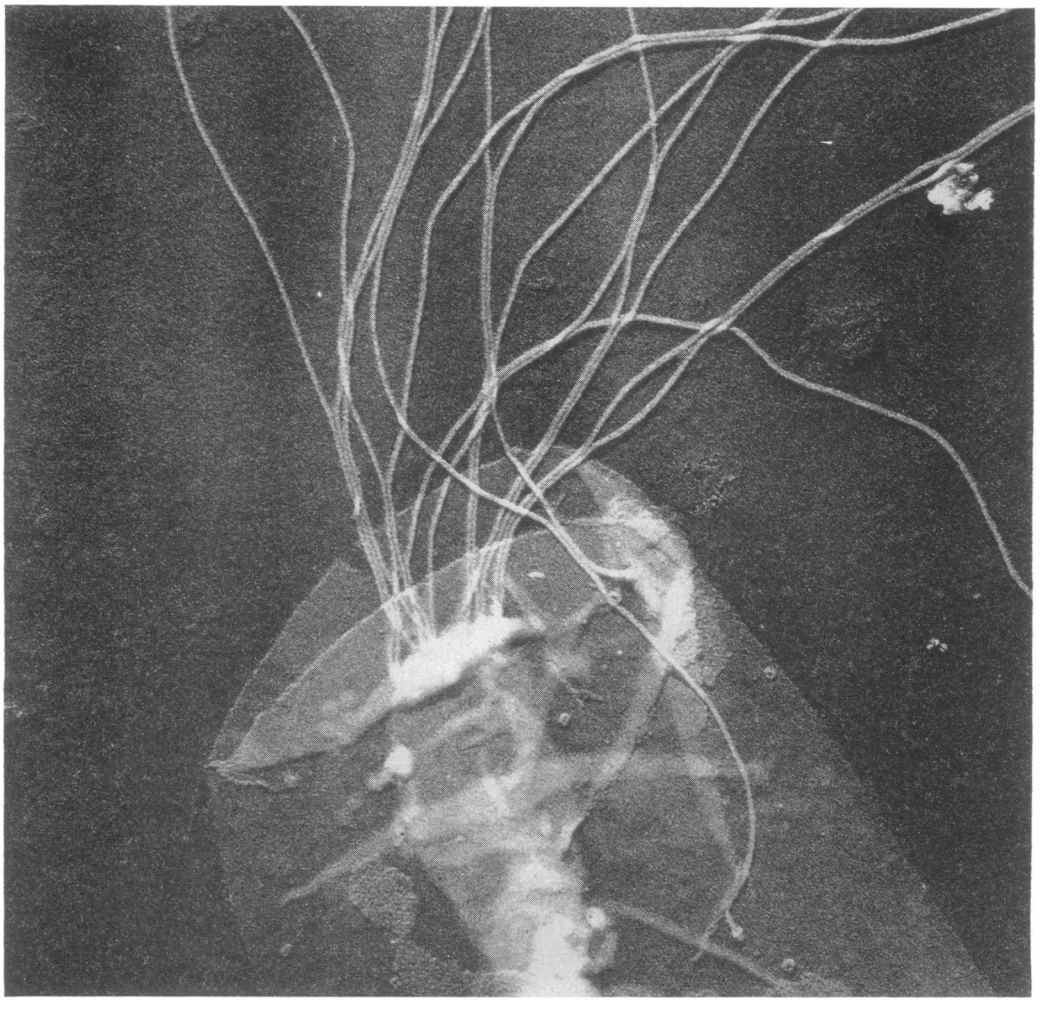

Figure 1. Spirillum serpens cells autolyzed and trypsin digested, showing flagella attached to basal granules inside the cell (unpublished electron micrograph by R. W. Horne and M. R. J. Salton). ×60,000.

The surface structures of the bacterial cell can be conveniently classified (88) as surface appendages, surface adherents, rigid cell wall, and protoplasmic membrane.

II. Surface Appendages

Two main surface appendages of bacterial cells are the flagella, the organs of locomotion of the cell (121), and the fimbriae (27, 29). The flagella have now been well characterized chemically (58, 121) and there is generally no difficulty in distinguishing between the appearance of flagella and fimbriae in electron micrographs of bacteria, as illustrated in figures 1 and 2. The term fimbriae was first proposed by Duguid and his colleagues (27, 29) as the Latin equivalent of "threads," "fibers," and "filaments" used in earlier descriptions of these structures (3, 49). This term has gained wide acceptance although Brinton (13) has suggested the word *pili* (Latin for "hair") as a synonym. The only other surface appendage that should be mentioned is that encountered in the iron bacterium, *Gallionella ferruginea*, which possesses long strands of material that are obviously very different from flagella and fimbriae (113). All these surface structures may have intracellular origins;

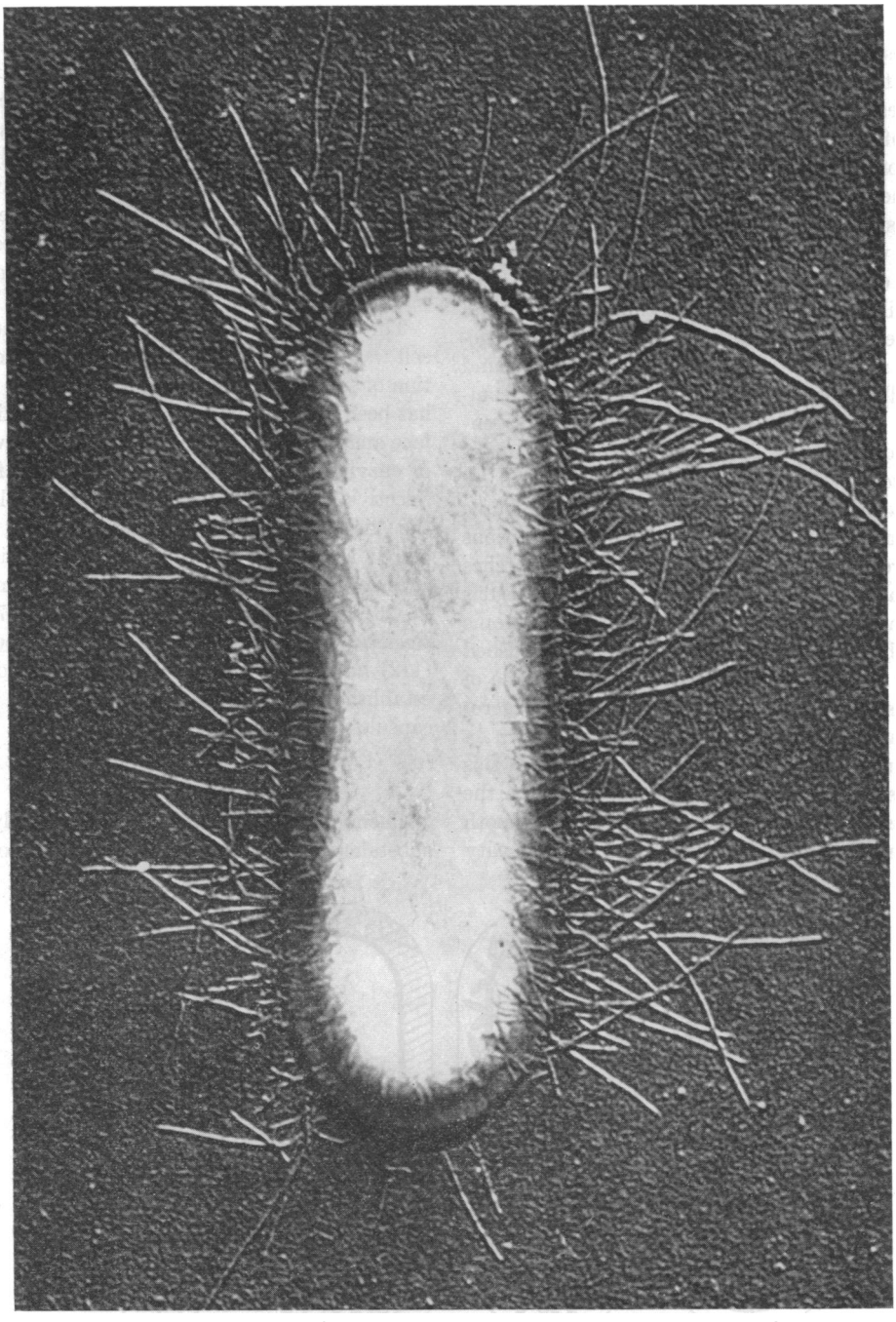

Figure 2. Fimbriae arranged around the cell surface of *Shigella flexneri* (Duguid and Gillies (28)). ×45,000.

flagella apparently pass through the wall and membrane and terminate in a spherical organelle located in the bacterial protoplasm, as illustrated in figures 1 and 2. Although the fimbriae are responsible for the adhesive properties of certain bacteria (28), the functions they may confer on the cell have not been clearly established.

III. Bacterial Capsules

Capsules were among the first surface structures to be removed from bacterial cells and chemically characterized. The classical investigations of Avery and Heidelberger (see Heidelberger (43)) on the chemistry and immunochemistry of the pneumococcal capsular polysaccharides were the first extensive studies giving us some concept of the variety of compounds forming the surface layers of certain bacterial cells.

Until comparatively recent times, the bacterial capsule has generally been regarded as a homogeneous accumulation of viscous material around the cell. However, Tomcsik's (110) studies combining phase-contrast microscopy and antigen-antibody reactions have shown that the capsules of *Bacillus anthracis* and certain strains of *Bacillus megaterium* are by no means of simple physical structure. Indeed, these investigations have revealed the presence of localized patches of a capsular polysaccharide in a gel of capsular glutamyl peptide. The electron microscopic study by Labaw and Mosley (59) has established a very complex physical state for the capsule of the Lisbonne strain of *Escherichia coli*. Macromolecular components were embedded in a structureless gel forming the capsule of this organism. Stained preparations usually give the impression that the capsular surface is smooth and continuous but a further physical complexity was brought to light when Ivanovics and Horvath (55) detected fairly regular indentations along the surface of the capsule of a *Bacillus megaterium* strain. There is probably little doubt that the capsules of many bacteria are physically homogeneous structures of one type of polymeric substance, but with refined methods of studying the anatomy of the bacterial surface, some of the more complicated structures, illustrated diagramatically in figure 3, have now become well established.

The relationship of the capsules to the rigid cell wall has been of great interest and the isolation of specific capsule-degrading enzymes (6, 23) has been of the utmost value in studying the surface anatomy of bacteria. There is now a variety of enzymes or enzyme systems available for the selective removal of bacterial capsules, leaving the viability of the decapsulated cells unimpaired as well as the ability to synthesize the capsular material. Encapsulated pneumococci (6, 23), klebsiellae (1), streptococci (63, 67), and *Bacillus* spp. possessing γ-glutamyl peptides (112) have been enzymically decapsulated, thus establishing the anatomical relationships of the capsules and walls of these organisms.

A. Chemical Composition

Chemically, bacterial capsules are polymeric substances of either polysaccharide or polypep-

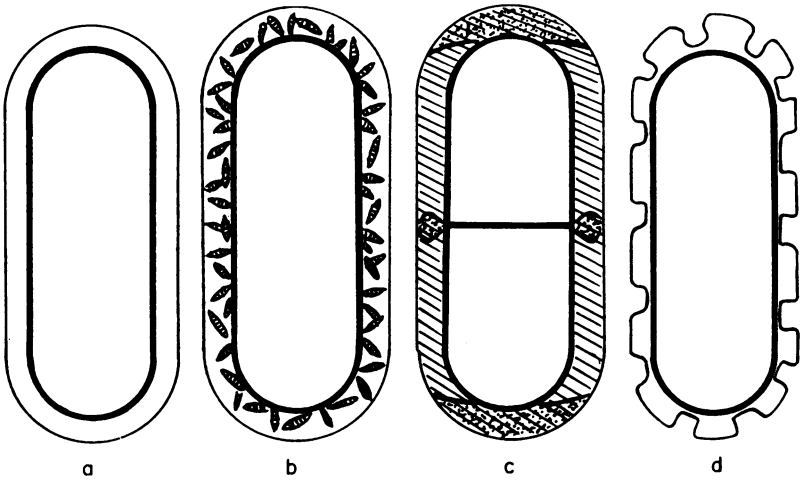

Figure 3. A diagrammatic representation of the types of capsular structure (taken from reference 88): (*a*) capsule forming continuous layer around cell; (*b*) capsular layer with banded fibrils (59); (*c*) complex capsule with localized patches of polysaccharide and polypeptide (10); (*d*) discontinuities in capsular surface (55).

TABLE 1

Chemical nature of bacterial capsular substances

Organism	Class of Substance and Products of Acid Hydrolysis
Gram-positive:	
Bacillus anthracis	Polypeptide: γ-D-glutamyl peptide
Bacillus megaterium	Polypeptide: γ-glutamyl peptide Polysaccharides: amino sugars; sugars
Bacillus circulans	Polysaccharide: glucose, mannose, uronic acid
Pneumococci	Polysaccharides: sugars, amino sugars, uronic acids, ribitol phosphate
Streptococci	
Groups A and C	Polysaccharide: hyaluronic acid-glucosamine, glucuronic acid
Rumen species	Polysaccharide: galactose, rhamnose, uronic acid
Gram-negative:	
Acetobacter capsulatum	Polysaccharide: dextrin-glucose
Haemophilus influenzae	Polyribophosphate
Aerobacter-Klebsiella group	Polysaccharides: complex polyuronides-glucose, fucose, glucuronic acid
Escherichia coli	Polysaccharide: fucose, galactose, hexuronic acid

Data summarized from references 88 (giving original references), 42, 78, 127.

tide nature. So far as I am aware, heteropolymers containing polysaccharide and peptide residues covalently linked (as in bacterial walls) have not been encountered as capsular substances. Of all the bacterial capsular polysaccharides studied, those of the pneumococci have been investigated in greatest detail and the chemical structure of a number of different types has been determined (43). Both amino sugars and uronic acids are widely distributed in capsular polysaccharides from both gram-positive and gram-negative bacteria. The capsular polysaccharides of gram-negative bacteria must not of course be confused with the polysaccharide moieties of the protein-lipid-polysaccharide complexes constituting the macromolecular components of the wall or envelope. The variety of materials forming bacterial capsules (briefly summarized by Salton (88)) is illustrated in table 1 and includes hyaluronic acid, polyuronides, various polysaccharides, and the γ-glutamyl peptides. The list of capsular substances presented in table 1 is by no means comprehensive. It does not include, for example, other interesting surface polymers such as the amino uronic acid forming the Vi antigen (16), the M proteins of streptococci (60), and other components that are not demonstrable by the methods usually employed for detecting capsules (26).

In general, there is little chemical relationship between capsular substances and the cell-wall structures, there being distinctive compounds in the wall enabling us to differentiate one from the other. However, Nature has prevented us from putting everything into tight little compartments and there are now two instances in which capsular substances have been found to contain compounds that we have come to regard as exclusively wall substances. An extremely interesting example of this situation has arisen from the discovery of ribitol phosphate in the specific polysaccharide of type 6 pneumococcus by Rebers and Heidelberger (78). Up to the time of this report, ribitol phosphate compounds in polymeric form had been found only in the teichoic acids of the bacterial wall, discovered by Baddiley and his colleagues (4, 9). The second example of a material of capsular origin containing a wall substance, probably the cell-wall amino sugar, muramic acid, is the polysaccharide derived from *Bacillus megaterium* by Guex-Holzer and Tomcsik (42). This material appeared to be immunologically identical to the cell-wall substance and upon isolation the capsular polysaccharide was found to contain glucosamine, galactosamine, and an unknown amino sugar presumed to be muramic acid. Whether this material should be regarded as a true capsular polysaccharide, or whether it represents a local accumulation of wall material being "over-produced" by the dividing cell, could only be decided by further investigation. At least these two cases illustrate

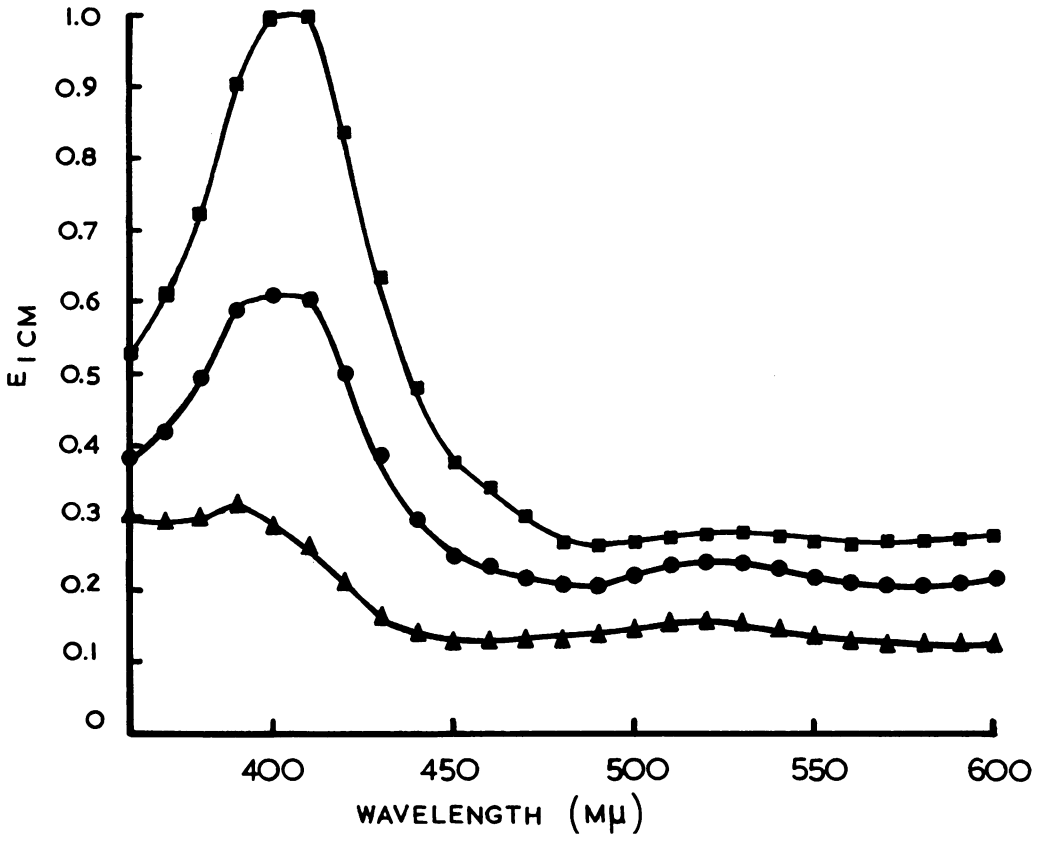

Figure 4. Absorption spectra of the products of Dische (22) reactions on *Aerobacter (Klebsiella) aerogenes* strain A3 untreated walls (●—●); walls after extraction of capsular polysaccharide (▲——▲); and the hot-water extractable polysaccharide (■——■). $E_{1\ cm}$ for 0.45 mg each fraction (89).

the point that there may be a greater chemical overlap between capsular substances and walls than we had hitherto suspected. Although both the cell walls and the capsular polypeptides from *Bacillus anthracis* and *Bacillus megaterium* contain the D-isomer of glutamic acid (52, 54, 84), the capsular peptide is a simple polymer, whereas in the wall the D-glutamic acid is chemically linked in a more complex mucopeptide.

The retention of capsular and surface substances on the cell wall during its isolation presents a problem in establishing the chemical anatomy of the surface structures. Thus the M protein was retained on isolation of the walls of a group A streptococcus and it could be removed enzymatically from the walls by digestion with trypsin (80). Further studies with a capsulated strain of *Aerobacter (Klebsiella) aerogenes* have also shown that during wall isolation some of the polysaccharide is retained and, on hydrolysis of the "wall" fractions, monosaccharides characteristic of the capsular substance are also present. Fortunately with this strain it is possible to distinguish between constituents of the capsule (127) and the wall polysaccharide components, for Dudman and Wilkinson (25) had shown that the polysaccharide could be extracted from intact cells with hot water. When the "wall" fractions were extracted in this way, the fucose and uronic acid of the capsular polysaccharide were found in the water-soluble fractions, leaving the insoluble wall fraction devoid of these sugars (89). The Dische (22) reaction for methyl pentose can be used to follow the distribution of these sugars in "wall" and capsular polysaccharide fractions as illustrated in figure 4. The monosaccharide constituents detected in "wall" fractions of encapsulated, slime-producing, and nonen-

TABLE 2

Monosaccharide constituents of "walls" of encapsulated, slime-producing, and nonencapsulated strains of Aerobacter (Klebsiella) aerogenes

Strain	Preparation	Monosaccharides
A3 capsulated	Cell walls	Galactose, glucose, fucose, uronic acid
	Wall extracted with hot water	Galactose, glucose
	Extracted capsular polysaccharide	Galactose, glucose, fucose, uronic acid
A3 (Sl) slime-producing	Walls	Galactose
A3 (O) non-slime, non-capsular	Walls	Galactose

Reference 89.

capsulated strains of *Aerobacter* (*Klebsiella*) *aerogenes* are summarized in table 2. These results emphasize the need for specific removal of capsular substances when a differentiation of wall and capsular polysaccharides is being sought.

IV. The Cell Wall

The major structural component of the bacterial cell is the rigid wall, which may account for about 10 to 40 per cent of the weight of the cell (88). The excellent studies of Shockman, Kolb, and Toennies (100) have demonstrated how the wall contribution to the weight of the cell depends on the growth phase, rising from 27 per cent in the exponential phase to 35 per cent in the stationary phase of the organism, *Streptococcus faecalis*. Furthermore, the nutritional status of the organism can also influence the amount of cell-wall substance formed, as shown in the amino acid depletion studies by Shockman (99).

The isolation and characterization of bacterial cell walls has in recent years presented us with some fascinating details of the comparative anatomy and chemistry of gram-positive and gram-negative bacteria. From the variety of walls examined in the electron microscope, it is now apparent that, in general, the walls of gram-negative bacteria are physically more complex than those of gram-positive organisms (90). The presence of spherical macromolecules in a cell wall was first reported by Houwink (48). Since then a number of spirilla and other gram-negative bacteria have been shown to possess fine structure in the walls (see Salton (90)). Layers of hexagonally packed spherical macromolecules have been encountered most frequently. Although the isolated wall of *Escherichia coli* has a homogeneous appearance on examination of shadowed preparations in the electron microscope (48, 93), it is evident from the beautiful thin sections, prepared by Kellenberger and Ryter (57) and presented in figure 5, that the wall is multilayered. The studies of Weidel and his colleagues are now beginning to correlate the physical and chemical complexity of the wall of this organism (125). A type of fine structure differing from that commonly found in many of the gram-negative bacteria has been observed in the complex wall or "envelope" of the organism, *Lampropedia hyalina*. This macromolecular structure observed independently by J. A. Chapman and M. R. J. Salton (*unpublished observations*) and R. G. E. Murray (*unpublished observations*) is illustrated in figure 6.

A. Chemistry of Cell Walls

The first attempt to discover the chemical composition of a bacterial cell wall was made as long ago as 1887 by Vincenzi (116). Apart from finding substantial amounts of nitrogen and deducing that the wall was not composed of cellulose, Vincenzi (116) was unable to suggest anything of a more definite nature. However, during the past ten years a great deal of information on the chemical composition of bacterial cell walls has become available and several outstanding features have emerged. The walls of gram-negative bacteria were found to be chemically more complex than those of gram-positive organisms (81). Walls of gram-positive bacteria contained a small variety of amino acids, amino sugar, and sugar components (81). It was thus apparent that the walls of gram-positive bacteria were made up of a new structural class of polymers differing from the structural polysaccharides commonly encountered in the walls of fungi and higher plants.

The study of the chemistry of bacterial cell walls has attracted many investigators in the past decade and only the briefest account can be given of some of the essential features. Several

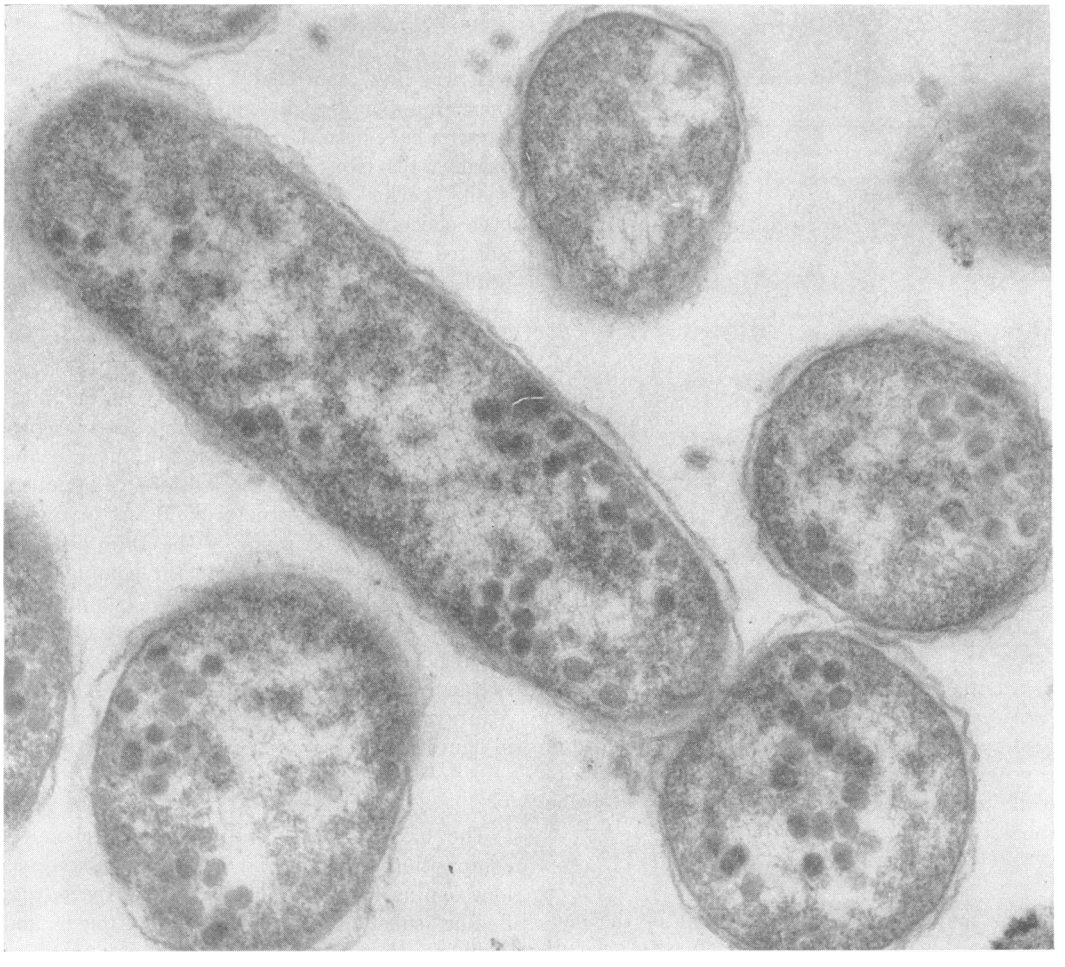

Figure 5. Thin sections of *Escherichia coli* strain B infected with T2 phage showing multilayered cell wall and a separate electron-dense layer, the cytoplasmic membrane (Kellenberger and Ryter (57)). ×63,000.

substances not generally encountered in the cells of higher organisms have been found in bacterial cells and shown to be localized in the rigid wall structures. Thus muramic acid (104), discovered first in spore peptides by Strange and Powell (106) and later in walls by Cummins and Harris (19), Strange and Dark (105), and Salton (83), and α,ϵ-diaminopimelic acid, isolated and characterized by Work (128), are two substances confined to bacteria and closely related microorganisms such as blue-green algae (90, 130). Another fascinating feature of cell-wall chemistry has been the widespread occurrence of the D-isomers of the amino acids, alanine, glutamic acid, and aspartic acid (52, 53, 84, 99, 109), in the wall mucopeptides. If we can endow Nature with purpose, it seems eminently sensible of Her to have designed walls containing D-amino acids in their peptides, thus providing structures resistant to many of the commoner proteolytic enzymes and peptidases.

The walls of many gram-positive bacteria may be made up entirely of the mucocomplex substances, mucopeptides and mucopolysaccharides, or both. The mucopeptides (64) invariably contain the amino sugars glucosamine and muramic acid, and peptides composed of a variety of 3, 4, or 5 principal amino acids. In addition to mucopeptides, some walls contain polysaccharide or oligosaccharide residues (46,

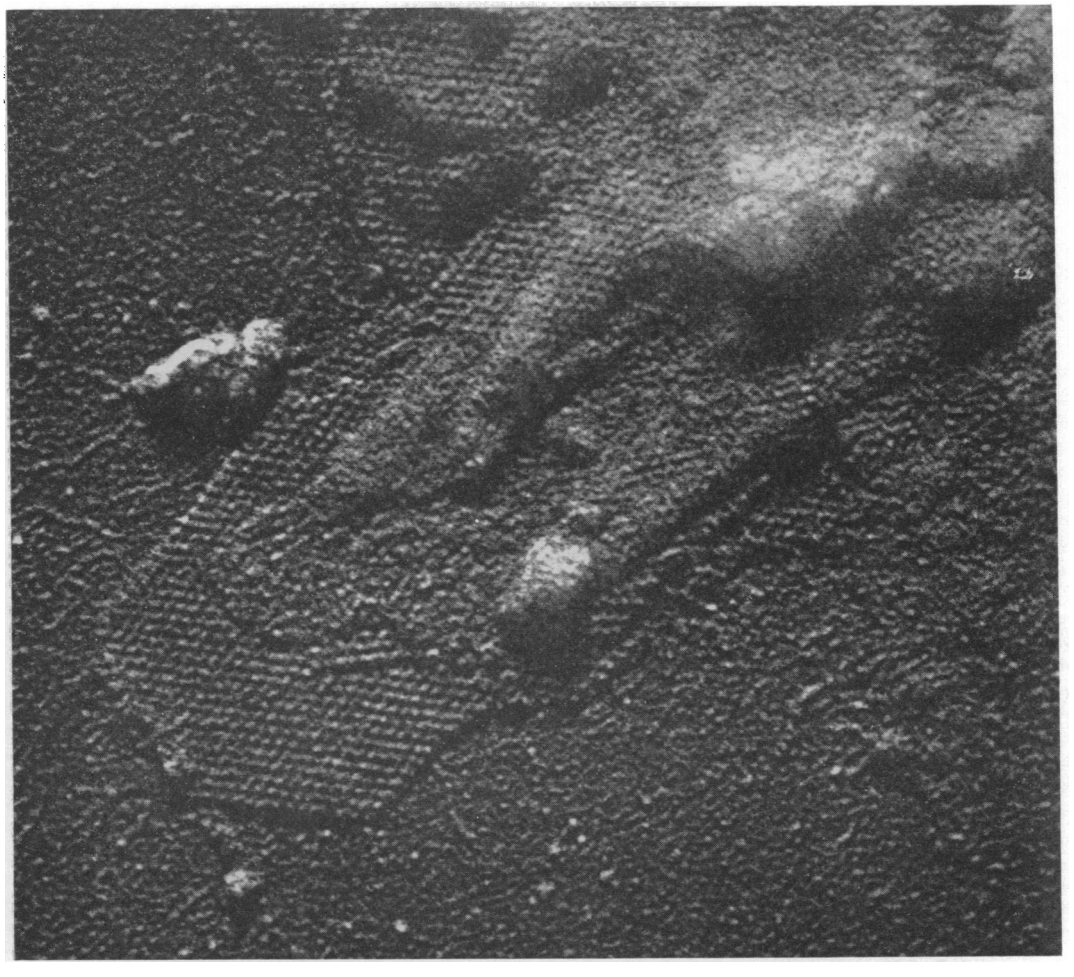

Figure 6. Fine structure in a wall layer obtained from disintegrated cells of *Lampropedia hyalina* (unpublished observations by J. A. Chapman and M. R. J. Salton). ×110,000.

68, 79) covalently linked to the mucopeptides. A new class of cell-wall polymer differing from the mucocomplex substances was discovered several years ago by Baddiley and his colleagues (4, 5, 9). Following the characterization of two nucleotides, cytidine diphosphoglycerol and cytidine diphosphoribitol (8), a search for a biosynthetic function for these nucleotides led to the detection of ribitol and glycerol-phosphate polymers in bacteria and ultimately to the localization of the teichoic acids (from Greek *teichos* = wall) in the isolated cell walls. It will be recalled that in an earlier study Mitchell and Moyle (72) had found polyolphosphates in their "envelope" fractions of *Staphylococcus aureus*. In general the two types of teichoic acid (glycerol- and ribitol-teichoic acid) do not occur together (5) and they seem to be absent from a number of gram-positive bacteria. One interesting feature of the teichoic acids is the presence of ester-linked alanine in both types. The relationship of the glycerol-teichoic acid to the polyglycerophosphate compound found in a number of gram-positive bacteria by McCarty (69) has not been established. The latter could conceivably arise from the glycerol-teichoic acid if the labile ester-linked alanine was lost during isolation and purification.

Studies on the molecular structure of wall mucopeptides have advanced rapidly in the past few years. Much information has been gained from several sources, including the elucidation

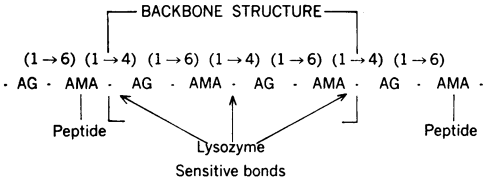

Figure 7. Type of molecular structure proposed for the wall of *Micrococcus lysodeikticus* (91), showing the arrangement of peptide side-chains on an acetyl amino sugar backbone possessing alternating 1→4, 1→6 bonds between *N*-acetylmuramic acid (*AMA*) and *N*-acetylglucosamine (*AG*).

of the structure of the nucleotides accumulaitng in the presence of penicillin and other antibiotics (77, 107) and investigations of the products of enzymatic hydrolysis of isolated cell walls (83). One type of structure emerging from the early work on the products of lysozyme digestion of walls (83) was suggested by Salton (85) and further expanded from the knowledge of the nucleotide structure by Brumfitt, Wardlaw, and Park (14). The isolation of di- and tetrasaccharides and peptide-amino sugar complexes (mucopeptides) in lysozyme digests of walls (35) and their chemical characterization (34, 91) enable us to suggest (92) the type of structure for the wall of *Micrococcus lysodeikticus* illustrated in figure 7.

The cell walls of gram-negative bacteria are much more complex. In addition to containing mucopeptides in common with gram-positive bacteria (87, 124) they also possess major protein, lipid, and polysaccharide constituents. The mucocomplex or mucopeptide components of the walls of gram-negative bacteria may account for only 10 to 20 per cent of the weight of the wall, but it is apparent that it is this class of chemical constituent which is responsible for the structural rigidity of the wall (87, 88, 90, 124, 125). It seems likely that the protein, lipid, and polysaccharide components of the wall are present as a macromolecular complex, with the mucopeptide forming links in a rigid layer (125) or a reinforcing network throughout the entire wall (90). The various antigenic substances isolated as the O, or somatic smooth-phase, antigens are probably derived from the macromolecular complexes of the cell walls (89). It is not known at present whether the wall is made up of a variety of chemically and immunologically different macromolecular subunits.

TABLE 3
Chemical composition of bacterial cell walls

	Principal Classes of Constituents and Products of Acid Hydrolysis
Gram-positive bacteria	1. Mucopeptides-glucosamine; muramic acid; 3, 4, or 5 amino acids. 2. Mucopolysaccharides-amino sugars, monosaccharides 3. Teichoic acids-ribitol, phosphate, glucose or glucosamine, alanine; glycerol, phosphate, alanine Walls may have compositions with the following combinations: 1, 1 + 2, 1 + 3, 1 + 2 + 3.
Gram-negative bacteria	Mucopeptides as above. (Teichoic acids not as yet isolated from this group.) Protein, Lipid, Polysaccharides } Probably present as complexes of all three classes

References 88, 90.

Some of the principal features of the chemical composition of bacterial walls are summarized in table 3. More extensive accounts of cell-wall chemistry are available in earlier reviews by Cummins (18) and Work (129) and in more recent contributions by Salton (88, 90).

V. Protoplast Membranes

One of the most important advances contributing to the further study of the anatomy of the surface structures of the bacterial cell was made when Weibull (118) isolated and characterized the protoplasts of *Bacillus megaterium* in 1953. This enabled a direct examination of the functional and chemical properties of the bacterial membrane to be made. That the isolated protoplast presented a surface different from that of the original intact cell was demonstrated in a number of ways. It could not be infected with bacteriophages (118), which require a specific receptor in the cell wall (94). The antigens on the surface of the protoplasts differed from those of the isolated walls and intact cells (111, 114, 115). Unlike walls and intact cells, the protoplasts of *Bacillus megaterium* and *Micrococcus lysodeikticus* and their membranes are extremely susceptible to disaggregation with sodium dodecyl sulfate

and other anionic detergents (36, 38, 86). These various properties based on the characteristics of the protoplast membrane can be used as criteria in defining *protoplasts*, a term reserved for the organized protoplasmic elements of bacterial or microbial cells deprived completely of the cell-wall structure (12). Most of the true protoplasts have been obtained by enzymatic degradation of the wall (12, 20, 32, 40, 70, 118). With organisms such as *Bacillus megaterium*, *Micrococcus lysodeikticus*, and *Sarcina lutea*, wall degradation with lysozyme may be complete and leave none of the characteristic wall compounds in the protoplasts or the protoplast membranes (12, 70). There are, however, several instances in which protoplasts have been obtained without complete digestion of the cell wall. An autolytic enzyme from *Staphylococcus aureus* cuts the wall of that organism into two hemispheres, thus releasing the intact protoplast when the enzymatic action is allowed to take place in a suitable stabilizing medium (75). Similarly, partial breakdown of the wall of *Neurospora crassa* permits the extrusion of an intact protoplast (7).

Unfortunately, not all organisms are amenable to such elegant enzymatic manipulations designed for the stepwise "peeling off" of the surface layers of the cell. The walls of many gram-positive bacteria are only partially degraded by lysozyme (95) and, consequently, attempts to isolate and characterize the protoplast membranes of these organisms will have to await the development of more specific enzyme preparations. The incomplete removal of wall components is especially conspicuous with the formation of spherical cells ("protoplasts," spheroplasts (12, 70)) of gram-negative bacteria following treatment with lysozyme and ethylenediaminetetraacetic acid (EDTA), or the growth of these

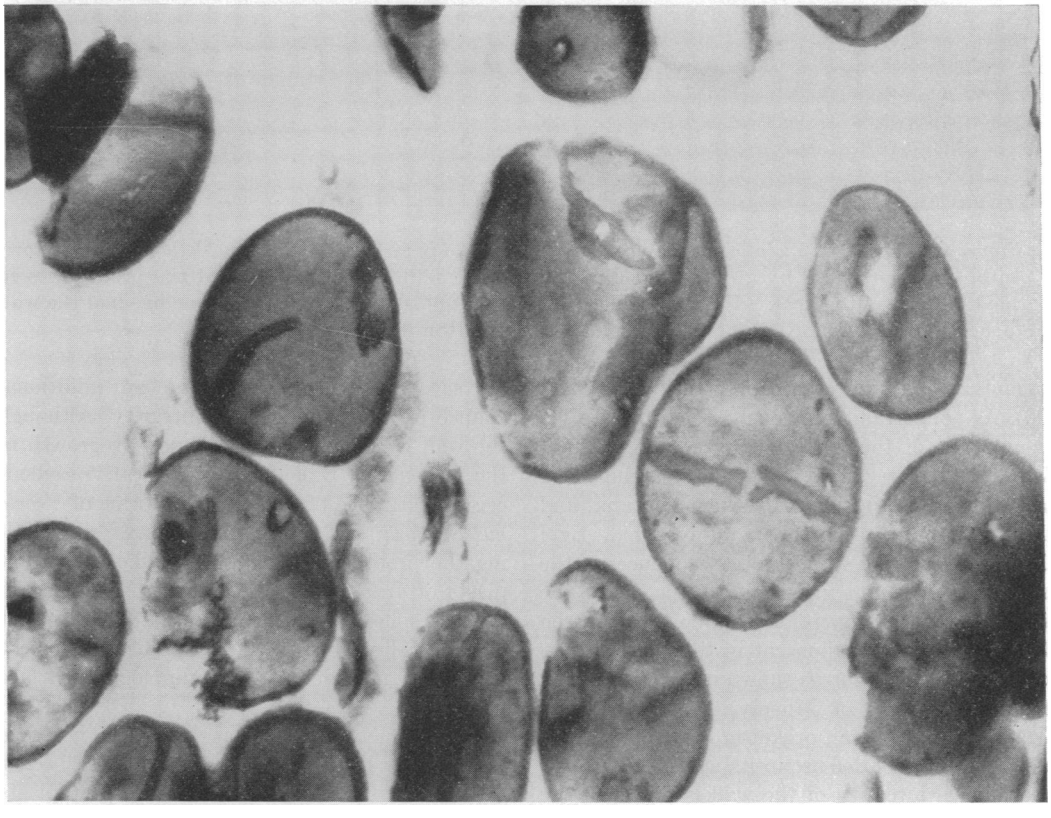

Figure 8. Some anatomical consequences of the action of pencicillin on bacteria. Thin sections of *Staphylococcus aureus* exposed to penicillin. Gross distortion at the points of cross wall formation clearly visible (Murray, Francombe, and Mayall (76)). ×46,000.

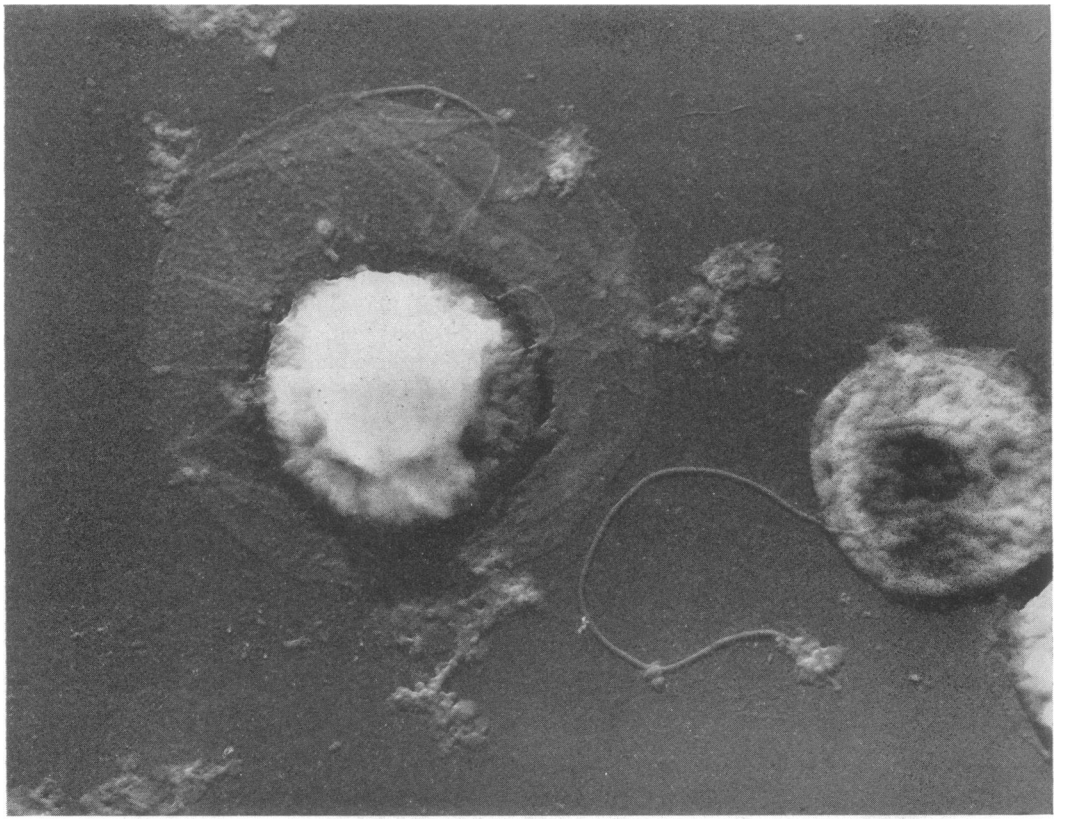

Figure 9. Some anatomical consequences of the action of penicillin on bacteria. *Vibrio metschnikovii* "protoplasts" prepared by growth in the presence of penicillin. The majority of the protoplasts so formed have an outer weak wall as in the left-hand "protoplast"; the right-hand one has had the wall detached during preparation for electron microscopy (Salton (90)). ×16,500.

organisms in the presence of penicillin (61, 70, 96). As pointed out previously, the mucopeptide component of the wall of gram-negative organisms can be regarded as either a reinforcing network or, as suggested by Weidel, Frank, and Martin (125), as part of an organized, rigid layer. At least the structural consequences of the action of penicillin in its interference with the formation of mucopeptide can be clearly seen from the "lesions" apparent in the thin sections of *Staphylococcus aureus* shown in figure 8, taken from the studies of Murray, Francombe, and Mayall (76). Such an organism has, so to speak, no second line of defense in its wall, for once the mechanical integrity of the wall is breached the protoplast membrane will be unable to withstand the high osmotic pressure and lysis will ultimately ensue. By way of contrast, the walls of the gram-negative bacteria with their protein-lipid-polysaccharide complexes have an additional chance of maintaining their integrity. Although the wall is considerably weakened by growth in the presence of penicillin, the typical "poached-egg" appearance of the spherical forms of *Vibrio metschnikovii* still shows an outer wall surrounding the protoplast (figure 9). Unlike the protoplasts of gram-positive bacteria, these spherical cells or spheroplasts are agglutinated by intact cell and cell-wall antisera, thus indicating a very similar, if not identical, immunological surface (47, 96, 98).

The structural analysis of gram-negative bacteria has thus been hampered by the absence of suitable methods for isolating protoplasts analogous to those of gram-positive bacteria and as a consequence the evidence for the existence of a separate, functional protoplasmic membrane structure is still largely circumstantial. However,

the appearance of thin sections of gram-negative bacteria strongly supports the conclusion that they too possess a well defined protoplast membrane (see figure 5).

The behavior of the "envelope" or "hull" of certain gram-negative organisms has suggested to several investigators that separate wall and membrane structures may not exist in them (65, 66). It is likely that this question could be resolved with an enzyme system capable of degrading the wall sufficiently to allow the isolation of a protoplast if such exists. Attempts to isolate such enzymes have been disappointing and as pointed out by Salton (82) the production by a single organism of all the enzymes required to break down the wall may be an infrequent event in Nature. It is of course apparent to even the elementary student of microbiology that there must be organisms producing enzymes capable of breaking down *everything* (used only in the sense of all macromolecular components and their building blocks), otherwise we would be knee deep or even further immersed in walls of gram-negative bacteria! When such enzymes become available it may be possible to decide whether there is a structure analogous to the membrane of the gram-positive bacteria, or whether the "envelope" of the gram-negative organisms is an integrated structure possessing both "wall" and transporting functions of an osmotic membrane. The success of this approach would be dependent on there being enough difference in the chemical constitution of wall and membrane to permit the selection of specific enzymes. It is quite conceivable, however, that walls and membranes of gram-negative bacteria may be sufficiently similar to make the selective removal of the wall very difficult or even impossible.

A. Chemical Composition of Membranes

That the protoplast membrane would be chemically more complex than bacterial walls was suspected from Weibull's (118, 119) demonstration of the presence of the cytochrome system in the isolated membranes of *Bacillus megaterium* and the detection of a number of enzymes of the electron transport system in these structures by Storck and Wachsman (103). Two groups of investigators have isolated and characterized chemically the protoplast membranes of *Bacillus megaterium* (122) and *Micrococcus lysodeikticus* (39). Both bacterial membranes are made up largely of protein and lipid. A comparison of wall and membrane composition of each species is presented in tables 4 and 5. The lipid of the membranes of these two organisms appears to be mainly phosphatidic acid; accordingly, Gilby and Few (38) have suggested that cationic detergents may act on this lipid component of the protoplast membrane. The membrane fractions have frequently been found to form a characteristic yellow layer on centrifugation of lysed protoplasts (39, 70, 75, 118). This pigmentation can at least in part be accounted for by the presence of carotenoids (37, 39). Nucleic acids (both ribonucleic (RNA) and deoxyribonucleic (DNA)) have been detected in isolated membranes (114, 122). Weibull and Bergström (122) found that the RNA contents of batches of membranes of *Bacillus megaterium* varied from about 0.5 to 2 per cent but substantially higher values have been observed by Vennes and Gerhardt (114). It

TABLE 4

Comparison of composition of cell wall and protoplast membrane of Bacillus megaterium

Constituent	% Dry Weight	
	Wall	Membrane
Nitrogen	7.4–7.8	10.3–10.9
Phosphorus	3.4–3.5	
Lipid	4.2–5.9	15.9–20.9
Hexose	0.3–0.9	1.8–9.8
Amino sugar	20–23	<0.7
Diaminopimelic acid	7–9	<0.1

Reference 122.

TABLE 5

Comparison of composition of cell wall and protoplast membrane of Micrococcus lysodeikticus

Constituent	% Dry Weight	
	Wall	Membrane
Nitrogen	7.6	8.4
Phosphorus	0.22	1.16
Lipid	0	28.0
Mannose	0	18.9
Glucose	3.5–5.8	0
Amino sugar	16–22	2.7

Reference 39.

is possible that the nucleic acid material present in the membrane fraction may represent contaminating matter from the bacterial protoplasm (122).

VI. Localization of Enzymes in Bacterial Cells and a Summary of the Comparative Chemical and Biochemical Anatomy of Gram-positive and Gram-negative Bacteria

With the methods available for the release of protoplasts of gram-positive bacteria and the isolation of the protoplast membranes it has been possible to come to some conclusions about the localization of certain enzymes in the major surface structures of the bacterial cell. The biosynthetic capabilities of the bacterial protoplast (70, 120) are so similar to those of intact cells that it appears unlikely that the cell wall contributes much more than mechanical stability to the bacterial cell. Thus the complete loss of the wall during protoplast formation does not seriously impair the functioning of the osmotic barrier, the biosynthesis of complex molecules such as proteins and nucleic acids, or the synthesis and assembly of bacteriophages and spores (20, 31, 120).

The distribution of enzymes in protoplast and soluble and particulate fractions of *Bacillus megaterium* has been studied by several investigators and there is very good agreement between the results of Storck and Wachsman (103) and those of Weibull, Beckman, and Bergström (123) for several strains of this organism. Some of the results for the localization of enzymes in the study of Weibull *et al.* (123) are presented in table 6. Investigations with *Bacillus megaterium* (123) and *Staphylococcus aureus* membranes (74) have confirmed the presence of cytochromes, the reduced diphosphopyridine nucleotide (DPNH) oxidase, the succinic dehydrogenase, and the malic dehydrogenase systems in these structures. Mitchell and Moyle (74) found acid phosphatase mainly in the membrane of *Staphylococcus aureus*, whereas this enzyme and hexokinase were largely in the "soluble" protoplasmic fraction of *Bacillus megaterium* (123) as shown in table 6. There is no information about the detection of enzymes in wall fractions of gram-positive bacteria. The chemical composition of these structures would, however, lead one to suspect that whatever enzymes were present could be derived from contamination with membrane fragments or adsorbed protoplasm. This of course does not preclude the possibility that enzymes are located on the wall of the intact bacterial cell.

TABLE 6

Relative amounts of enzymes in membrane and soluble protoplasmic fractions of Bacillus megaterium

Enzyme	Membrane Fraction	Soluble Protoplasm
Succinic dehydrogenase	145.0	4.5
Malic dehydrogenase	32.6	15.7
Lactic dehydrogenase	41.2	57.2
Isocitric dehydrogenase	3.5	112.0
Fumarase	32.2	101.8
DPNH oxidase	261.0	1.0
Catalase	1.3	100.0
Hexokinase	5.5	87.5
Acid phosphatase	3.0	99.6

Data from reference 123.

TABLE 7

Enzyme distribution in fractions from a strain of Pseudomonas disintegrated in the Mickle apparatus

Enzyme	Crude Wall Fraction	Protoplasmic Fraction
Succinic dehydrogenase	+	±
Malic dehydrogenase	+	±
Fumarate dehydrogenase	−	+
Alanine dehydrogenase	−	+
DPNH oxidase	+	Trace

Unpublished data, A. D. Brown, S. Jeffery, and M. R. J. Salton.

Owing to the difficulty of isolating membranes of gram-negative bacteria as separate structural entities, the question whether certain enzymes are localized in the wall or membrane will have to await further investigation. However, there have been several studies of the distribution of enzymes in soluble, particulate, and "envelope" or "hull" fractions of gram-negative bacteria (2, 17, 50, 51, 62). A number of the enzymes of the electron transport system have been found in the "envelope" fractions (17, 51). Hunt, Rodgers, and Hughes (51) isolated a "cell wall-membrane" fraction from mechanically disintegrated cells of a strain of *Pseudomonas fluorescens*. From their excellent studies they concluded that the nicotinic acid hydroxylase

and succinic acid dehydrogenase systems were located in this complex structure. Further shaking of these "wall-membrane" fractions with glass beads did not release much of the enzyme activities. However, lysozyme and EDTA treatment at 25 C for 8 to 10 min released the total activity into the supernatant fraction. A. D. Brown, S. Jeffery, and M. R. J. Salton (*unpublished observations*) studied the distribution of enzymes in crude "wall," small particle, and protoplasmic fractions of a *Pseudomonas* sp. disintegrated in buffer at 0 C in the Mickle apparatus. The qualitative results presented in table 7 again confirm the presence of succinic acid dehydrogenase, malic dehydrogenase, and DPNH oxidase in the crude wall or "envelope" fractions. Owing to the greater difficulty in obtaining clean wall fractions of gram-negative bacteria, further investigations will be needed before it is possible to arrive at firm conclusions on the distribution of enzymes in the wall and membrane structures or particles derived from both. At the moment, all we can decide about the gram-negative organisms is whether an enzyme is present in the "envelope" (cell wall-membrane) fraction or in the particulate or soluble fractions.

Although there are still many serious gaps in our knowledge we can summarize a number of the essential features of the comparative anatomy of the surface structures of gram-positive and gram-negative bacteria. Some of the chemical and biochemical properties are presented in figure 10.

VII. The Gram Stain and the Bacterial Surface

With our present increased knowledge of the nature of the bacterial surface, is it possible to conclude anything more definitive about the mechanism of the Gram stain reaction? I believe we are much closer to an understanding of this stain procedure which divides the bacterial world into two broad groups separable not merely on their response to the Gram stain, but also on the basis of biochemical and chemical properties (10). It should of course be pointed out that it is now almost impossible to propose any *new* theory to explain the Gram stain reaction, as most of the possibilities have been covered at some time or other during the long history of this staining procedure. Moreover, every major class of the macromolecular components of the bacterial cell has been implicated in the mechanism of the

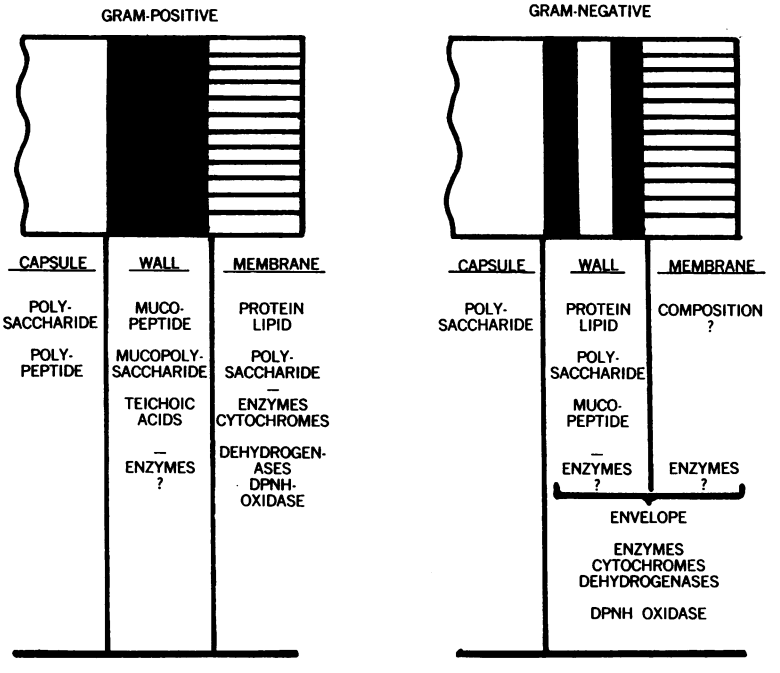

Figure 10. Summary of the chemical and biochemical anatomy of the surface structure of gram-positive and gram-negative bacteria.

TABLE 8

Theories and cellular substances involved in the Gram stain reaction

Nucleoproteins Nucleic acids	Deussen (21) Dubos and MacLeod (24) Henry, Stacey, and Teece (44, 45)
Lipids Special lipids Lipo-protein	Eisenberg (30) Schumacher (97) Stearn and Stearn (102)
Carbohydrate and nucleic acid	Webb (117)
Glycerophosphate complex Polyglycerophosphate	Schumacher (97) Mitchell and Moyle (71)
Permeability	Burke and Barnes (15) Kaplan and Kaplan (56) Wensinck and Boevé (126) Bartholomew, Cromwell, and Finkelstein (11)

TABLE 9

Cell-wall composition and Gram stain reaction

Organism	Gram Reaction	Major Chemical Components of Cell Walls
Saccharomyces cerevisiae	+	Polysaccharide, protein, lipid
Candida spp.	+	Polysaccharide, protein
Staphylococcus aureus	+	Mucopeptide, teichoic acids
Bacillus subtilis	+	Mucopeptide, teichoic acids
Streptococcus faecalis	+	Mucopeptide, mucopolysaccharide, teichoic acids
Micrococcus lysodeikticus	+	Mucopeptide
Escherichia coli	−	Protein, polysaccharide, lipid, mucopeptide
Salmonella gallinarum	−	As for *E. coli*
Proteus vulgaris	−	As for *E. coli*
Spirillum serpens	−	As for *E. coli*

stain procedure. Table 8 gives a brief, selected summary of the principal theories and substances alleged to be involved (10, 11, 15, 21, 24, 30, 44, 45, 56, 71, 73, 97, 101, 102, 117).

So often in the studies of the mechanism of the Gram stain, attempts have been made to isolate specific substances that may be responsible for the retention of the crystal violet (CV)-iodine (I) complex. This approach led Henry, Stacey, and Teece (45) to "restore" the Gram stain with extracted Mg-ribonucleate and to conclude that the RNA of gram-positive bacteria, coupled to basic proteins, was responsible for the stain reaction. Mitchell and Moyle (71) could find no correlation between the Gram stain and nucleic acid contents but they believed the Gram reaction was related to the presence of polyol phosphates in the bacterial envelope (71, 73). Here again a convincing correlation has broken down, as we now know that various strongly gram-positive bacteria are devoid of the teichoic acids in the wall. That many substances likely to occur in bacterial cells can stain as gram-positive material has been shown by Shugar and Baranowska (101). Lipids, polysaccharides, RNA, and certain proteins can all retain the CV-I complex to a greater or lesser extent (101). It therefore seemed unlikely that the Gram stain could be correlated with the presence of any one specific substance in the cells of those bacteria showing a positive reaction.

To what extent the chemical constituents of the bacterial wall are correlated with the Gram stain can be judged from the data presented in table 9. The main features which emerge from comparative studies of the chemistry of microbial walls are the presence of mucopeptide, mucopolysaccharide, and polysaccharide complexes in the walls of all gram-positive bacteria and the relatively high (up to 20 per cent) lipid contents in the walls of gram-negative organisms. It therefore seemed conceivable to the writer that on the one hand the high lipid content of the walls of gram-negative bacteria might be a factor contributing to their negativity, and that on the other hand the dehydration of the wall mucocomplexes during the decolorizing step of the Gram stain might reduce the "pore size" in the wall and render the CV-I complex relatively inaccessible to the solvent. Both of these possibilities would be amenable to experimentation and any results should give an indication of the

importance of "permeability" factors in the Gram stain.

The extractability of lipids from the walls with 95 per cent ethanol, the concentration employed in the differentiation step in Hucker's modification of the stain (10), was tested. From 40 to 50 per cent of the lipid content of isolated walls of *Escherichia coli* or *Proteus vulgaris* could be extracted under these conditions. The direct removal of wall lipid from these organisms could thus contribute to the extractability of the CV-I complex.

If there is a differential response of the wall of the two groups to the passage of substances across it in ethanol, then some further evidence could be gained from "permeability" (not in a physiological sense of course) or leakage studies. With organisms grown on media containing P^{32}, it has now been possible to show a differential passage of intracellular metabolites containing P^{32} across the walls or "envelopes" (cell wall-membrane) when cells were placed in graded concentrations of ethanol. A comparison of typical results obtained with two gram-positive and two gram-negative bacteria is afforded by the results shown in figures 11 and 12. This differential effect of ethanol concentration on the leakage of intracellular substances from gram-positive and gram-negative bacteria is very similar to the extractability of the CV-I complex reported in the excellent studies of Wensinck and Boevé (126). The leakage studies thus suggest that the pore size of the wall is sufficiently reduced during dehydration in high concentrations of ethanol to trap a large fraction of the intracellular constituents within the cells of gram-positive bacteria. A series of gram-positive and gram-negative bacteria have been studied in this manner and the ethanol-induced release of P^{32} (expressed as a percentage of the maximal release for each organism) is summarized in table 10.

Exposure of gram-positive bacteria labeled with P^{32} to iodine solutions (as used in the Gram stain) prior to suspension in ethanol reduced the leakage even further in the ethanol concentrations between 80 to 100 per cent (v/v). Such a pretreatment was without effect on the gram-negative bacteria. Leakage experiments were then performed on washed cells using the Gram stain conditions for bacterial suspensions described by Wensinck and Boevé (126), *i.e.*, suspension in crystal violet solution, washing with water, and treatment with iodine. When the cells were taken up in graded ethanol concentrations and the P^{32} release was determined, the differential pattern of extractability for the gram-positive *Staphylococcus aureus* and the gram-negative *Escherichia coli* (as shown in figures 11 and 12) persisted. These results are in accord with "permeability" differences being responsible for the

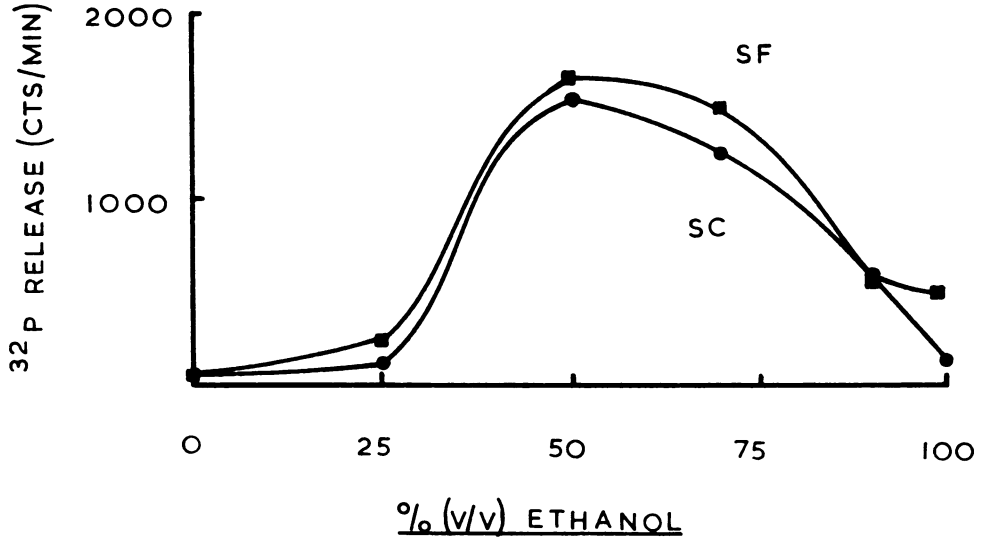

Figure 11. Effect of ethanol concentration on the release of P^{32} compounds from *Streptococcus faecalis* (*SF*) and *Saccharomyces cerevisiae* (*SC*) cell suspensions at 20 C.

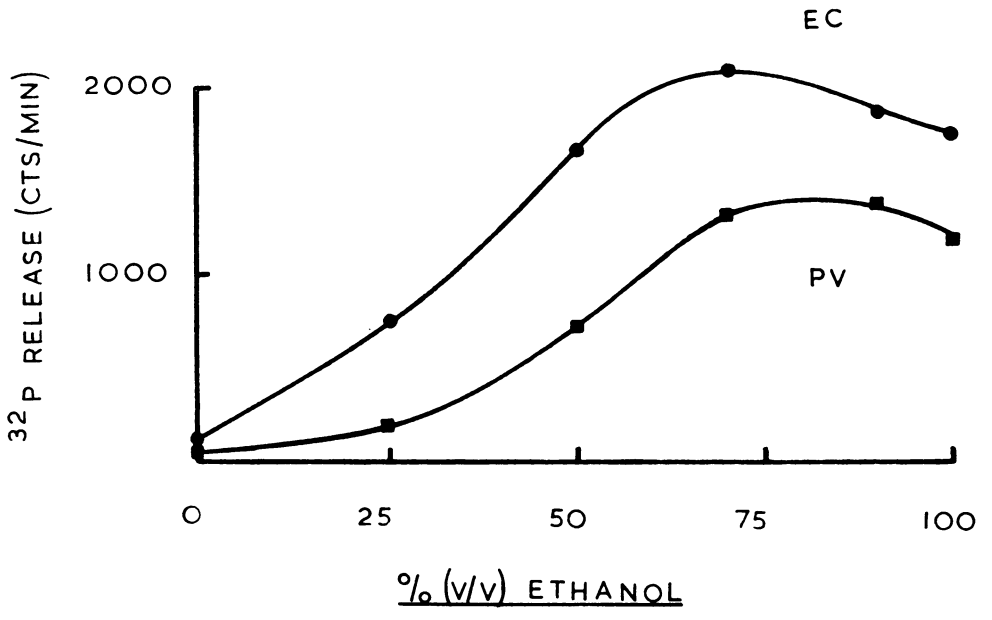

Figure 12. Effect of ethanol concentration on the release of P^{32} compounds from *Escherichia coli* (*EC*) and *Proteus vulgaris* (*PV*) cell suspensions at 20 C.

Gram stain. The evidence suggests not only that the CV-I complex is largely inextractable but also that even small molecular weight metabolites containing P^{32} are also trapped within the cell when the wall is dehydrated with 95 per cent ethanol.

It has of course been known for some time that cells treated with lysozyme become gram-negative (Webb (117)) and in more recent years isolated protoplasts have also been found to give a gram-negative reaction as shown by Gerhardt, Vennes, and Britt (33). These workers have also shown that crushed protoplasts from *Bacillus megaterium* Gram-stained prior to wall removal with lysozyme, as well as crushed whole cells, could be decolorized, thus reaffirming that structural integrity of the organism is a prerequisite for Gram positivity. Finally, the most convincing evidence that it is the wall of the gram-positive organism which is the barrier to removal of the CV-I complex has been obtained with suspensions of several lysozyme-sensitive bacteria Gram-stained by the procedure described by Wensinck and Boevé (126). When stained cell suspensions of gram-positive organisms are incubated with lysozyme, the cell walls are digested and the residual protoplast retains the CV-I complex, which is then completely accessible and is extracted quantitatively by a single treatment with 95 per cent ethanol. These results thus support the view that Gram positivity is due to the reduced accessibility of the CV-I complex to the solvent, resulting probably from a reduction in the pore size by dehydration of wall mucocomplexes by 95 per cent ethanol and possibly also from the presence of the large iodine atom, which may of course become associated with parts of the molecules as it does in other polysaccharides (41). However, as iodine is an inhibi-

TABLE 10

Release of P^{32} from bacteria in 100 per cent ethanol

Gram Reaction	Organism	% Released Relative to Maximum
−	*Pseudomonas* sp.	96
−	*Proteus vulgaris*	90
−	*Escherichia coli*	84
−	*Salmonella gallinarum*	75
−	*Neisseria catarrhalis*	65
+	*Micrococcus lysodeikticus*	35
+	*Streptococcus faecalis*	33
+	*Staphylococcus aureus*	26
+	*Bacillus megaterium*	22
+	*Saccharomyces cerevisiae*	10

tor of lysozyme (108), it seems that the backbone in the wall is sufficiently free of CV-I complex or I to permit the enzyme to degrade the wall. These results also indicate that the CV-I complex is "attached" to the bacterial protoplast, either at the surface or distributed throughout the protoplasm.

In the short space of the past decade a great deal has been learned about the anatomy of the bacterial surface and, fortunately for the microbiologist, the chemist and the biochemist have been attracted by some of the unusual substances present in bacterial cells. The incursion of the chemist into the microbial world has prompted at least one group of microbiologists to remark:

"Bugs: Slurp macromolecular goo
And chemists will make a pet of you."[2]

It is likely that the "macromolecular goo" will continue to attract many into the fascinating world of the microorganism and will add a great stimulus to our understanding of the structure and functioning of the organized microbial cell.

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