Microbial Cell Walls and Membranes

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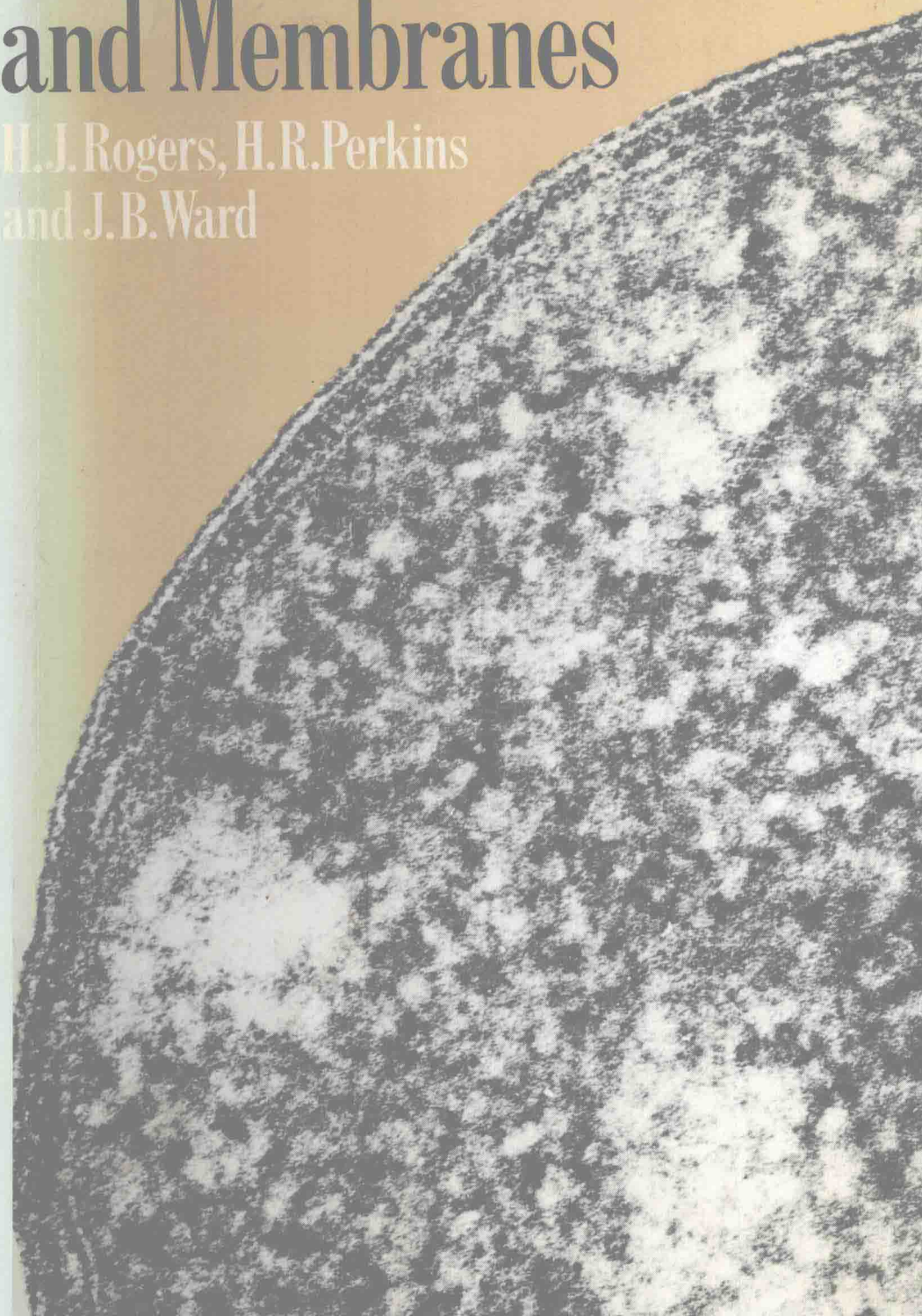

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Preface

In 1968 when *Cell Walls and Membranes* was published it was still reasonable to attempt to write a book covering the whole subject. Accordingly this edition of the book had something to say about walls from micro-organisms and plants as well as about membranes from bacteria and animal cells. A decade later this is manifestly impossible. Knowledge about almost all the subjects has grown explosively, particularly about membranes and the biosynthesis of macromolecules. Moreover aspects of the subject that were still in a relatively primitive state ten years ago have grown into highly sophisticated subjects worthy of extended treatment. The result is that the present book has had to be confined to structures and functions relating to only one division of the biological kingdom, namely micro-organisms. Even then severe limitations have had to be made to keep the task within the time available to the authors and their expertise. A few of the titles of chapters such as those on the isolation of walls and membranes, the structure of the components of bacterial and micro-fungal walls and their biosynthesis remain from the earlier book. These chapters have been almost completely rewritten and a number of quite new chapters added on topics such as the action of the antibiotics that inhibit bacterial wall synthesis, on the function of bacterial membranes, and the bacterial autolysins. The vast majority of the work described in the book has been published in the last ten to fifteen years; and much of it in the last two or three years. An attempt has been made to summarize subjects in which there is by now some degree of consensus about the general structures or mechanisms involved. Other important areas, which some might have liked to have seen included such as, for example, the biosynthesis and export of proteins, particularly into the outer membrane of Gram-negative species of bacteria, or the inter-relations between surface growth, cell division and DNA replication in bacteria have been deliberately omitted. It was felt that these subjects are still at the growing points of the science and that it was impossible to make statements about them that would not be extremely evanescent.

Clearly the levels to which the various chapters in the book have been written are not the same. Some are much more detailed and deal very much more with the frontiers of knowledge than others. This is partly a reflection of the nature of the subjects, and partly of the nature of the authors and their professional expertise in the subjects. In general, the readers the authors have had in mind are final year undergraduate students in microbiology and postgraduate students working on the appropriate subjects. Some chapters, however, may be of interest to research workers,

particularly those whose interests border the topics dealt with in the book.

The authors would like to thank all who have been kind enough to supply pictures for the text of the book and in particular, Dr. I. D. J. Burdett of the National Institute for Medical Research who was also kind enough to read and comment on the various drafts of the chapter on ultrastructure. They would also like particularly to thank Mrs. H. B. Sharp whose dedicated labours in disentangling the written manuscripts and the errors in referencing of two of the authors (H. J. R. and J. B. W.) and turning them into beautifully typed pages has added much to any value the book may have. We should also like to thank Miss J. White and Mrs. J. L. Marsh for doing the same services for the remaining author (H. R. P.).

Contents

1

Ultrastructure of bacterial envelopes

1.1 Introduction

The majority of the text of this book will be concerned with the chemistry, biochemistry and physiology of the envelopes and intracytoplasmic membranes of bacteria. As a background to this discussion, something needs to be said about the appearance and arrangement of these structures in the cell, even if this serves no other purpose than to allow people some idea of the complexity of present day aims in trying to understand the more complicated functions of bacteria, such as their ability to divide. This chapter should, however, be regarded only as a topological guide to what follows, not as a thorough review of the ultrastructure of bacteria.

The last two decades have seen steady progress in the resolution of the layers that surround bacteria and of the inclusions they contain. That bacteria had 'walls' was recognized long before the advent of the electron microscope. Even, however, with the design and application of special wall stains, the level of resolution under the light microscope was too low to allow reliable dimensions, much less infrastructures to be seen. The isolation of wall preparations [39, 45, 91, 111, 116, 150] and the ensuing chemical studies did little more from the ultrastructural point of view than to confirm the work with the light microscope. The preparations were monitored by metal shadowing under low-powered electron microscopes. Collapsed structures of the same shape as the original bacteria were seen, but these techniques yielded no information about internal wall structure and little about external structures. The preparation and examination of 'ultra-thin' sections of bacteria was in its infancy at this time [6, 30] and staining with molybdate and tungstate, the so-called negative stains, had not been applied. The development and application of methods for the preparation, embedding, fixing and staining of material, along with the increased resolving power of electron microscopes has revolutionized our understanding of the ultrastructure of biological material, including the layers of the envelopes of bacteria.

1.2 The Gram-positive cell wall

The structural and molecular differences between the outer layers of bacteria that retain the purple iodine–gentian violet complex, despite extraction with polar solvents

such as alcohol and acetone, compared with those that do not do so, are usually quite clear and very few anomalies among true bacteria have been brought to light (eg. *Butyrivibrio fibrisolvens* [32, 158]), with the exception of the *Halobacteria* and the methanogenic bacteria which have envelopes fitting neither category. The mechanism of Gram's [76] empirical method of staining is still only partially understood [148]. The general, rather featureless appearance of the walls of Gram-positive bacteria was apparent at a relatively early stage of electron microscopic studies, whereas the complex nature of the Gram-negative envelopes demanded all the skill of biological workers in designing better fixation and staining conditions, and of physicists in designing electron microscopes giving greater resolution to solve their structure. Even as late as 1975, a considerable advance was still possible in our understanding of the envelope of the experimentally ubiquitous *Escherichia coli.*

The walls of most Gram-positive bacteria appear thick and relatively structureless in section (Fig. 1.1), irrespective of the methods for fixation and staining. Their thickness is capable of great variation, but during steady state growth or within the exponential phase in ordinary batch cultures these are rather slight. Wall synthesis is not coupled to protein or nucleic acid synthesis other than through the formation of the wall biosynthetic enzymes themselves and the supply of a very limited number of amino acids and nucleotides (see Section 8.2), so that variation in wall thickness, outside steady-state or nearly steady-state growth conditions is in no way surprising [73]. If protein synthesis by bacteria is deliberately stopped, either by the exhaustion of an essential amino acid or by the addition of antibiotics, such as chloramphenicol, wall synthesis is not necessarily affected and great thickening can occur [161]. For example, walls of *Staphylococcus aureus* have been increased in thickness from 30 nm to 100 nm by incubation in the presence of chloramphenicol [67]. Likewise, the thickness of walls of bacilli doubles when a tryptophan auxotroph of *Bacillus subtilis* is incubated in the absence of tryptophan for an hour at 37° C [98]. The precise measurement of wall thickness is not easy, however, [82, 120] since it demands that sections should be exactly median whether cut longitudinally or radially in rod-shaped organisms. Often the inner and outer edges of the wall are difficult to define with precision. The problem of measuring wall thickness precisely has been carefully studied [82, 83] using *Streptococcus faecalis* and *B. subtilis* [14]. In seven separate exponential phase cultures of *S. faecalis*, the thickness measured in sections of whole cells was found to vary between 26.7 and 28.3 nm with an average of 27.2 nm and with an error of about 10–15%. These authors' criterion, for a precisely antitangential section, was that the wall should appear sharply tribanded after glutaraldehyde–osmium tetroxide fixation and staining according to Ryter and Kellenberger [145] (see Fig. 1.2). The thickness of walls in exponentially growing cultures of *B. subtilis* was studied in cross-fractured cells in freeze-fracture preparations. The value obtained was 27.62 ± 2.75 nm. The thickness, however, of fragments of isolated walls varied considerably according to the staining procedure used. For example, treatment with uranyl acetate following glutaraldehyde fixation altered the mean value to 25.13 nm. The excellent use to which the biosynthetic process of wall thickening has been put in studying cell

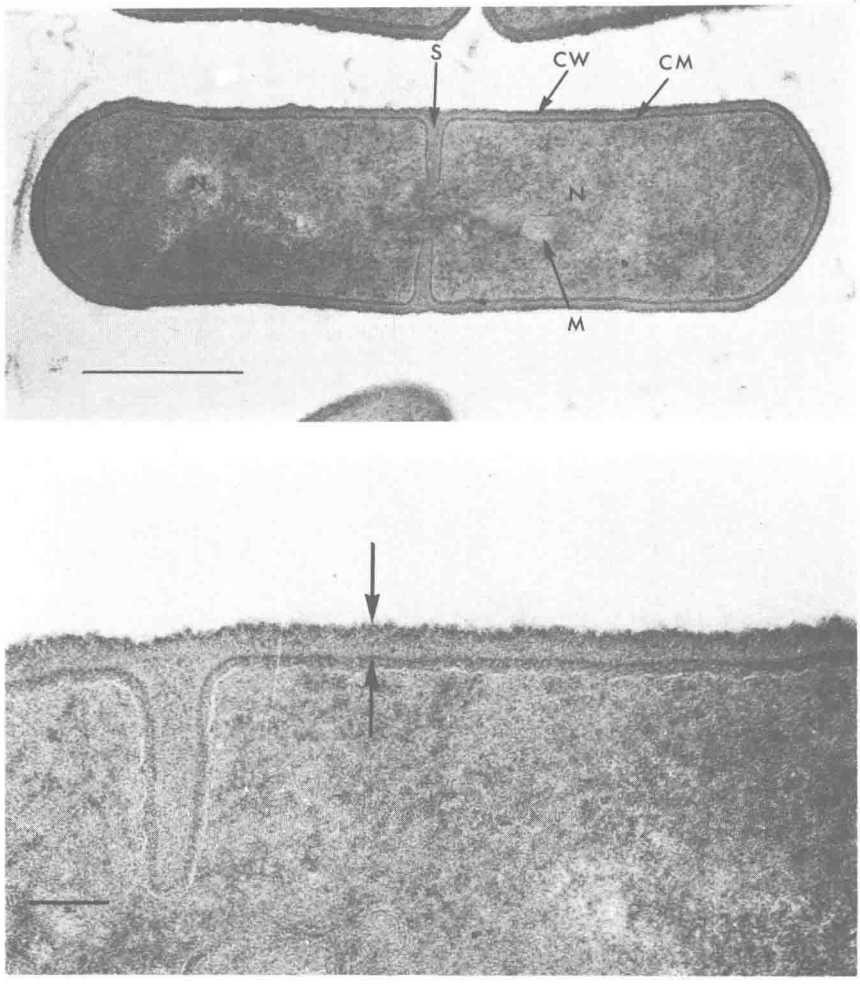

Figure 1.1 (a) A longitudinal section of *Bacillus subtilis* fixed and stained with glutaraldehyde and osmium, showing the absence of structure within the walls apart from their tribanded appearance. N, nuclear bodies; CM, cytoplasmic membrane; CW, cell wall; S, septum; M, mesosomes. The bar is equivalent to 0.5 μm. (We are indebted to Dr. I. D. J. Burdett of the National Institute for Medical Research for this photograph.) (b) A higher magnification where the bar represents 0.1 μm.

division of bacteria will be described in Chapter 15. Too few reliable measurements have otherwise been made of wall thickness in micro-organisms growing exponentially, or in a steady state, to comment on the results quoted. It would appear that many rapidly growing Gram-positive bacteria have walls that are usually of the order of 30 nm in thickness but values as high as 50–80 nm have been quoted [70, 149] and

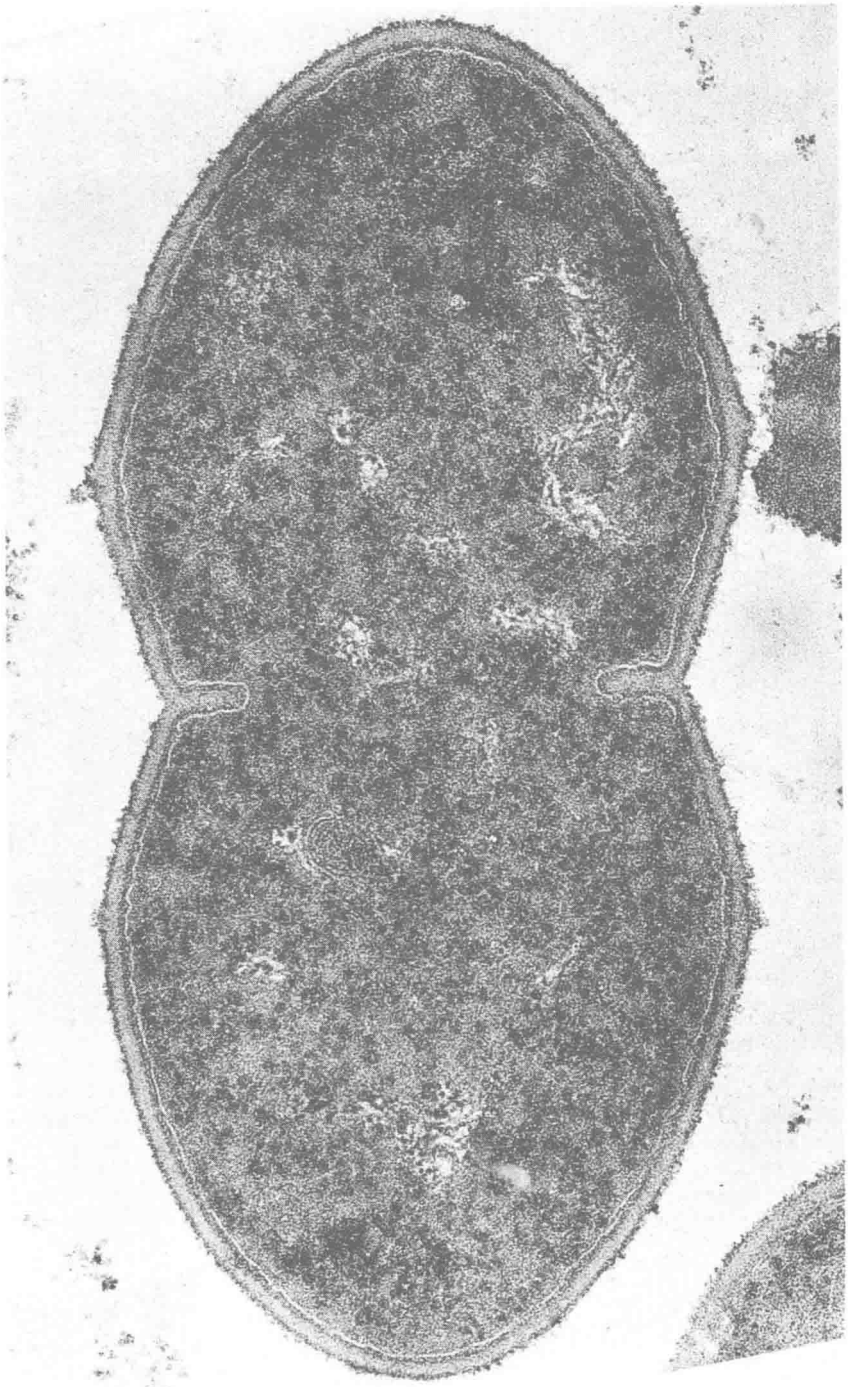

Figure 1.2 An exactly longitudinal section of *Streptococcus faecalis* showing the tribanded appearance of the wall. Magnification is 96 720. (We are indebted to Prof. M. Higgins of the Department of Microbiology, Temple University, Philadelphia, U.S.A. for this photograph.)

as low as 15.17 nm for *B. fibrisolvens* [32]. This latter organism although having a Gram-positive type wall stains Gram-negatively.

Claims that are made from time to time, for the presence of meaningful detailed structure in the depth of walls of Gram-positive species from the examination of very highly magnified sections of stained preparations or of negatively stained material, may be regarded with suspicion. The former would often appear to be due to chance distribution of stain in sections and the latter to the vagaries of the negative staining method itself. Repeated observations from many laboratories have nevertheless shown banding of walls from Gram-positive species in sections subjected to various fixing and staining procedures, and in freeze-fractured material. The sections usually have dark bands on the outer and inner limits of the wall and the effects of using a variety of fixation and post-staining methods on this phenomenon in the walls of *B. megaterium* and *B. subtilis* have been examined [14, 125]. In the former organism a triple-layered appearance was obtained when either isolated walls or whole organisms had been stained with osmium, permanganate or lead citrate. When uranyl acetate, phosphomolybdate or phosphotungstate had been applied the 'stains' were deposited evenly throughout the wall and no banding was seen. Unfortunately our knowledge of the physical and chemical reasons for the deposition of stain in biological material is not sufficiently detailed to allow deductions from this difference. It may nevertheless represent some real difference in properties between the innermost and outermost layers of the wall compared with that in its depth. When wall thickening had occurred during inhibition of protein synthesis in *B. subtilis,* the dark innermost layer seen after prefixation with glutaraldehyde, post-fixation with OsO_4 and staining with uranyl acetate, moved into the interior resulting in a striped appearance of the walls (Fig. 1.3) [98]. This suggests a chemical or physical distinction between the original innermost wall layer and the central region. In general, the low atomic number of the elements of greatest abundance in nature has precluded serious attempts to use unstained material for electron microscope examination. However, by adjusting the photographic recording process to give pictures of maximum contrast, Weibull [200] succeeded in obtaining rewarding pictures of the envelopes of bacteria that had been treated only with 4% glutaraldehyde at pH 7.0. A clearly triple or double-banded wall overlying the cytoplasmic membrane was seen in sections of *B. subtilis.* Results such as this suggested that the outer layer of the wall may differ in composition as far as the electron scattering power of its atoms is concerned and the results obtained with stained preparations reflect this difference. Since the atom with the highest atomic number in the wall is phosphorus, Weibull's [200] picture appeared to provide support for the suggestion that teichoic acid (see Section 7.1.1) is concentrated in the outer layers of the wall [125]. However, Millward and Reaveley [117] concluded that the trilamellar appearance of unstained walls of *B. licheniformis* and *S. aureus* was more likely to be due to variable packing of wall material than to the distribution of teichoic acid. They thought that the latter was, in fact, diffusely scattered through the thickness of the wall. This result is also supported by some aspects of a more complex study of the contrast patterns across bacterial walls produced by a variety of techniques [60]. Staining

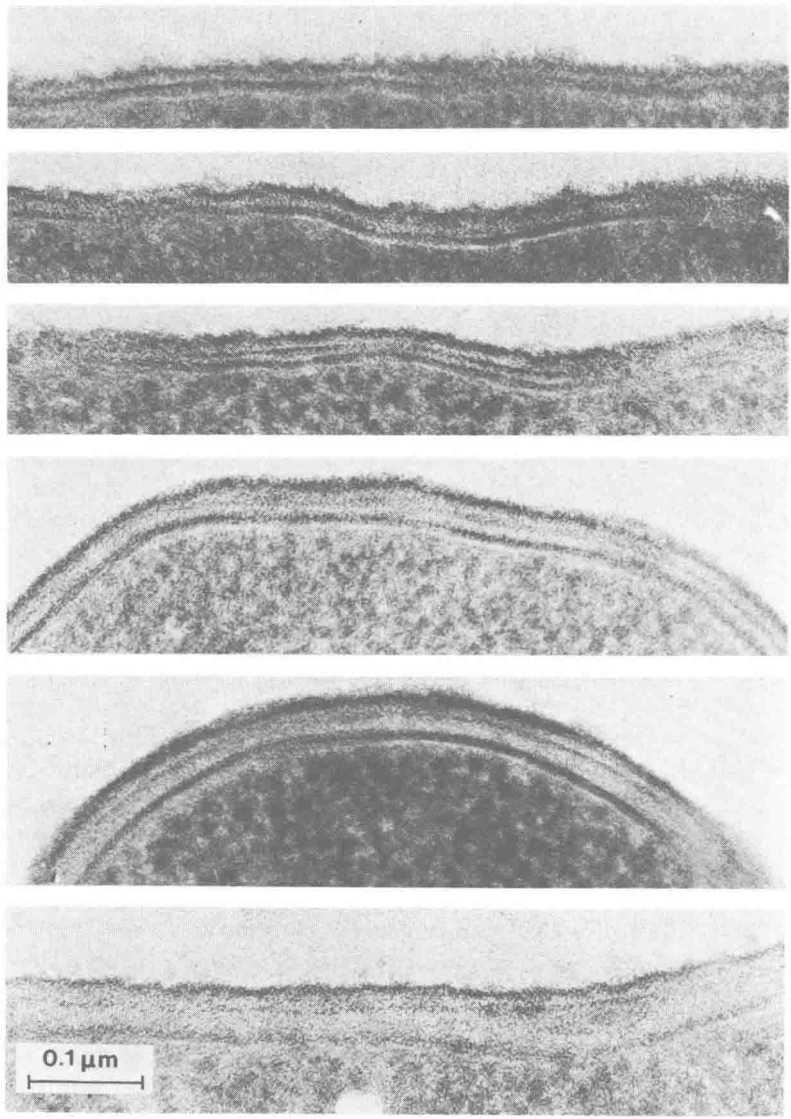

Figure 1.3 Progressive wall thickening in *B. subtilis trp* during incubation in the absence of tryptophan but presence of wall amino acids and glucose. The movement of the inner darkly staining band of the original wall into the interior, during the thickening process, can be seen (from [98]).

of osmium-fixed cells and walls of *S. aureus* with various combinations of lead citrate, uranyl acetate and ruthenium red were compared with pictures of sections of walls obtained after treatment with gold-coupled concanavalin A [59] and by a concanavalin A–peroxidase method. The concanavalin A studies were interpreted to show that teichoic acid was distributed throughout the thickness of the wall. This study illustrates some of the difficulties of reaching unambiguous conclusions about the distribution of teichoic acid in walls.

Evidence from the effects of extracting the accessory polymers from walls by reagents such as acids or formamide upon the tribanded appearance of stained preparations of walls from the same species of bacteria, has been conflicting. For example, Swanson and Gotschlich [177] found that the inner electron-dense layer of streptococcal walls was lost after treatment with HNO_2 and concluded that this was due to the loss of teichoic acid. Wagner *et al.* [192], on the other hand, extracted cells of Group A and Group C streptococci with eight different reagents that were found to remove proportions of the rhamnose-containing polysaccharides varying from 20–95%. The trilaminar appearance of the walls of the organisms was not altered after treatment with 10% trichloroacetic acid for 24 h at 4° C, despite a reduction in their thickness by 85%. Likewise, when wall preparations from *S. faecalis* and *Lactobacillus arabinosus* were treated with TCA, a very high proportion of the accessory polymers was removed and the walls were thinner but the trilaminar appearance persisted even though it was weaker [4, 186]. Thus, the peptidoglycan component of streptococcal walls, at least, can itself express the trilaminar appearance in sections after the application of standard Ryter–Kellenberger treatment. Wagner *et al.* [192] also showed that ferritin-labelled antibody produced against peptidoglycan would react with both sides of the wall. Antibodies raised against peptidoglycan will also react specifically with homologous organisms in a number of different species. Thus, even if any layering of the walls is present, peptidoglycan is accessible to antibody molecules, at least on the outer surface, and in streptococci apparently, on the inner surface as well. On the other hand a conditional mutant of *B. subtilis* with grossly reduced amounts of teichoic acid in its walls, did not have trilamellar walls when examined in section, whereas when grown under the appropriate conditions it regained both its wall teichoic acid and its wall staining pattern [38]. A different type of explanation of staining patterns has been suggested by considering the chemistry of the various stains used for electron microscopy [14]. The outer 'stained' bands might, according to this explanation, result from the mutual electrostatic charge properties of the wall and the stain. Some stains may have difficulty in entering the wall, and thus combine with the surfaces preferentially.

Although it is still not possible to be dogmatic about the relationship between the tribanded appearance of walls from Gram-positive bacteria and the distribution of different polymers within them, a simple layering seems highly unlikely. Nevertheless study of freeze-fracture material [186] again emphasizes that the stain distribution may correspond to some real differences possibly in the physical properties of the different parts of the wall. In whole cells, the walls appeared amorphous but in wall preparations first extracted with sodium dodecyl sulphate to