Electron Microscopy of Proteins

Volume 3

edited by

JAMES R. HARRIS

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Preface

This book follows the pattern established by the two previous volumes in this series, in that it contains chapters dealing with diverse subject areas in which electron microscopy has made a significant contribution over the past ten to twenty years. Each chapter is written by an internationally recognized worker or group of workers in the particular field, who are based in laboratories in Europe and the U.S.A. I have been extremely impressed with the quality of the manuscripts submitted by the authors, which certainly equal, and possibly even exceed, the high standard of those incorporated into the previous volumes.

Apart from the application of the more widely used specimen preparation techniques for conventional transmission electron microscopy, i.e. thin sectioning, freeze-fracture and negative staining, the more recent use of image analysis and scanning transmission electron microscopy receive considerable attention. Most chapters include some emphasis on biochemical aspects of the topic under discussion, which serves to re-emphasize the main theme of the series as a whole, namely the application of electron microscopy alongside other approaches used for the study of biological macromolecules.

The first chapter, on algal cell walls, is from Keith Roberts and his colleagues, at the John Innes Institute in Norwich, who have made such a significant contribution to this field over the past few years. Their chapter is an excellent example of the progression through the more usual specimen preparation techniques to their recent studies on image processing. This is followed by the chapter on bacterial cell walls and membranes, by two eminent electron microscopists, Uwe B. Sleytr of the Centre for Ultrastructure Research, University of Agriculture, Vienna and Audrey M. Glauert of the Strangeways Research Laboratory, Cambridge. The regular arrays of proteins and glycoproteins within a range of different bacterial cell walls is given detailed coverage and the recent important studies on the purple membrane of *Halobacterium halobium* performed by Unwin and Henderson is given due emphasis. When the series was initially planned, it was proposed

that there would be a single chapter dealing with arthropodan, molluscan and annelidan respiratory proteins. However, on the advice of Professor E. F. J. van Bruggen it was decided to separate the phylum annelida as a separate chapter, which appears here. (The chapter on arthropodan and molluscan respiratory proteins appears in Volume 1 of the series, by van Bruggen and his colleagues). Serge N. Vinogradov and his colleagues of the Department of Biochemistry, Wayne State University, Detroit, have collated a comprehensive account on annelidan haemoglobins and chlorocruorins, which deals in detail with the physical properties of these molecules as well as their study by electron microscopy. The recent application of scanning transmission electron microscopy to biological molecules is exemplified by its use to study annelidan haemoglobins. James R. Paulson, of the MRC Laboratory of Molecular Biology in Cambridge (now of the University of Michigan), has produced a thorough survey of eukaryotic chromatin and chromosomal proteins, which emphasizes the importance of the nucleosome concept and deals in detail with the chromatin fibre and the structure of the metaphase chromosome. The chapter on amyloid proteins is contributed by Alan S. Cohen and his colleagues of Boston University School of Medicine, who have been leading workers in this field for many years. A discussion of the biochemical and immunological aspects of the amyloid proteins is followed by a detailed consideration of the ultrastructure of amyloid fibrils and the P-component. The final chapter, on tubulin and associated proteins, is by Linda A. Amos of the MRC Laboratory of Molecular Biology in Cambridge. Dr Amos has been one of the foremost investigators of tubulin over the past few years, and presents a thorough account of the subject which emphasizes both the biochemistry and electron microscopy.

I would like to thank all the contributors for presenting their manuscripts so promptly and for the enthusiasm they have shown on being involved in the series. I would also like to thank the staff of Academic Press for the efficient and courteous manner in which they have dealt with the production of this volume, for their continued support for the series and their interest in its future development. The continued support for and interest shown in the series by Dr W. J. Jenkins, former Director and Dr J. F. Harrison, Director, North East Thames Regional Transfusion Centre, has been of lasting value. Finally, and perhaps of greatest importance, I wish to acknowledge the unending encouragement and help given by my wife, Carol, without whose backing the series might never have started.

January 1982

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1. The Structure of Algal Cell Walls

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I. INTRODUCTION

There are two broad types of extracellular matrix in algae. The first is that in which microfibrils, often of cellulose or chitin, are arranged within an apparently amorphous matrix of other polysaccharide molecules. Most algae have such conventional cell walls and our knowledge of them derives largely from a chemical approach. There is also a second kind of wall, which usually contains considerably more protein, in which the molecules are arranged in a far more regular manner. Two groups of algae illustrate this second type: the Haptophyceae which possess a cell covering of ordered scales, and the Volvocales, whose cells are covered with a thin crystalline glycoprotein cell wall. The electron microscope has played a very large part in determining the nature of these cell coverings and in this chapter we will concentrate on the methods used, and the results obtained, for both these groups of algae. Our aim in this case is to derive a description of the shape and precise three-dimensional location of all the molecules in the cell wall to a resolution limited only by the electron optical and specimen preparation methods used. This aim has not yet been achieved for any one cell wall, but there is every

reason to think that, using the methods and approaches to be outlined, we will soon be able to provide such a description, which we believe is an essential preliminary to understanding cell wall function in detail. It is clear that electron microscopy alone cannot provide all the answers, and much of the information we need to build up our picture of the cell walls is derived from other sources. Electron microscopy only becomes meaningful when it is used in conjunction with biochemical, cytochemical and genetic data, and this chapter will, we hope, illustrate this. We will also concentrate on the fact that the electron microscope is more powerful at elucidating structures in which there is a high degree of order than those in which more random or amorphous components occur. Both kinds of cell wall, which we will discuss, display ordered arrays of molecules and this means that new methods of specimen preparation, the minimization of radiation damage, and digital image processing become relatively more important; an importance reflected in the emphasis of this chapter. Of the work to be described, that on the Haptophyceae has been done by other workers, while that on the Volvocales has largely been carried out by ourselves.

The reasons for concentrating a relatively large amount of our effort into determining the structure of the cell walls of a relatively minor group of green algae, the Volvocales, are threefold. First, they form a model system for studying the processes of self assembly. It is possible, *in vitro*, to disassemble and reassemble the crystalline lattice of glycoproteins which cover the cell wall of *Chlamydomonas reinhardii* making possible a study of the parameters affecting their self assembly. Secondly, they form convenient model systems for investigating the structure, synthesis and function of the similar hydroxyproline-rich glycoproteins present in the cell walls of higher plants, from which they are hard to isolate intact. Thirdly, they afford an ideal model system (thin biological crystals with good long-range order) for developing methods of computer image analysis, methods which will be usefully applied to more common biological structures such as membranes.

As the emphasis of these volumes is inevitably methodological, we have followed this approach in the organization of this chapter. Each section discusses a particular method or approach in electron microscopy, its advantages, its limitations and the contribution that it has made to our knowledge of the structure of algal cell walls.

II. THIN SECTIONING

A considerable amount of information about the structure of algal cell walls is, in theory, obtainable by conventional thin section electron microscopy. The questions that will concern us here are what sort of information we can obtain, to what resolution is the information reliable and how should we

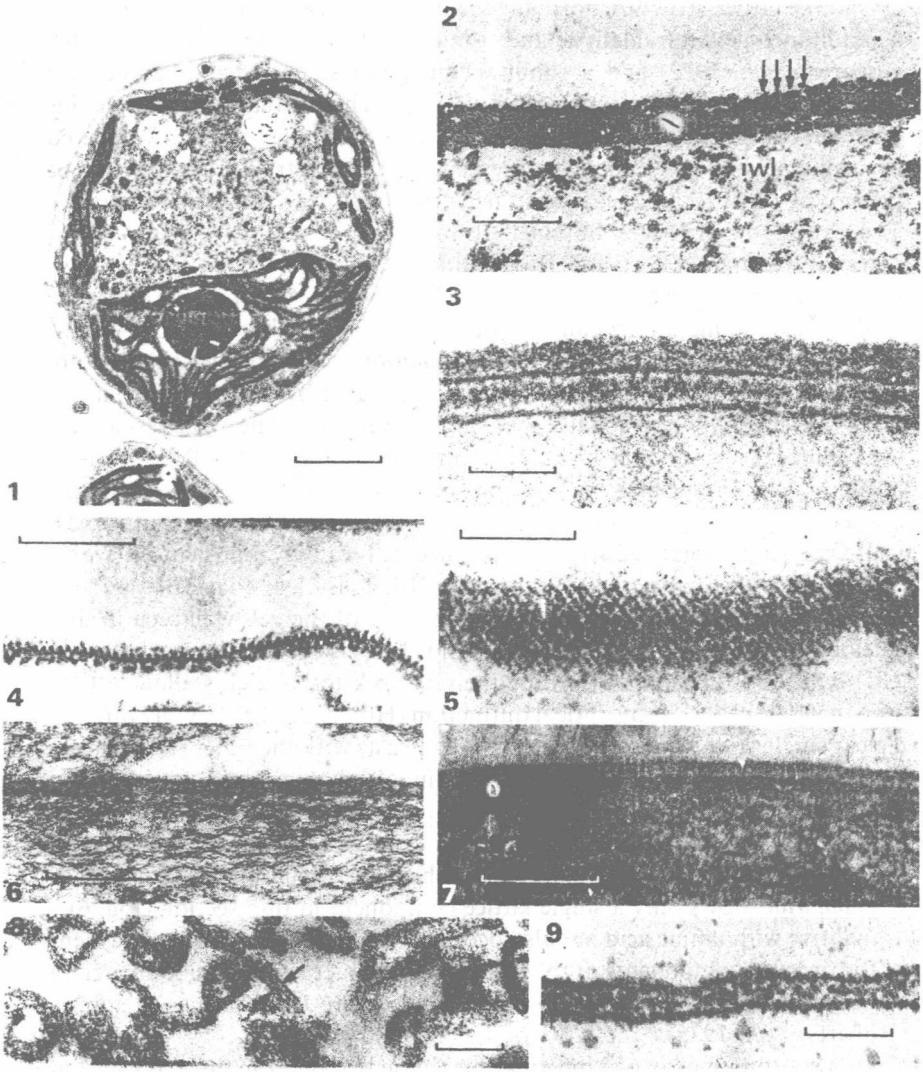
interpret it in relation to observations with other techniques? These questions can perhaps best be illustrated by considering results for the cell walls of the Volvocales.

These walls are thin laminar structures and thin sectioning can provide an estimate of the thickness and relative positions of the various wall layers. Different fixatives tried with *Chlamydomonas reinhardii* resulted in very different images of the cell wall, so we monitored fixation by examining the fixed cell walls using a negative stain. Only two fixatives did not disrupt the crystalline lattice structure normally seen in negatively-stained unfixed walls: a simultaneous glutaraldehyde and osmic-acid fixative (Franke *et al.*, 1969; Roberts *et al.*, 1972) and a simultaneous glutaraldehyde and tannic acid fixative with post-osmication (Catt *et al.*, 1978). This means that at least in this one case conventional glutaraldehyde fixation (in whatever buffer) followed by osmic acid (Fig. 1) does not preserve a delicate structure known to be present by other techniques such as negative staining and shadowed replicas. The thin section images obtained using the two successful fixatives are markedly different (Figs 2 and 3). The tannic acid fixative alone clearly shows in thin section the periodic components which reveal in which layers of the cell wall the crystalline lattice component resides (Fig. 2). Furthermore, it also reveals that the wall in the case of *C. reinhardii* is a sandwich-like structure with two crystalline layers back-to-back, separated by an associated amorphous layer. Only occasionally is a single layer seen. The thickness of the crystalline layers is about 7 nm and the thickness of the total wall "sandwich" is 40 nm. This tannic acid fixative enabled us to monitor the process of wall self-assembly *in vitro* with *Chlamydomonas reinhardii*. Figures 8 and 9 show wall fragments assembled from the purified cell wall glycoprotein 2BII with and without the associated glycoprotein 2BI. It is clear that 2BII alone is capable of producing the repeating component of the cell wall seen in thin section (Catt *et al.*, 1978). This information has enabled us to conclude that 2BII by itself is capable of forming the two back-to-back crystalline lattices seen in whole cell walls by optical diffraction (Hills *et al.*, 1973). Only rarely is a clear single crystalline layer seen. This contrasts with the other related algae examined which all appear by negative staining and optical diffraction to have a cell wall containing only a single crystalline layer, a fact confirmed by tannic acid fixed, thin sectioned material. Some examples are shown in Figs 4–7. Interestingly, *Chlamydomonas angulosa*, which has a cell wall lattice related to *C. reinhardii* shows only a single lattice component in thin section (Fig. 6).

Fixative with tannic acid has also been useful in clarifying the arrangement of the ordered components of the flagellar collar, a crystalline tube encircling the base of the flagellum, which varies in structure from species to species (Roberts *et al.*, 1975).

Thin sectioning provided information on the two other main layers seen in the wall (Fig. 3): first the inner wall layer, which is amorphous and therefore is

not clearly seen in negatively-stained images can be seen both in reassembled cell walls, and in walls in which the crystalline components have been removed (Hills *et al.*, 1975). This stable cross-linked component is responsible for giving the whole cell wall its shape, and thin sectioning has shown that it is the first component to be laid down, adjacent to the plasma membrane, around the naked daughter cells after cell division, or around cells that have had their walls removed (Roberts and Hills, 1976; Robinson and Schlosser, 1978). The



components of the crystalline lattice must then diffuse through this meshwork and assemble themselves on its outer surface.

Secondly, the outer amorphous layer seen in Fig. 3 is not seen in tannic acid fixed material (Fig. 2), nor in cell walls that have been reassembled *in vitro* (Figs 8, 9), nor in cells that have been grown on defined inorganic medium. The conclusion we have reached is that this material is not a genuine cell wall component but results from the adsorption of macromolecules from the normal rich growth medium to the negatively-charged cell wall surface (Catt *et al.*, 1976).

Sectioning studies have therefore told us two main things. One is that the cell wall construction of the Volvocalean algae follows a common plan, consisting of a stable amorphous inner wall layer of variable thickness, on the outer face of which is assembled a crystalline wall layer of constant thickness. The other is that great care must be taken to monitor fixation with other techniques if possible, and that we should not rely too much on the fidelity of images obtained using only one fixative.

One of the strengths of thin sectioning is in revealing the relationship between various cell components perhaps better than any other technique. A good example here is *Pleurochrysis*, a haptophycean alga that secretes ordered scales which form the main component of the extracellular matrix or cell wall. By thin sectioning (Fig. 10) it can be clearly demonstrated that the scales have their origin in the Golgi apparatus and that each scale is progressively

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Fig. 1. *Chlamydomonas reinhardii* cell fixed in conventional glutaraldehyde fixative and postfixed in osmic acid. Scale bar=2 μm.

Fig. 2. Cell wall of *Chlamydomonas reinhardii*, fixed in tannic acid containing fixative (Catt *et al.*, 1978). The two other components of the triplet layer are seen and the outer one is clearly periodic (→). iwl=inner wall layer. Scale bar=100 nm.

Fig. 3. Cell wall of *Chlamydomonas reinhardii*, fixed in simultaneous glutaraldehyde and osmic acid (Roberts, 1974). The inner wall layer and central triplet layer are clearly seen together with the adsorbed material on the outer surface of the wall. Scale bar=100 nm.

Figs 4 and 5. Cell wall of *Chlorogonium elongatum* fixed in tannic acid containing fixative (Roberts and Hills, 1976). The periodic structure within the wall is clearly seen both in cross-section (Fig. 4) and glancing section (Fig. 5). Scale bar=200 nm.

Fig. 6. Cell wall of *Chlamydomonas angulosa* fixed in a tannic acid containing fixative showing the typical Volvocalean wall structure of a regular component overlying an amorphous inner wall layer. Scale bar=2 μm.

Fig. 7. Cell wall of *Lobomonas piriformis* fixed in a tannic acid containing fixative (Roberts *et al.*, 1981). Scale bar=2 μm.

Fig. 8. Thin section of pellet of reassembled fragments of purified glycoprotein 2BII from *Chlamydomonas reinhardii* (Catt *et al.*, 1978). Complete triplet layers are seen in short lengths (→). Scale bar=200 nm.

Fig. 9. A portion of a fragment reassembled from a mixture of glycoproteins 2BI and 2BII (Catt *et al.*, 1978). Large, clearly structured fragments are produced. Scale bar=100 nm.

elaborated within a vesicle which eventually exocytoses, depositing the scale in a defined orientation outside the cell (Brown and Romanovicz, 1976; Romanovicz and Brown, 1976; Brown *et al.*, 1970, 1973; Brown, 1969). Thin sectioning has also revealed details of the ontogeny of the cell wall in the Volvocalean algae. In *Chlorogonium* (Roberts and Hills, 1976), *Chlamydomonas* (Robinson and Schlosser, 1978) and *Pandorina* (Fulton, 1978a, b) it has been clearly demonstrated that the amorphous inner wall layer is the first of the cell wall components to be assembled during the growth of a new cell wall.

Thin sectioning has severe disadvantages for high resolution structure determination and with good reason has contributed relatively little to our detailed knowledge of the algal cell walls (its great advantages are in conjunction with cytochemical or autoradiographic techniques; see the next section). What are the limitations on the information that thin sectioning can

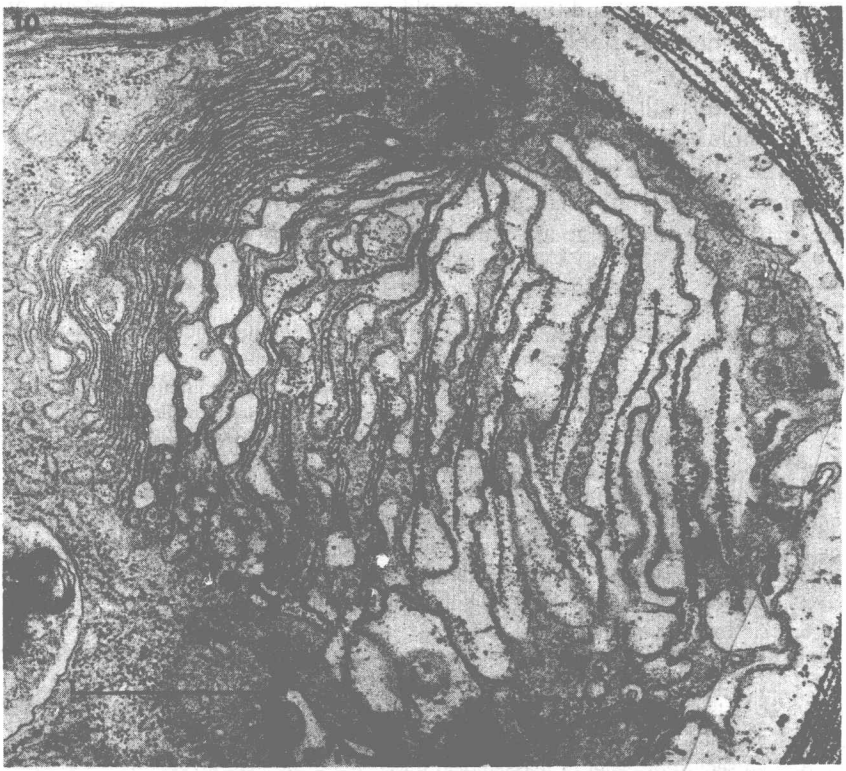

Fig. 10. Median section through the Golgi apparatus of *Pleurochryss scherfellii*. Each cisterna contains a cell wall scale in various stages of assembly. A completed scale prior to exocytosis is seen at the right. Localization of polysaccharide is by periodic acid–silver methanamine followed by lead citrate. Scale bar=1 μm. Micrograph supplied by Dr Dwight K. Romanovicz.

provide? A major problem is fixation; it is quite clear from the discussion above that there is no ideal fixative and fixatives which do not preserve delicate structures, or which allow them to be extracted later during processing, obviously create problems of image interpretation. Another problem is contrast. Not only does a thin section have at least 50 nm of weakly electron-scattering resin for the beam to penetrate but contrast then has to be provided by the successive depositions of heavy metal crystallites (omium, uranium and lead) onto the original specimen. This alone limits the effective resolution to 2–3 nm. This is a problem further compounded by the difficulty of relating small objects to each other within the depth of a thin section. Without tilting information, two 3-nm protein molecules separated by 50 nm might appear as such, or touching, depending on their orientation within the section.

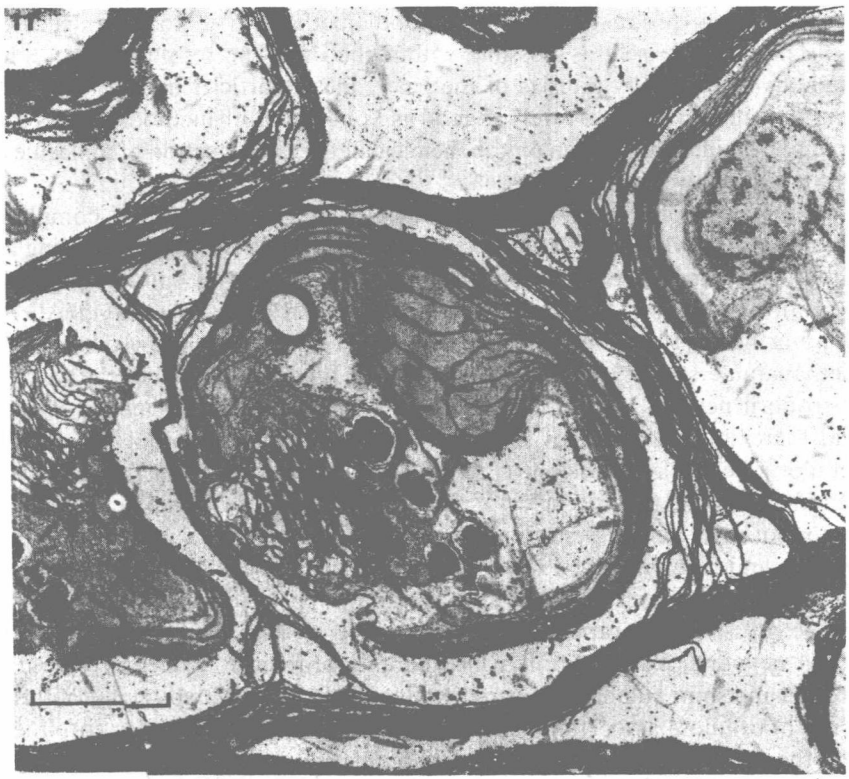

Fig. 11. A section of actively growing cells of *Pleurochrysis scherfellii*, cytochemically stained as in Fig. 10. The dense silver deposition on the wall is evident, as are the Golgi vesicles containing scales. Scale bar = 2 μm. Micrograph supplied by Dr Dwight K. Romanovicz. Both Figs 10 and 11 originally appeared in *Journal of Applied Polymer Science* (Brown and Romanovicz, 1976). Copyright John Wiley and Sons.

A clear indication of the resolution limit of sectioned material, in a favourable specimen, is seen in the study of Akey *et al.* (1980) of crystals of cytochrome oxidase from *Pseudomonas*. Here diffraction data from sections of tannic acid embedded crystals was sharply limited to 2 nm although electron diffraction data from unstained glucose embedded crystals extended out to 0·9 nm.

III. CYTOCHEMISTRY

Cytochemical electron microscopy may follow two different general approaches. One is to ask if specific chemical reagents or enzyme reactions remove structures seen by conventional EM methods and the other is to specifically react cell components in such a way that they form an electron dense reaction product. The idea of both methods is to localize specific molecules as precisely as possible within the cell. The resolution of such methods is limited by the size of the heavy metal particles used to provide contrast and in practice is rarely as good as 3 or 4 nm. Both methods have been used to provide information on the chemical nature and location of molecules within the cell wall of various algae.

In the haptophycean algae, the cell covering is composed of complex organic scales, which each contain an amorphous matrix material housing a radial array of microfibrils, on which is spun a web of spiral cellulosic microfibrils (see Fig. 35). Silver hexamine staining in *Chrysochromulina* (Allen and Northcote, 1975) and periodate oxidation of the scales followed by silver methanamine staining in *Pleurochrysis*, both preferentially stain the radial microfibril network (Fig. 12). These radial microfibrils are removed if the scales are treated with 5 M KOH, pronase, or dilute acid, but the spiral cellulosic network is resistant (Fig. 13). Specific cytochemical staining has demonstrated the presence of aryl sulphatase and alkaline phosphatase in the region of the Golgi apparatus where radial microfibril synthesis occurs (Brown and Romanovicz, 1976). These and other cytochemical observations have led to our present picture of the basic haptophycean scale. This includes the progressive elaboration within Golgi vesicles (Figs 10, 11) of a quadriradial network of microfibrils which contain arabinose, galactose, fucose, protein and some sulphated sugars. On this is laid down the spiral microfibril array of cellulose associated with a glycoprotein containing hydroxyproline and the whole is embedded in a water soluble coating substance containing galactose, glucose, mannose and protein.

In the Volvocalean algae the cytochemical approach to electron microscopy has been less used, and one example will suffice. Two lines of evidence had suggested that terminal mannose residues on the *Chlamydomonas* cell wall