THE BACTERIAL CELL WALL

by MILTON R. J. SALTON

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PREFACE

The first attempts to obtain some direct information about the chemical composition of bacterial cell walls or envelopes were made in several laboratories during 1950. At that time it seemed inconceivable that the stage would be reached when it would be difficult to condense the new knowledge about cell walls into a monograph of the size of the present volume. Indeed, so much information with direct and indirect bearings on the problems of the nature and structure of microbial surfaces has now accumulated that the writing of a completely exhaustive record of the field would be a lengthy task. Some degree of selectivity has therefore been inevitable and this book represents an attempt to summarize the results of certain facets of the investigations into bacterial cell walls from the development of suitable methods of isolation to the current interest in the biosynthesis of these complex and fascinating structures. The treatment of each specialized part of this volume has been largely 'historical' and accordingly I have not hesitated in including some of the earlier electron micrographs and illustrative material which will convey the sequence of events in the development of this field and which has formed the basis for more recent investigations. In the opening chapter, an effort has been made to put the 'bacterial cell wall' into anatomical perspective by discussing its relationship to the cell surface structure as a whole.

Although the book is primarily concerned with walls of bacteria, some mention has been made of the closely related blue-green-algae. The walls of yeasts, fungi and other microbial groups have not been specifically dealt with in this volume for, apart from chemical studies on fungal walls carried out in the laboratories of Dr. W. J. Nickerson, little additional material on fine structure and biosynthesis has appeared since the summary presented in the published account of the 1960 CIBA Lectures on 'Microbial Cell Walls'. It is hoped that this will stimulate rather than deter new researches into the nature of the surface structures of the more neglected groups of microorganisms.

With the rapid growth of scientific literature even in this small specialized field, it is inevitable that this book will be 'out of date' in some sections by the time it is published. The two major problems in the cell wall field, the structure and biosynthesis of wall 'mucopeptides' are attracting increasing attention and rapid progress is bound to be made in solving some of the obvious gaps in our knowledge. The inclusion of new important material presents a constant headache to publisher and author alike. Indeed, since the completion of the original manuscript much new material has appeared. Fortunately it has been possible to insert brief references to several important aspects of wall structure and biosynthesis and I would especially like to thank Drs. J. T. PARK, R. W. JEANLOZ, N. SHARON, J. M. GHUYSEN, J. L. STROMINGER

and P. MEADOW for kindly giving me permission to refer to their results prior to their publication.

The preparation of a monograph is rarely a 'one man show' and I would like to express my thanks to many friends and colleagues who have helped in a direct way, for their valuable discussions, interest and contributions to the development of this field over the years. I should also like to thank especially, Miss Gay LYNCH for the excellence of the typing of the manuscript and tables and for her valuable assistance throughout the preparation, checking and proof reading of the book.

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CHAPTER 1

The anatomy of the bacterial surface

INTRODUCTION

It is now just a little over twenty years ago that the application of electron microscopy to the problems of cell structure heralded two very fruitful decades of research culminating in our present knowledge of the fine structure and anatomy of cells derived from a wide variety of organisms. Because of the small dimensions of bacteria, electron microscopic studies have assumed special importance in resolving their detailed anatomy. Some of the early electron micrographs of bacterial cells were indeed little better than the photomicrographs a good cytologist could produce with staining techniques and the light microscope, but now even the most ardent bacterial cytologist would have to concede that many of the fine structures of microorganisms could not have been detected without the combined use of shadow casting, thin sectioning and negative staining with electron microscopy. Thus in two decades we have emerged from the rather vague world of the bacterial cytologist and entered the exciting world of the bacterial anatomist where cell structures and functions can be more accurately resolved and described at the macromolecular level.

The growth of our detailed knowledge of the anatomy of microorganisms has of course been but one facet of the general advance in cell biology. With the perfection of the thin sectioning technique it has become possible to compare the principal anatomical features of cells derived from a wide variety of animal, plant and microbial species. The information gained from such comparative investigations of cellular structure together with our biochemical and chemical knowledge of cells presents us with a clear picture of some of the essential similarities and differences between cells from the major classes of organisms.

All types of cells capable of undergoing division and growth possess certain common structures and organelles. These include ribonucleic acid (RNA) – protein particles (ribosomes) of about the same dimensions, plasma membranes of the so-called 'unit membrane' type with an overall thickness of approximately 75 Å (ROBERTSON, 1959; SJÖSTRAND, 1960) and a nucleus or chromatinic body. Bacteria and blue-green algae differ from animal and plant cells in several respects, viz.: the structure of the nuclear body, the absence of organized mitochondria possessing a limiting membrane and enclosed cristae and the absence of an endoplasmic reticulum. The chromatinic bodies or nuclear structures of bacteria and blue-green algae are not surrounded by nuclear membranes (KELLENBERGER, 1960; HOPWOOD AND GLAUERT, 1960; RIS and SINGH, 1961; MURRAY, 1962) a feature which distinguishes them from those of higher plant and animals cells, yeasts and fungi. A well-defined mitochondrial structure similar to that found in animal and plant cells as well as in fungi and yeasts has not been detected in bacteria

The tables are printed together at the end of the book.

and blue-green algae although organelles with equivalent biochemical functions are undoubtedly present. The 'mesosome' structures of bacteria (FITZ-JAMES, 1960; SALTON AND CHAPMAN, 1962) also referred to as the 'intracytoplasmic membranous elements' by GLAUERT (1962) and a 'remarkable organelle' (VAN ITERSON, 1961) appear to possess the enzyme systems normally found in mitochondria isolated from other types of cells. It was formerly believed that a single plasma or protoplast membrane system in bacteria was the mitochondrial equivalent but recent investigations have shown that so-called plasma membrane preparations contain the mesosome membranes as well (SALTON AND CHAPMAN, 1962). As FITZ-JAMES (1960) has pointed out the mesosome is produced by the invaginated growth of the plasma membrane and it may well be that both membrane elements form a continuous and homogeneous system. The closest resemblance to the membrane-mesosome system of bacteria so far reported in other microbial groups is the multimembrane system recently observed by LINNANE, VITOLS AND NOWLAND (1962) in anaerobically grown cells of the yeast *Torulopsis utilis*. In aerobically grown *Torulopsis utilis* the mitochondria were structurally and enzymically normal. It was suggested that the membrane system of the anaerobic cells was concerned with the morphogenesis of the mitochondria. At the chemical level diphosphatidyglycerol has been found in the membrane-mesosome fractions of *Micrococcus lysodeikticus* as well as in mammalian mitochondria (MACFARLANE, 1961; MARINETTI, ERBLAND AND STOTZ, 1958) and it will be of great interest to see if this type of lipid is a characteristic component of membranes and membranous organelles.

A comparison of the anatomy of the photosynthetic apparatus (chloroplast) in plants with the equivalent organelle (chromatophores) in photosynthetic bacteria and blue-green algae bears a similar structural relationship to that seen for mitochondria. The complex chloroplast structure of plants is replaced in bacteria and blue-green algae by a membrane system of lamellae or a chromatophore network, both of which are probably derived from the invagination of the plasma membrane (GIESBRECHT AND DREWS, 1962). Thus in bacteria and blue-green algae the biochemical functions of the highly organized mitochondria and chloroplasts are found in the simple 'unit membranes' with a lower degree of structural complexity.

At the chemical level, bacteria differ from animal, plant and other types of microbial cells in that they have not so far been shown to contain sterols. One possible exception to this general rule seemed to be found in the *Mycoplasma* spp. which require an exogenous supply of cholesterol for growth. However, recent investigations (RODWELL, personal communication) indicate that cholesterol is taken up by the cells but is not chemically modified in any way. The presence of a sterol in *Chlorobium limicola* has been suggested by AARONSON AND BAKER (1961) but this report awaits further confirmatory evidence. The other major chemical difference between most bacteria and blue-green algae, and cells derived from other groups of organisms is the possession of the cell-wall mucopeptides (glycosaminopeptides, glycopeptides) the characteristic and most conspicuous components of which are the substances muramic acid, α, ε-diaminopimelic acid (DAP) and D-isomers of certain amino acids.

Thus structurally and chemically bacterial cells possess much in common with other types

of cells. There are, however, several outstanding differences which place the majority of bacterial species apart from other microorganisms and cells of plant and animal origin. One of the very interesting chemical and biochemical differences is the inability of bacteria to produce sterols. So far there have been no reports of sterols in blue-green algae (FOGG, 1953), a fact which again emphasizes their general similarities to the bacteria. Many of the other structural and chemical differences arise from the characteristics of the surface components of the bacterial cell, in particular the mucopeptide nature of the walls. The remaining portion of this chapter will therefore be devoted to a detailed discussion of various aspects of the anatomy of the bacterial surface.

Some of the principal structural, chemical and biochemical properties of bacteria and blue-green algae are contrasted with those of cells of animals, plant and other microbial groups in Table 1. It is of considerable interest that bacteria, once regarded as the most primitive forms of life, are almost as structurally complex and are generally as biochemically sophisticated as 'higher' cells.

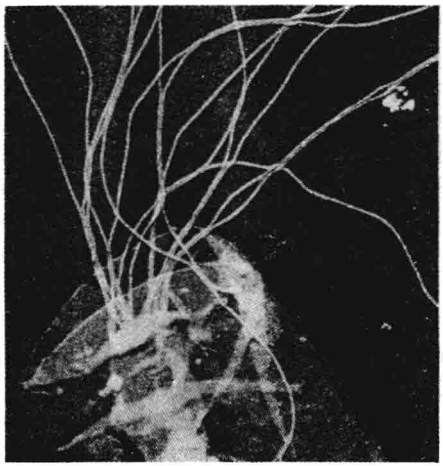
CELL SURFACE STRUCTURE

Electron microscopy has not only added a tremendous amount of detail to our knowledge of the fine structure of the bacterial cell as a whole, but it has also contributed to a more precise definition of the anatomy of the cell surface structures. Although the early cytologists had established the presence of flagella and the principal surface layers of capsules, walls and membranes (KNAYSI, 1951), without the electron microscope the isolation and chemical characterization of many of these cellular components would not have been possible. The detection of fine structure in bacterial walls, capsules and flagella and the discovery of fimbriae (DUGUID, SMITH, DEMPSTER AND EDMUNDS, 1955) was of course entirely dependent on the resolution of the electron microscope.

The anatomy of the bacterial surface has been the subject of detailed discussions in 'The Bacteria' (GUNSALUS AND STANIER, 1960) and by WILKINSON (1958); WILKINSON AND DUGUID (1960); DUGUID AND WILKINSON (1961); SALTON (1961). The surface components of the bacterial cell can be separated anatomically into two groups: (1) the surface appendages, (2) the surface layers.

SURFACE APPENDAGES

In general the surface appendages can be readily distinguished from the surface layers. Thus flagella and fimbriae of various bacteria and the filamentous appendages of *Gallionella ferruginea* (VAN ITERSON, 1958) are seen as quite separate entities as illustrated in Fig. 1. Of these three types of surface appendage more is known about the nature and structure of bacterial flagella following the classical work of WEIBULL (1948). He isolated homogeneous preparations of these structures and laid the foundations of our present knowledge of the chemistry



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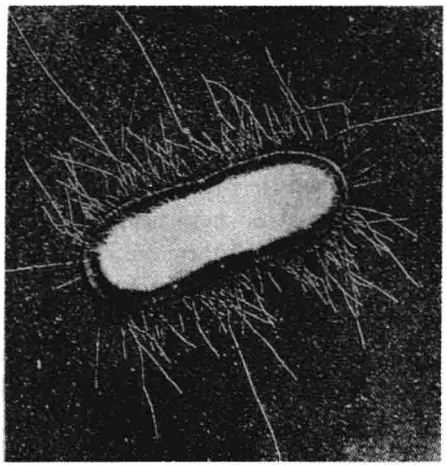

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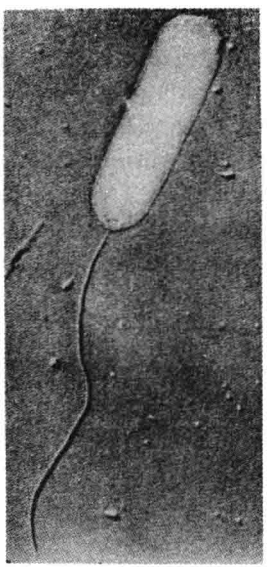

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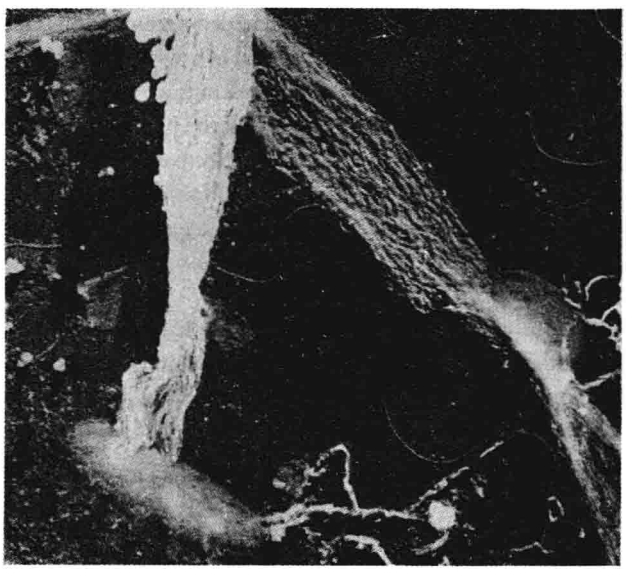

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FIGURE 1. *Surface appendages of bacterial cells.*
(a) Flagella tuft attached to envelope of autolysed *Spirillum serpens.* × 30,000.
(b) Flagellum of *Pseudomonas aeruginosa.* × 12,500.
(c) Fimbriae surrounding the cell surface of *Shigella flexneri.*
(d) Ferruginous strands attached to cells of *Gallionella ferruginea.* × 8,000.

of flagella (STOCKER, 1956; WEIBULL, 1960; KERRIDGE, 1961). Bacterial fimbriae have also been isolated but at present so far as the author is aware they have not been clearly defined in chemical terms (BRINTON, 1959). The filamentous appendages of *Gallionella ferruginea* are known to contain some organic material as well as iron oxide (VAN ITERSON, 1958).

The decision as to whether the stalks of the *Caulobacter* spp. should be regarded as 'surface appendages' or more properly as specialized extensions of the surface layers and cell contents must await further investigations.

SURFACE LAYERS

The surface layers of the bacterial cell can be visualized as a series of simple or complex concentric shells differentiated into the following principal regions:

1. ionic layer
2. capsules, microcapsules
3. adsorbed slimes and gums
4. cementing layer in cell aggregates
5. cell wall or outer envelope component
6. cell membranes, plasma membranes.

The cytologically demonstrable surface components will be discussed in some detail but passing mention must be made of the ionic layer of the bacterial cell.

The outermost 'layer' of the bacterial surface is an ionic one with the various charged substances of the surface components contributing to the net charge. Although studies of the electrophoretic mobilities of bacteria before and after treatment with various drugs, antibiotics and antibacterial agents and enzymes have yielded interesting information (MCQUILLEN, 1951a, b; DOUGLAS, 1957; GEBICKI AND JAMES, 1962) they have only been of limited value in elucidating the precise nature of surface components and the fate of these components during the various treatments. Investigations of the surface charge of bacteria have thus been largely of diagnostic value and of use in substantiating more direct chemical studies on the outer layers. One interesting example of the latter use of electrophoresis was the demonstration of the differences in mobilities of whole cells of *Bacillus megaterium* and the isolated protoplasts derived by treatment with lysozyme (DOUGLAS AND PARKER, 1958). Such a difference in surface charge found by DOUGLAS AND PARKER (1958) was compatible with the known differences in chemical composition of walls and membranes of *Bacillus megaterium* (WEIBULL AND BERGSTRÖM, 1958).

Capsules and microcapsules

Capsules form the outermost layer of certain bacterial species. It has long been recognized that a capsule is not an essential structural element of the bacterial cell and its production is subject to both phenotypic and genetic variation. Thus environmental conditions may markedly affect the ability of a given bacterial species to produce a capsule detectable by the usual cytological methods (DUGUID AND WILKINSON, 1961). Under suitable conditions of nitrogen and phosphorus deficiency the capsule of *Aerobacter aerogenes* (*Klebsiella aerogenes*) possessed a diameter of up to 4.3μ (DUGUID AND WILKINSON, 1953, 1954). The retention of a capsule may

also be dependent on other factors such as the absence of capsule-degrading enzymes. In certain streptococcal groups (especially Groups A and C) capsules of hyaluronic acid are detectable in the early exponential phase, but as the organisms continue to grow logarithmically, hyaluronidase is produced and the capsules are no longer detectable (BAZELEY, 1940; KASS AND SEASTONE, 1944).

In addition to the role of environmental factors on capsule production, DUGUID AND WILKINSON (1953, 1954) have shown that mutant strains derived from the fully encapsulated strain of *Klebsiella aerogenes* (A3) may possess slime layers of identical polysaccharide, readily removable from the cell surface by washing with water; other mutants may be completely devoid of capsule or slime. The latter strains would be equivalent to the 'rough' variants of the Gram-positive pneumococci.

AVERY AND DUBOS (1931) were the first to demonstrate the selective removal of a capsular layer from bacterial cells. Removal of the capsular polysaccharide from the pneumococcal cells was achieved without impairing the viability of the cells and thus established the anatomical and functional differentiation of the capsule and wall of these organisms (AVERY AND DUBOS, 1931). Similar studies have been extended to other bacterial species and enzymic 'decapsulation' without loss of cell viability has been achieved with *Klebsiella pneumoniae* (ADAMS AND PARK, 1956), *Bacillus anthracis* and *Bacillus megaterium* (TORII, 1955). Although the surface M and T proteins of group A streptococci can also be removed by digestion with trypsin without loss of viability (LANCEFIELD, 1943) these components are not present as recognizable capsular or slime layers.

The selective removal with enzymes of the capsular, slime and other layers external to the rigid cell wall therefore offers an extremely valuable method for investigating the anatomical relationships of the surface layers of the bacterial cell. The prior enzymic removal of capsular structures when chemical investigations are to be performed on the cell wall proper has obvious advantages in determining the nature of the bacterial wall. It can be concluded then that bacterial capsules and slime layers are morphological entities physically distinguishable from the underlying cell envelope structures of many bacteria. By growing bacteria under conditions which prevent capsule formation and by enzymic removal of the fully formed capsular and slime layers it has been shown that the cells retain their morphological integrity. These observations have established the dispensability of capsules, slimes and sheaths and indicate that the walls or envelopes are responsible for cell shape and are more intimately involved in the viability of the cell.

Based largely on stained preparations, it has been widely believed that capsules are homogeneous accumulations of amorphous, viscous gel-like materials around the bacterial cell-wall surface. The possibility that they may be physically and chemically heterogeneous was first suggested when TOMCSIK (1951) and TOMCSIK AND GUEX-HOLZER (1951) applied immunological reactions to *Bacillus anthracis* and other members of the genus Bacillus and examined the antibody-treated cells under the phase-contrast microscope. Cells exposed to antibody against isolated capsular γ-glutamyl polypeptide and antibody to capsular polysaccharide

showed a complex disposition of the latter within the glutamyl capsular polypeptide (TOMCSIK, 1951, 1956). LABAW AND MOSLEY (1954) examined encapsulated cells of the Lisbonne strain of *Escherichia coli* in the electron microscope and detected striated fibrillar structures embedded in an amorphous capsular matrix. Discontinuities in the capsular surface of *Bacillus megaterium* were reported by IVANOVICS AND HORVATH (1953). These variations in physical structure of bacterial capsules are illustrated diagrammatically in Fig. 2 (taken from SALTON, 1960).

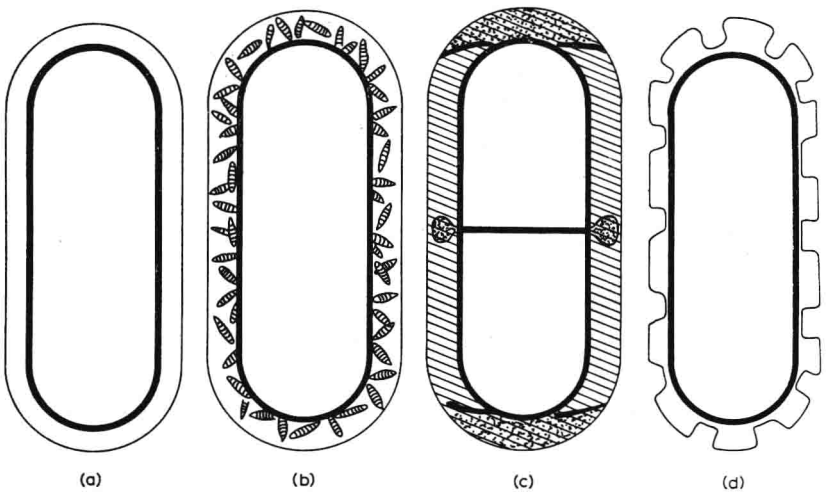

FIGURE 2. A diagrammatic representation of the types of capsular structures found in bacteria.
(a) Capsule forming a continuous layer around the cell.
(b) Capsular layer with banded fibrils as in *Escherichia coli* Lisbonne.
(c) Complex capsule with localized patches of polysaccharide and polypeptide as in *Bacillus megaterium* M.
(d) Discontinuities in the capsular surface as in *Bacillus megaterium*. (SALTON, 1960a).

So far the discussion has referred largely to capsules and slime layers as defined and demonstrated cytologically by DUGUID (1951). Bacteria producing loose slime and extracellular gums and polysaccharides may have such materials adsorbed on the cell wall or cell envelope surfaces. Many of these substances have been easily removed from the cells by repeated washing, Some components may be strongly adsorbed and it was of interest to note that certain halophilic organisms possessed a layer of deoxyribonucleic acid (DNA) which could not be washed from the surface (SMITHIES AND GIBBONS, 1955). This strongly adsorbed DNA was later shown to be of intracellular origin, its presence upon the surface being a consequence of the instability of the cell walls when the organism was grown on media containing less than 0.7 *M* sodium chloride (TAKAHASHI AND GIBBONS, 1957). Both the loosely adherent surface polysaccharides and the more strongly bound DNA of intracellular origin have a different anatomical status to the components described as 'microcapsular' materials (WILKINSON, 1958).

To overcome some of the difficulties of differentiating and defining certain surface components of the bacterial cell WILKINSON (1958) has collectively defined the smooth antigenic substances of Gram-negative bacteria and other related materials as 'microcapsules'. The term microcapsule has thus been taken to indicate the presence of surface components (usually detectable by immunological reactions) which are often difficult to differentiate from other layers of the bacterial envelope. In addition to the smooth O somatic antigens of Gram-negative bacteria, the M-proteins of haemolytic Streptococci have also been placed within this general grouping. It should be kept in mind however, that the latter never assume the dimensions of capsules and it is even difficult to detect any difference in the appearance of isolated streptococcal walls after removal of the M-protein substances (SALTON, 1953; SLADE, 1957).

In discussing the surface layers of the bacterial cell SALTON (1960a) also used the term 'microcapsule' to describe the O antigens of Gram-negative bacteria. Although there is little doubt that the O antigens are anatomically on the surface of the bacterial cell it is now extremely doubtful that the term 'microcapsule' has any valid meaning for these components. The immunologically specific polysaccharides are part of lipid-polysaccharide-protein complexes localized in the cell envelope fraction of Gram-negative bacteria. It is only after treatment with warm 45% phenol solution that the lipopolysaccharides are released from the complexes in the bacterial surface (WESTPHAL, LUDERITZ AND BISTER, 1952). Moreover the investigations of WEIDEL and his colleagues have clearly established the differentiation of the isolated wall or envelope of *Escherichia coli* into a phenol-insoluble rigid layer (mucopeptide, glycosaminopeptide) and the phenol-soluble layers of the lipopolysaccharide-protein complexes (WEIDEL, FRANK AND MARTIN, 1960). These features, together with the present knowledge that there appears to be no enzyme systems available for the selective release of the O antigens from the surface of Gram-negative bacteria (contrast with the capsules of both Gram-positive and Gram-negative bacteria discussed above) lead to the conclusion that the smooth O antigens are part of the multilayered wall or envelope structure. The author therefore feels that the term 'microcapsule' confuses rather than clarifies the anatomical status of the lipo-polysaccharide-protein complexes of Gram-negative bacteria and that these components should not be described as 'microcapsular'. However, in describing the Vi antigens and components such as the M-proteins of Streptococci as 'microcapsules', it may serve to distinguish them from other well-defined capsules or slime.

Although the M proteins and Vi antigens have not been detected in electron micrographs, evidence for a microcapsular layer in *Nocardia calcarea* has been presented by GLAUERT (1962). In her excellent thin sections of *Nocardia calcarea*, GLAUERT (1962) has resolved an extremely uniform layer of 50 Å thickness surrounding the cell wall. The microcapsular layer is much more electron transparent than the underlying wall.

At the chemical level there is usually little confusion between the capsular substances and the constituents of the cell wall. The chemical composition of bacterial capsules, especially the polysaccharides has been discussed in monographs and review articles from time to time and the reader is referred to HEIDELBERGER, 1956; WILKINSON, 1958; SALTON, 1960; STACEY AND