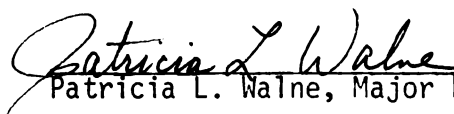
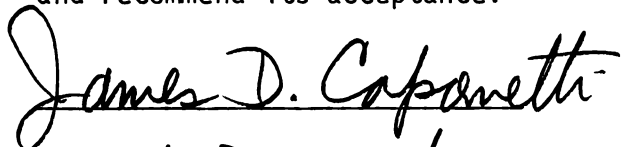


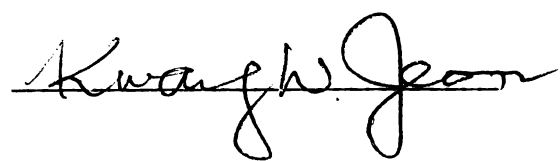
To the Graduate Council:

I am submitting herewith a dissertation written by Linda Ann Pocratsky entitled "Nutritional, Chemical and Ultrastructural Characterization of the Lorica and Extracellular Mucilage of Phacotus lenticularis (Phacotaceae, Volvocales)." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.


Patricia L. Walne, Major Professor

We have read this dissertation
and recommend its acceptance:





Accepted for the Council:


Vice Chancellor
Graduate Studies and Research

NUTRITIONAL, CHEMICAL AND ULTRASTRUCTURAL
CHARACTERIZATION OF THE LORICA AND
EXTRACELLULAR MUCILAGE OF
PHACOTUS LENTICULARIS
(PHACOTACEAE,
VOLVOCALES)

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Linda Ann Pocratsky

March 1982

3058062

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Patricia L. Walne, for her guidance during my research program and for many valuable editorial comments during the preparation of this dissertation. I also wish to thank my other committee members, Dr. James D. Caponetti, Dr. James N. Dumont, Dr. Kwang W. Jeon and Dr. Elisabeth Gantt, for their careful reading of my dissertation and many helpful comments.

I am grateful to Mr. Dick Williams for the training he provided in both scanning and transmission electron microscopy. His patience and technical assistance were greatly appreciated. I would like to thank Dr. Charlie R. Brooks and Mr. Bob L. McGill in the Department of Metallurgical Engineering for instrument training and helpful discussions during the use of the AMR 900 scanning electron microscope. Dr. Les Hickok provided access to the Olympus Universal Research Microscope and photomicrographic system. The use of the Beckman DU spectrophotometer and Gilford gel scanner was provided by Dr. Otto J. Schwarz. I would also like to thank Dr. James D. Caponetti for the use of the gel electrophoresis equipment and for many helpful technical discussions. I am very grateful to Dr. Alan S. Heilman for his excellent technical advice on black and white photography and for his help in the preparation of the color photographic plates.

I am deeply indebted to my parents, Mr. and Mrs. Stanley Pocratsky, for their love and support throughout my graduate program. Without their continued faith in me, this degree would not have been

possible. Finally, I would like to thank my friend and companion, Mark E. Reeves, for many things, too numerous to mention. His enthusiasm and confidence in me helped to make the road much smoother.

ABSTRACT

The nutrition, chemical composition and ultrastructure of the unicellular chlorophycean flagellate Phacotus lenticularis are investigated, with special emphasis on the lorica and extracellular mucilage. Cells of P. lenticularis are distinguished by the presence of a bivalved lorica, which can vary in size, shape and color depending upon culture conditions.

Elemental analysis of isolated loricas with SEM-EDS reveals differences in dark and colorless loricas. Dark loricas have manganese as the principal element, whereas in colorless loricas, calcium is the predominant element. The presence of nitrogen in the medium influences both the size and elemental composition of the lorica.

The lorica matrix is estimated to contain ca. 36% carbohydrate, 26% protein and 4% ester-linked sulfate, with the amino acid hydroxyproline accounting for ca. 1% of the protein. Chemically, the lorica is composed of a structural polymer of glucose, with a supporting acid mucopolysaccharide matrix.

The lorica is composed of two structural elements. A granulo-fibrillar component, deposited first during development, is closely appressed to the surface of new cells. Dense stellate cores are then deposited in an orderly array external to the granulo-fibrillar layer and are subsequently interconnected to form a continuous structure. Production of lorica precursor materials occurs in the Golgi apparatus. Loricas undergo an autolytic process in which core and fibrillar

components dissociate, thereby allowing for the release of daughter cells after cytokinesis.

Extracellular mucilage, produced by the cells at all times during culture, is ca. 50% carbohydrate, 12% protein, 16% sulfate and 5% uronic acid, by weight. Seven sugars and 12 amino acids are detected by thin-layer and gas-liquid chromatographic methods, with hydroxyproline as the most abundant amino acid in the mucilage.

Ultrastructurally Phacotus differs from previously described phacotacean algae in the structure of the pyrenoid and stigma. A chloroplast lamellar lattice is described for the first time in any volvocalean alga.

The phylogenetic position of Phacotus is re-evaluated in terms of its mode of cell division and type of cell covering. That Phacotus may have diverged from the chlamydomonad line before the evolution of a true cell wall is discussed.

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LIST OF ABBREVIATIONS

Measurements and Other Symbols

α	alpha	N	normal/normality
AB	Alcian Blue	nm	nanometer
ara	arabinose	p	para
AY	Alcian Yellow	PA-TCH-SP	periodic acid-thiocarbohydrazide-silver proteinate
β	beta		
C	centigrade	PAS	periodic acid-Schiff's
ca.	approximately	PASH	periodic acid-silver hexamine
cc	cubic centimeters		
cm	centimeter	psi	pounds per square inch
CPC	cetylpyridinium chloride	R _f	ratio of fronts
CPD	critical point dried	rib	ribose
ECM	extracellular mucilage	rha	rhamnose
fruc	fructose	RR	Ruthenium Red
fu	fucose	SDS	sodium dodecyl sulfate
g	grams	SEM	scanning electron microscope/microscopy
gal	galactose		
gal acid	galacturonic acid	SEM-EDS	scanning electron microscope-energy dispersive X-ray analysis
GLC	gas-liquid chromatography		
glc acid	glucuronic acid		
glu	glucose		
HMF	hydroxymethyl-furfuraldehyde	sor	sorbose
KeV	kilo-electron volt	SW	soil-water
kV	kilovolt	SWE	soil-water extract
L	liter	TBO	Toluidine Blue O
LD	light-to-dark	TCA	trichloroacetic acid
LM	light microscope/microscopy	TEM	transmission electron microscope/microscopy
<u>m</u>	meta		
M	mole/molar	TFA	trifluoroacetic acid
mA	milliamp	THC	thick-layer chromatography
man	mannose	TLC	thin-layer chromatography
MB	Methylene Blue	TMS	trimethylsilyl
mg	milligram	v/v	volume per volume
min	minute	W/m ²	Watts/meter ²
ml	milliliter	w/v	weight per volume
mm	millimeter	X g	times gravity
mM	millimole	xyl	xylose
mos	months		
MVM	Modified Volvox Medium		
μ g	microgram		
μ L	microliter		
μ m	micrometer		

Organelles

bb	basal body
c	chloroplast
ch	chromatin
d	dictyosome
ER	endoplasmic reticulum
f	flagellum
lor	lorica
m	mitochondrion
mu	mucilage
n	nucleus
np	nuclear pore
nu	nucleolus
py	pyrenoid
s	stigma
sg	starch grain
v	vesicle

CHAPTER I

INTRODUCTION

Members of the algal family Phacotaceae (order Volvocales, class Chlorophyceae) are motile unicells which are distinguished by the presence of a non-living surface layer or encasement, the lorica. These organisms may have two or four flagella; biflagellate members, such as Phacotus, have two axial flagella inserted at an oblique angle with respect to the long axis of the cell. The protoplast of phacotacean genera has a typical chlamydomonad organization, with a cup-shaped chloroplast with an eyespot and one or more pyrenoids and contractile vacuoles at the base of the flagella (Smith, 1950; Bourrelly, 1966; Bold and Wynne, 1978). The lorica is commonly impregnated with various compounds such as iron, manganese and calcium (Smith, 1950; Huber-Pestalozzi, 1951; Bourrelly, 1966; Gerard, 1980; Porcella and Walne, 1980) but recent studies have shown the presence of other substances such as silicon and aluminum (Gerard and Walne, 1978; Gerard, 1980). In some genera, the lorica is larger and not the same shape as the protoplast, with a space between the two; in others, the two are tightly appressed (Smith, 1950). At cytokinesis, the lorica separates or fragments irregularly (Bourrelly, 1966). Pertinent literature concerning the Phacotaceae is summarized in Table I.

The genus Phacotus has a compressed lorica composed of two overlapping halves. The lorica is reportedly impregnated with some form of calcium and often has a sculptured appearance (Smith, 1950). The

TABLE I

A PARTIAL SUMMARY OF PERTINENT LITERATURE
ON THE PHACOTACEAE

Date	Author	Contribution
1899	Dangeard, P. A.	Wrote the original description of <u>Phacotus</u> .
1916	Takeda, H.	Wrote the original description of the new genus and species <u>Dysmorphococcus variabilis</u> .
1925	Skvortzow, B. B.	Described the new genus and species <u>Wislouchiella planktonica</u> .
1927a	Pascher, A.	Described and illustrated <u>Phacotus lenticularis</u> var. <u>sphaerica</u> and <u>Wislouchiella planktonica</u> in a report of rare algae.
1927b	Pascher, A.	Described and illustrated 8 species of <u>Phacotus</u> and 7 species of <u>Pteromonas</u> found in Europe.
1930	Conrad, W.	Described 2 new species of <u>Pteromonas</u> .
1938	Bold, H. C.	Reported the occurrence of <u>Dysmorphococcus variabilis</u> for the first time in the United States.
1941	Singh, J.	Isolated 2 species of <u>Phacotus</u> from the soil.
1942	Brinley, F. J. and L. J. Katzin	Reported the widespread occurrence of <u>Phacotus lenticularis</u> and <u>Wislouchiella planktonica</u> throughout the Ohio River system.
1942	Higinbotham, N.	Wrote the original description of the genus <u>Cephalomonas</u> .
1942	Lackey, J. B.	Described 2 species of <u>Phacotus</u> and 1 of <u>Wislouchiella</u> from the watersheds of the Cumberland and Duck Rivers in Tennessee. Commented on the need for a critical taxonomic examination of some members of the Phacotaceae.
1944	Jane, F.	Described the new species <u>Pteromonas varians</u> and <u>Coccomonas platyformis</u> .

TABLE I (Continued)

Date	Author	Contribution
1949	Hortobagyi, T.	Described the new species <u>Coccomonas eberii</u> .
1950	Kampter, E.	Investigated the chemical composition of the lorica of <u>Phacotus</u> and noted the lack of iron.
1950	Smith, G. M.	Described the Phacotaceae, including species of 8 genera found in the United States.
1953	Bold, H. C. and R. C. Starr	Described the new species <u>Dysmorphococcus globosus</u> .
1955	Cole, G. A.	Described <u>Phacotus lenticularis</u> as one of the most abundant profundal forms encountered in a survey of Minnesota lakes, with a high tolerance for anoxic waters.
1956	Bourrelly, P.	Described members of the Phacotaceae, with descriptions of both <u>Phacotus</u> and <u>Wislouchiella</u> .
1957	Fott, B.	Described the new species <u>Pteromonas condiformis</u> .
1961	Huber-Pestalozzi, G.	Described and illustrated 14 species of <u>Phacotus</u> .
1963	Swale, E.M.F.	Documented the taxonomic history of <u>Pteromonas angulosa</u> .
1965	Peterfi, L. S. and S. Peterfi	Detailed the morphology of 5 species of <u>Pteromonas</u> .
1966	Evans, J. H.	Discussed the taxonomic features and cultural characteristics of <u>Pteromonas varians</u> .
1967	Belcher, J. F. and E.M.F. Swale	Described the species <u>Pteromonas tenuis</u> with light and electron microscopy.
1970	Prescott, G. W.	Described the 8 Phacotacean genera found in the United States.
1975	Porcella, R. A. and P. L. Walne	Described the ultrastructure of the vegetative cells and the development of the lorica in <u>Dysmorphococcus globosus</u> .

TABLE I (Continued)

Date	Author	Contribution
1976	Porcella, R. A.	Described the ultrastructure and lorica development in <u>Dysmorphococcus globosus</u> .
1978	Gerard, D. A. and P. L. Walne	Described the surface configuration and chemical composition of the lorica of <u>Pteromonas protracta</u> .
1979	Dawson, J. T. and D. O. Harris	Reported a new species, <u>Dysmorphococcus africana</u> , from South Africa.
1979	Gerard, D. A. and P. L. Walne	Described lorica development in <u>Pteromonas protracta</u> .
1979	Good, B. H. and R. L. Chapman	Reported the possible presence of a sporopollenin in the lorica of <u>Dysmorphococcus</u> .
1980	Gerard, D. A.	Described the ultrastructure, elemental composition and development of the lorica of <u>Pteromonas protracta</u> .
1980	Porcella, R. A. and P. L. Walne	Described the structure of the lorica of <u>Dysmorphococcus globosus</u> .
1981a	Pocratsky, L. A. and P. L. Walne	Reported the chemical composition of the lorica and extracellular mucilage of <u>Phacotus lenticularis</u> .
1981b	Pocratsky, L. A. and P. L. Walne	Described the chemical, nutritional and ultrastructural characteristics of the lorica and extracellular mucilage of <u>Phacotus lenticularis</u> .
1981	Shyam, R.	Studied the life cycle of <u>Dysmorphococcus sarmaii</u> .

protoplast is ovoid and is in contact with the lorica at the anterior end only. Asexual reproduction results in 2, 4 or 8 daughter cells which are released by a longitudinal division of the lorica. Resting stages (such as aplanospores) and palmelloid forms are common. Sexual reproduction is known in certain species (Bourrelly, 1966).

Lorica construction has been studied most extensively in the Chrysophyceae in which several types of loricas have been defined. Taxonomic re-evaluations of lorica structure have led to the characterization of several types of loricas. They are (1) individual imbricate scales, each composed of microfibrils (Hilliard and Asmund, 1963); (2) microfibrillar structure sometimes thickened and obscured by mineral deposits and usually with a helical or annular construction (Belcher, 1968; Wujek, 1969; Hilliard, 1971; Kristiansen, 1972); (3) a less-patterned microfibrillar arrangement with both single- and double-walled loricas (Kristiansen, 1969, 1972); (4) a "primitive" microfibrillar arrangement (Schnepf et al., 1975; Herth et al., 1977) and (5) non-microfibrillar, consisting of a mucilaginous matrix impregnated with needle-shaped crystals (Belcher, 1969; Belcher and Swale, 1972). Other criteria used in lorica classification include its shape and size, its chemical or elemental composition and whether it is entire or valved. A summary of literature pertaining to protistan loricas is presented in Table II.

In the Phacotaceae, the loricas of only two genera, Dysmorphococcus and Pteromonas, have been examined at the ultrastructural level (Porcella, 1976; Gerard, 1980; Porcella and Walne, 1980). These studies revealed

TABLE II

A PARTIAL SUMMARY OF PERTINENT LITERATURE
ON PROTISTAN LORICAS

Date	Author	Contribution
1950	Kampter, E.	Reported an absence of iron in the lorica of <u>Phacotus</u> .
1950	Middlehoek, A.	Described and illustrated the lorica of <u>Bicosoeca planktonica</u> .
1953	Pringsheim, E. G.	Described the loricas of 13 species of <u>Trachelomonas</u> grown in culture and discussed their reliability as a taxonomic trait.
1956	Singh, K. P.	Discussed the morphology of the lorica in six species of <u>Trachelomonas</u> .
1958	Petersen, J. B. and J. B. Hansen	Discussed the loricated neuston organisms <u>Hyalobryon minutum</u> , <u>Hyalocyclix stipitata</u> and <u>Chromophyton rosanoffii</u> .
1961	Fjerdingstad, E. J.	Described the ultrastructure of the collar of the choanoflagellate <u>Codonosiga botrytis</u> .
1963	Hilliard, D. K. and B. Asmund	Examined the morphology of <u>Dinobryon</u> , <u>Hyalobryon</u> and <u>Epipyxis</u> with electron microscopy. Combined some species of <u>Hyalobryon</u> and <u>Dinobryon</u> into the genus <u>Epipyxis</u> on the basis of lorica structure.
1967	Karim, A.G.A. and F. E. Round	Detected the presence of fine cellulose-like microfibrils in the lorica of several species of <u>Dinobryon</u> by electron microscopy.
1967	Belcher, J. H. and E.M.F. Swale	Described the structure of <u>Pteromonas tenuis</u> by light and electron microscopy, with reference to the lorica.
1968	Belcher, J. H.	Discussed the lorica construction of <u>Pseudokephyrion pseudospirale</u> . Found the Torica was composed of a microfibrillar skeleton and mineralized deposits.
1968	Gold, K.	Detected the presence of proteins in the lorica of the ciliated protozoan <u>Tintinnida</u> .

TABLE II (Continued)

Date	Author	Contribution
1968	Tappan, H. and A. R. Loeblich	Compared the lorica composition of modern and fossil <u>Tintinnida</u> .
1969	Gold, K.	Described the incorporation of radioactive amino acids into the loricas of the ciliated protozoans <u>Tintinnopsis tubulosa</u> and <u>Favella campanosa</u> . Also speculated on the manner of lorica deposition in these organisms.
1969	Kristiansen, J.	Described the lorica structure in <u>Chrysolykos planktonicus</u> . Found that the lorica was composed of interwoven microfibrils with no matrix between them.
1969	Peterfi, L. S.	Described the fine structure of the lorica of <u>Poterioochromonas malhamensis</u> . Found the lorica is a microfibrillar meshwork.
1969	Wujek, D. E.	Postulated a mechanism for lorica development in <u>Dinobryon sertularia</u> .
1970	Gold, K., R. M. Pfister and V. R. Liguori	Described the loricas of 2 choanoflagellates, <u>Acanthoecopsis</u> sp. and <u>Diaphanoeca grandis</u> with electron microscopy.
1970	Kramer, D.	Described the fine structure and morphogenesis of the lorica fibrils of <u>Ochromonas malhamensis</u> .
1970	Thronsdon, J.	Described the lorica of <u>Salpingoeca spinifera</u> .
1972a	Kristiansen, J.	Examined the fine structure of the lorica in 6 Chrysophycean species and discussed the arrangement of the cellulose microfibrils in each.
1972b	Kristiansen, J.	Examined the fine structure of the lorica of <u>Bicoeca crystallina</u> and described its organization and development. Showed that 2 types of lorica organization exist in this genus.
1973	Franke, W. W. and W. Herth	Described the fine structure of the lorica of <u>Dinobryon sertularia</u> with special reference to possible modes of lorica synthesis.

TABLE II (Continued)

Date	Author	Contribution
1973	Thomsen, H. A.	Described the structure of the lorica of several marine choanoflagellates.
1974	Leadbetter, B.S.C. and I. Manton	Identified silica as a component of the lorica of the collared flagellate <u>Stephanoeca diplocostata</u> .
1975	Gold, K. and E. A. Morales	Analyzed the composition of the lorica of the ciliated protozoan <u>Paravella gigantea</u> and concluded that it was composed of pseudochitin.
1975	Leadbetter, B.S.C.	Described the development of the lorica of <u>Savilla microspora</u> and <u>Stephanoeca diplocostata</u> .
1975	Leedale, G. F.	Reported the fine structure and development of the lorica in the genus <u>Trachelomonas</u> .
1975	Rosowski, J. R., R. L. Vadas and P. Kugrens	Described the surface configuration of the lorica of <u>Trachelomonas</u> by scanning electron microscopy.
1975	Schnepf, E., G. Roderer and W. Herth	Discussed the formation of microfibrils in the lorica of <u>Poterioochromonas stipitata</u> . Suggested that microtubules play a role in the microfibrillar orientation but not in their synthesis or secretion.
1976	Porcella, R. A.	Provided a fine-structural account of lorica development in <u>Dysmorphococcus globosus</u> .
1976	Thomsen, H. A.	Described the fine structure of the lorica of several silicified choanoflagellates.
1977	Herth, W., A. Kuppel and E. Schnepf	Studied the stalk fibrils of the lorica of <u>Poterioochromonas stipitata</u> by electron microscopy and chemical analyses. Found the fibrils had a composition similar to fungal chitin.
1977	Thomsen, H. A.	Described the fine structure of the lorica of the choanoflagellate <u>Acatheocopsis</u> .

TABLE II (Continued)

Date	Author	Contribution
1977	West, L. K.	Investigated the ultrastructure of <u>Trachelomonas hispida</u> var. <u>coronata</u> along with the substructure, composition and biomineralization of its cell envelope (lorica).
1977	West, L. K. and P. L. Walne	Demonstrated the presence of manganese in the envelope (lorica) of <u>Trachelomonas</u> .
1978	Gerard, D. A. and P. L. Walne	Reported the presence of calcium and silicon and a lack of iron in the lorica of <u>Pteromonas protracta</u> .
1978	Hibberd, D. J.	Described the structure of the lorica of <u>Bicosoeca accreta</u> with light and electron microscopy.
1979	Donnelly, L. S.	Described the ultrastructure and elemental composition of three species of <u>Trachelomonas</u> .
1979	Gerard, D. A. and P. L. Walne	Documented the involvement of the Golgi apparatus in lorica production in <u>Pteromonas protracta</u> .
1979	Good, B. H. and R. L. Chapman	Reported the possible presence of a sporopollenin in the lorica of <u>Dysmorphococcus</u> .
1979	Herth, W.	Documented the process of lorica formation in <u>Dinobryon divergens</u> with light microscopy.
1979	Moss, M. O. and G. Gibbs	Compared the levels of iron and manganese in the lorica of <u>Trachelomonas</u> from natural collections using microprobe analysis.
1980	Gerard, D. A.	Investigated the structure, development and elemental composition of the lorica of <u>Pteromonas protracta</u> .
1980	Herth, W.	Studied the process of chitin microfibril formation in the lorica of <u>Poteroiochromonas stipitata</u> using the stains Calcofluor white and Congo red.

TABLE II (Continued)

Date	Author	Contribution
1980	Porcella, R. A. and P. L. Walne	Described the structure and development of the lorica of <u>Dysmorphococcus globosus</u> .
1980	West, L. K. and P. L. Walne	Described the envelope (lorica) substructure of <u>Trachelomonas hispida</u> var. <u>coronata</u> .
1980	West, L. K., P. L. Walne and J. Bentley	Reported the mode of mineralization and elemental composition of the envelope (lorica) of <u>Trachelomonas hispida</u> var. <u>coronata</u> .
1981	Dunlap, J. R., P. A. Kivic and P. L. Walne	Compared the elemental composition of the envelopes (loricas) of <u>Trachelomonas</u> and <u>Strombomonas</u> .
1981	Dunlap, J. R., L. K. West, P. L. Walne and J. Bentley	Discussed the spatial segregation of manganese and iron in the envelope of <u>Trachelomonas lefevrei</u> .
1981	Laval-Peuto, M.	Described the construction of the lorica of the tintinnid <u>Favella ehrenbergii</u> .
1981a	Pocratsky, L. A. and P. L. Walne	Investigated the chemical composition of the lorica of <u>Phacotus lenticularis</u> .
1981b	Pocratsky, L. A. and P. L. Walne	Reported the nutritional and ultrastructural characteristics of the lorica of <u>Phacotus lenticularis</u> .
1981	Walne, P. L., J. R. Dunlap and P. A. Kivic	Compared the ultrastructure and elemental composition of the envelopes (loricas) of <u>Strombomonas</u> and <u>Trachelomonas</u> .

differences not only between the loricas of these two organisms but also between other cellular structures such as the pyrenoid and stigma.

Dysmorphococcus has a mono-layered entire lorica which becomes mineralized with ferric compounds, whereas Pteromonas has a bivalved, bi-layered lorica with a hexagonal surface pattern. The lorica of Pteromonas does not accumulate iron in large amounts, but does retain calcium and silicon under certain culture conditions. These data suggest that the Phacotaceae is not as homogeneous as was previously thought and may be divisible into two or more subgroups based on lorica structure and composition and on cellular organization.

The plasticity and pleomorphism of lorica shape in some members of this family depends on environmental and nutritional factors. For example, Wislouchiella planktonica and Pteromonas cruciata may intergrade in nature, so that their loricas are difficult to distinguish from one another (Lackey, 1942). Also, Wislouchiella commonly loses its characteristic winged lorica in culture and assumes a smooth shape with no projections, suggesting a possible nutrient-related phenomenon (personal unpublished observations). Such plasticity dictates the need for in-depth nutritional and developmental studies and a critical taxonomic evaluation of the genera in this family.

Recent investigations indicate that mucilaginous substances are released in copious amounts by some members of the Phacotaceae. Porcella and Walne (1980) reported that extracellular mucilage coating the surface of mature cells of Dysmorphococcus globosus was extruded through pores in the lorica and was retained as part of the lorica. Preliminary observations of the genus Phacotus with light microscopy revealed that

large amounts of mucilage were secreted into the culture medium (personal unpublished observations).

In addition to extracellular secretions, mucilages also serve as the main constituents of the continuous amorphous phase of cell walls and may serve as a type of cementing material for the water-insoluble elements of the wall (Preston, 1958). The cell walls of the algae contain between 30% and 70% of such water-soluble material, which is extractable from the tissues or cells by hot water, dilute acid and alkali (Percival, 1970, 1979). The extract, upon hydrolysis, will yield any of a number of simple sugars and acids, depending on the algal group examined. In the Rhodophyceae, for example, there is a repeating structural backbone of alternating α -1,3- and β -1,4-linked galactans, while in the Chlorophyceae there is a complex mixture of various simple sugars, with no obvious structural pattern (O'Colla, 1967; Percival, 1979).

Mucilages produced by the algae may aid the organism in several ways. The mucilages may provide mechanical protection when present in large amounts, function as ion reservoirs or exchangers (Percival, 1970; Ramus, 1972), form gas and water barriers to prevent desiccation (Percival, 1970) or function as an adhesive for attached forms (Retallack and Butler, 1972; Callow and Evans, 1974).

The study of extracellular mucilages has also gained attention because of the ecological impact such substances have on the planktonic organisms of lakes and rivers. Since mucilages are water-soluble, varying amounts can dissolve into surrounding waters and may affect nearby organisms. It is probable that even very small amounts of

released extracellular organics may be biologically active at low concentrations and may thus exert considerable ecological effects (Lewin, 1956; Nalewajko et al., 1980). These substances may affect the ecosystem directly by functioning as growth-limiting or promoting factors (Lefèvre and Jakob, 1949; Fogg, 1971; Hellebust, 1974) or indirectly by forming chemical complexes with other dissolved substances (Pollard and Smith, 1951; Fogg, 1966; Whitton, 1970).

While the release of simple substances, such as amino acids, organic acids and simple sugars, occurs primarily by diffusion across the cell membrane (Hellebust, 1974), the excretion of larger molecules such as polysaccharides takes place by a more complex process. A widely accepted mode for their transport to the cell surface is by way of reverse pinocytosis (exocytosis), whereby intracellular vesicles subsequently fuse with the plasma membrane (Whaley et al., 1972). Processing of most polysaccharide material within the cell takes place in the Golgi apparatus (Northcote, 1971; Whaley et al., 1972; Rothman, 1981). Recent studies with the rhodophycean unicells Rhodella and Porphyridium have shown that the carbohydrate components of algal mucilages are synthesized in the membrane system of the Golgi apparatus. They are then assembled into polysaccharides in the Golgi cisternae and are subsequently transported to the cell surface in secretory vesicles (Ramus, 1972; Evans et al., 1974). Pertinent literature on algal polysaccharides and mucilage production is summarized in Table III.

The following research was undertaken (1) to investigate the structure and composition of the lorica and to determine the mode of its synthesis, transport and deposition, (2) to determine the chemical

TABLE III

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON ALGAL
POLYSACCHARIDES AND MUCILAGE PRODUCTION

Date	Author	Contribution
1938	Miyaki, S., K. Hayasi and Y. Takino	Described the water-soluble polysaccharides of <u>Ulva lactuca</u> by biochemical methods. Found the major constituents to be uronic acid and rhamnose.
1939	Miyaki, S. and K. Hayaki	Described the water-soluble polysaccharides of <u>Enteromorpha compressa</u> by biochemical methods. Found the major constituents to be uronic acid and rhamnose.
1940	Norris, F. W.	Analyzed the mucilage of <u>Ulva lactuca</u> and detected polysaccharides containing rhamnose.
1944	Kylin, H.	Analyzed the mucilage of <u>Cladophora rupestris</u> and found it was a galactan sulfate.
1946	Kylin, H.	Investigated the cell wall constituents of <u>Ulva</u> and <u>Enteromorpha</u> . Found that the polysaccharides of both were sulfuric acid esters containing a methyl pentose.
1952	Dzhelileva, P. D.	Investigated the chemical components of green algae from the Black Sea. Estimated the seasonal variations of soluble polysaccharide content of <u>Enteromorpha intestinalis</u> , <u>Chaetomorpha aerea</u> , <u>Cladophora utriculosa</u> and <u>Codium tomentosum</u> .
1952	Hough, L., J.N.K. Jones and W. H. Wadman	Isolated polysaccharides from the freshwater algae <u>Nitella</u> , <u>Oscillatoria</u> and <u>Nostoc</u> . Found that those from <u>Nitella</u> and <u>Oscillatoria</u> were composed of polyglucosans, while the polysaccharide of <u>Nostoc</u> was a complex acidic mucilage composed of six different monosaccharides.
1954	Augier, J. and M.L.R. duMerac	Extracted a mucilage from <u>Codium dichotum</u> which, after hydrolysis, yielded galactose, mannose and arabinose.

TABLE III (Continued)

Date	Author	Contribution
1954	Brading, J.W.E., M.M.T. Georg-Plant and D. M. Hardy	Described the purification, hydrolysis and methylation of the sulfated polysaccharides from <u>Ulva lactuca</u> .
1956	Lewin, R. A.	Studied the extracellular soluble and capsular polysaccharides in 13 species of <u>Chlamydomonas</u> . Found the soluble polysaccharide constituted as much as 25% of the total organics produced, and the capsular mucilage represented 40-60% of the total organics. Found the predominant sugar components were galactose and arabinose.
1958	Guillard, R.R.L. and P. J. Wangerski	Investigated the correlation between cell numbers and extracellular carbohydrate production in several marine flagellates. Found polysaccharide production was most abundant during stationary or declining culture conditions.
1959	O'Donnell, J. J. and E. Percival	Analyzed the structure of water-soluble polysaccharides in <u>Cladophora rupestris</u> by means of Barry degradation and methylation.
1962	Fogg, G. E.	Wrote a comprehensive review of all types of extracellular products in algae, including carbohydrates.
1962	Jones, R. F.	Described the extracellular mucilage of <u>Porphyridium cruentum</u> as an acidic polysaccharide-protein complex.
1962	O'Colla, P. S.	Assembled existing information about mucilage production in all types of algae.
1965	Hirst, E. L., E. Percival and J. K. Wold	Described the site of ester sulfate groups and the manner of linkage between galactose residues in <u>Cladophora rupestris</u> and <u>Chaetomorpha</u> spp.
1965	Hoyt, J. W. and G. Soli	Demonstrated the ability of algal extracellular polysaccharides to reduce turbulence and friction in liquid culture.

TABLE III (Continued)

Date	Author	Contribution
1965	Marker, A.F.H.	Described extracellular carbohydrate release in two flagellates, <u>Isochrysis galbana</u> and <u>Prymnesium parvum</u> .
1969	Maksimova, I. V. and M. N. Pimenova	Studied the relationship between bacterial growth in algal cultures and the content of extracellular organic compounds in the medium. Found free sugars and organic acids produced by the algae were consumed by bacteria.
1972	Bourne, E. J., E. Percival and B. Smestad	Studied the extracellular carbohydrate production of <u>Acetabularia crenulata</u> .
1972	Ramus, J.	Characterized the encapsulating polysaccharides of <u>Porphyridium aerugineum</u> . Showed that the rate of polysaccharide production was greatest in stationary phase.
1972	Ramus, J. and S. T. Groves	Studied the accumulation of labelled sulfate by <u>Porphyridium aerugineum</u> and the subsequent appearance of solubilized capsular polysaccharide in the growth medium.
1972	Retallack, B. and R. D. Butler	Studied mucilage secretion in the zoospores of <u>Bulbochaete hiloensis</u> . Found that the zoospores secreted polysaccharides as an aid in attaching to the substratum.
1973	Ramus, J.	Reviewed current literature concerning polysaccharide production in <u>Porphyridium aerugineum</u> .
1974	Callow, M. E. and L. V. Evans	Characterized the process of glycoprotein adhesive formation in zoospores of <u>Enteromorpha</u> by cytochemistry and autoradiography.
1974	Evans, L. V., M. E. Callow, E. Percival and V. Fareed	Studied the synthesis and composition of extracellular mucilage in <u>Rhodella</u> using light and electron microscopy, autoradiography and various analytical means.

TABLE III (Continued)

Date	Author	Contribution
1974	Hellebust, J. A.	Reviewed available information on algal extracellular products.
1974	Ramus, J. and S. T. Groves	Described the kinetics of sulfur incorporation during <u>in vivo</u> sulfate esterification of <u>Porphyridium aerugineum</u> capsular polysaccharides.
1975	Tutumi, T. and K. Ueda	Carried out cytochemical studies on <u>Micrasterias americana</u> to determine the types of polysaccharides present in the cell and their origin.
1976	Callow, M. E. and L. V. Evans	Located sulfated polysaccharides within secretory cells by X-ray microanalysis in <u>Laminaria saccharina</u> .
1976	Lang, W. C. and M. J. Chrispeels	Studied the release of glycoproteins during the cell cycle of <u>Chlamydomonas reinhardtii</u> .
1976	Lange, W.	Speculated on the possible ecological significance of the mucilaginous sheaths of some blue-green algae.
1976	Menge, U.	Examined several developmental stages of <u>Micrasterias denticulata</u> with three different types of polysaccharide chemistry.
1977	Heaney-Kiernas, J., L. Roden and D. J. Chapman	Demonstrated a protein-carbohydrate linkage involving serine and threonine in the extracellular proteoglycan of <u>Porphyridium cruentum</u> .
1977	Rosowski, J. R.	Examined the mucilaginous surface of flagellated, creeping and palmelloid cells of several <u>Euglena</u> species with scanning electron microscopy.
1977	Rosowski, J. R. and R. I. Willey	Examined the mucilaginous stalk and envelope of <u>Colacium mucronatum</u> with scanning electron microscopy.
1977	Willey, R. I., K. Ward, W. Russin and R. Wibel	Carried out cytochemical studies of the extracellular carbohydrates of <u>Colacium mucronatum</u> .

TABLE III (Continued)

Date	Author	Contribution
1978	Fulton, A. B.	Examined the extracellular matrix of <u>Pandorina morum</u> with chemical analyses, cytochemistry and TEM.
1978	Tautvydas, K. J.	Isolated an extracellular hydroxyproline-rich glycoprotein and a mannose-rich polysaccharide from the mucilage and colony walls of <u>Eudorina californica</u> .
1978	Percival, E.	Reviewed the structure, function and biosynthesis of the polysaccharides found in green, red and brown seaweeds.
1979	Lamport, D.T.A.	Described the extracellular matrix of <u>Volvox carteri</u> as a hydroxyproline-rich glycoprotein.
1979	Percival, E.	Reviewed the structures of the sulfated polysaccharides metabolized by marine chlorophycean algae.
1980	Crayton, M. A.	Presented evidence for the presence of a sulfated polysaccharide component in the matrix of <u>Platydorina caudata</u> .
1980	Nalewajko, C., K. Lee and P. Fay	Investigated the influence of light on algal excretion and bacterial numbers both in culture and in lakes.
1980	Percival, E., M. A. Rahman and H. Weigel	Investigated the chemical composition of the extracellular polysaccharide of <u>Coscinodiscus nobilis</u> .
1980	Roberts, K., M. R. Gay and G. J. Hills	Found that the soluble extracellular glycoproteins of <u>Chlamydomonas reinhardtii</u> contain low levels of sugar-O-sulfate esters.
1981	Boney, A. D.	Discussed functional aspects of algal mucilages.
1981a	Pocratsky, L. A. and P. L. Walne	Detailed the chemical composition of the extracellular mucilage of <u>Phacotus lenticularis</u> .

TABLE III (Continued)

Date	Author	Contribution
1981b	Pocratsky, L. A. and P. L. Walne	Described the chemistry and ultrastructure of the extracellular mucilage of <u>Phacotus lenticularis</u> with the aid of several cytochemical stains.

composition of the extracellular mucilage and to investigate its sub-structure and mode of transport to the exterior of the cell, (3) to examine the role of nutritional factors in the morphology of the cell and lorica and (4) to compare the basic cellular organization of Phacotus lenticularis with that of other phacotacean and volvocacean organisms.

CHAPTER II

MATERIALS AND METHODS

1. CULTURE METHODS

Culture Conditions

Cultures of Phacotus lenticularis Ehr. (No. LB 61/1) were obtained from The Culture Centre of Algae and Protozoa, Cambridge, England. Stock cultures were maintained in 200-ml milk bottles containing a biphasic soil-water medium (Pringsheim, 1946; Starr, 1978) (Appendix A). Although cultures were maintained for long periods (i.e., 6 mos.) in this medium, cell density was very low. In order to attain rapid growth and high cell density, several defined media were tested, and those selected for use were Volvox medium (Starr, 1969) and Chu #10 (Chu, 1942). Best growth was established in a modified Volvox medium (MVM) (Appendix A). Cells were cultured in 250-ml flasks containing 150 ml of medium, in 2-liter flasks containing one liter of medium or in 15-ml test tubes containing 8 ml of medium. All cultures were maintained in a walk-in culture chamber under cool-white fluorescent illumination of ca. 10 W/m² (photosynthetically active radiation) at 18-20°C on a 12:12 LD cycle.

Axenic Cultures

For chemical analyses, axenic cultures were established by the purification of the cultures with antibiotics. Techniques employed were

essentially those of Droop (1967). Axenic cultures were maintained in MVM and were checked periodically in standard bacteriological media for contamination.

Axenic cultures were grown routinely in 250-ml flasks and transferred under sterile conditions every 7-10 days. For larger cultures, needed for growth studies and chemical analyses, 10-20 ml of actively growing cells (ca. 10^5 cells/ml) were transferred to 2-liter flasks containing one liter of medium.

Nutritional Studies

Preliminary studies with Phacotus lenticularis indicated that cell and lorica morphology varied greatly with the type of medium used. Nutritional studies were thus undertaken to investigate the effects of media composition on cell and lorica morphology.

Initial observations were made on cells cultured in biphasic soil-water, MVM and Chu #10 media. Combinations of defined media and soil-water extract (SWE) were also used (Appendix A). Differences in cell structure, lorica morphology and lorica color were noted. To investigate the effects of mineral supplements on cells grown in soil-water, various minerals were added as a soil-water overlay (Table XVIII, Appendix A). Cells were cultured in these media and the effects were noted at periodic intervals. Effects of different nitrogen sources on cells grown in both soil-water and MVM were also investigated. Nitrogen sources added to these media were $\text{Ca}(\text{NO}_3)_2$, NaNO_3 , NaNO_2 , KNO_3 , $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , $(\text{NH}_4)_2\text{HPO}_4$, $\text{CO}(\text{NH}_2)_2$ and casein hydrolysate (Table XIX, Appendix A).

Manganese supplements in the forms of manganese chloride and manganese acetate were added to various defined media and soil-water mixtures to observe their effects on lorica structure and composition. Manganous chloride, a component of MVM, was tested at normal, 2X, 5X and 10X concentrations. Manganese acetate was added at concentrations of 5, 10, 20, 40, 80, and 100 mg per 100 ml of medium.

To examine the effects on lorica composition of the absence of a metal chelator in the medium, Na₂EDTA was omitted from MVM medium. Preliminary experiments indicated that iron in the absence of a chelator was toxic to the cells. To overcome the effects of iron toxicity in such a medium, the amount of iron specified in the trace elements stock was reduced to one-quarter strength.

2. DESCRIPTIVE METHODS

Light Microscopy

For light-microscopic examinations, cells were observed in the living condition or were fixed by the addition of 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.1. Cells were viewed with an Olympus VANOX Universal Research Microscope, equipped with bright-field and oblique-illumination systems and an Olympus PM-10-A 35-mm photographic system. Photographic data were recorded on Kodak Panatomic X black and white film (ASA 32) and Kodak Ektachrome tungsten color slide film (ASA 50 or 160), using color balancing filters where necessary.

Transmission Electron Microscopy (TEM)

Thin sections. Cells were concentrated by centrifugation at 1000 Xg in a clinical centrifuge, then washed with culture medium 3 times, and fixed for one hour with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.1 in the dark at 4°C. After several washes in buffer, the cells were post-fixed for one hour in 2% OsO₄ in 0.05 M sodium cacodylate, pH 7.1, in the dark at 4°C. After fixation, cells were dehydrated in a graded ethanol series, followed by successive changes in propylene oxide, and were embedded in Spurr's low-viscosity resin (1969). Ultrathin sections were cut using a Porter-Blum MT-1 ultramicrotome outfitted with glass knives. Sections were mounted on uncoated 300- or 400-mesh copper grids and post-stained with either saturated aqueous uranyl acetate for 30 minutes or 1% potassium permanganate for 2 minutes (Bray and Wagenaar, 1978), followed by Reynold's lead citrate for 5 minutes (1963) (Appendix B). Thin sections were examined with Zeiss EM 9 S-2 and Hitachi H-600 transmission electron microscopes, and data were recorded on DuPont COS-7 Graphic Arts Film.

Thick sections. Thick sections of embedded cells were examined to determine the quality of the fixation and the position of the cells in the block. Sections, 0.5-1.0 µm thick, were cut with glass knives on a Sorvall (Porter-Blum) MT-1 ultramicrotome. The sections were flattened with xylene vapors, picked up with a glass loop and transferred to clean glass slides. Sections were subsequently stained with either methylene blue or toluidine blue and examined by light microscopy.

Whole mounts. Cell-free lorica preparations (see analytical electron microscopy section) were air dried on formvar-coated 300-mesh copper grids. Whole mounts were examined by transmission electron microscopy as previously described.

Ruthenium Red (RR) staining for acid mucopolysaccharides. Cells were concentrated by gentle centrifugation and then suspended in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.1, plus 0.1% RR and fixed for one hour at 4°C. After several buffer rinses, cells were post-fixed in 2% OsO₄ in 0.05 M sodium cacodylate buffer plus 0.1% RR for one hour. After buffer rinses, the cells were processed for electron microscopy by conventional methods. All fixation and dehydration steps were carried out in the dark at 4°C.

Alcian Blue (AB) staining for acid mucopolysaccharides. Cells were concentrated by gentle centrifugation and suspended in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.1, with Alcian Blue 8GX added to a concentration of 1%. Cells were fixed in this solution for one hour at 4°C, washed in buffer and post-fixed with 2% OsO₄ in 0.05 M sodium cacodylate buffer, pH 7.1, for one hour. Cells were then dehydrated and embedded according to standard procedures. All fixation and dehydration steps were carried out in the dark at 4°C.

Scanning Electron Microscopy (SEM)

Cells were collected by centrifugation and washed three times with media. The final pellet of cells was resuspended in 1% OsO₄ in 0.05 M sodium cacodylate buffer, pH 7.1, for 5-15 minutes at 4°C. Cells

were often covered by a heavy coating of mucilage that obscured cellular detail. The mucilage was removed by treating the cells either before or after fixation with the snail gut enzyme, glusulase, at a concentration of 2% for one hour. A drop of fixed cells was transferred to a poly-D-lysine-coated 12-mm round cover glass (Mazia et al., 1975). After one minute, the cover glasses were submerged in 10% acetone in a petri dish. All subsequent dehydration steps were done in this manner, with care taken not to let the cover slip dry out. After a dehydration series in acetone, the cover glasses were transferred to a 13-mm cover glass holder (Bomar Co.), which was then placed in an acetone-filled chamber of a Bomar critical point drying apparatus (Model No. SPC-900/EX). Cells were critical point dried (CPD) at 31°C and 1072 psi, using liquid CO₂ as a replacement liquid.

The cover glasses were mounted with double-stick tape on an aluminum SEM stub and coated with ca. 30 nm of gold/palladium (60/40) in a Denton Vacuum Evaporator (Model DV 515) at 6×10^{-5} torr. Specimens were examined in either an ETEC Autoscan or AMR-900 scanning electron microscope at an accelerating voltage of 20 kV. Photographic data were recorded on Polaroid Type 55 or 105 P/N film.

3. ANALYTICAL METHODS

Analytical Electron Microscopy

Loricas harvested from the bottoms of culture vessels were used for elemental analyses. Dark loricas were harvested from soil-water cultures and colorless loricas were harvested from culture tubes containing a mixture of Chu #10 defined medium and soil-water extract in

a ratio of 1 to 9. This mixture was especially useful for this technique since empty loricas accumulated in large amounts at the bottom of these tubes and could be gathered with little contamination from other cellular debris and soil.

All loricas were collected with Pasteur pipettes, concentrated by centrifugation and washed repeatedly with distilled water. The loricas were then treated with EDTA, EGTA or left untreated. They were subsequently air-dried on spectroscopically pure SEM graphite stubs. At least 5 loricas were routinely analyzed for elemental composition by scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS) in an AMR-900 scanning electron microscope equipped with an Ortec X-ray detector, a model 6200 multichannel analyzer and a Hewlett Packard X-Y recorder.

Demineralization. To study the metals bound to the lorica matrix, isolated loricas were treated with several demineralizing agents. These included 10 mM ethylenedinitrilotetraacetic acid (EDTA) and 1 mM ethyleneglycol-bis-(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA). Isolated dark loricas were mixed with 2 ml of one of the above in a 13 X 100 mm test tube at room temperature. At five minute intervals, samples were removed and observed with a light microscope and photographed as described above.

Cytochemistry

Several cytochemical dyes were used in this study to determine possible cellular and/or lorica components: 1) Alcian Blue, 2) Alcian Yellow, 3) Mercuric Bromphenol Blue, 4) Methylene Blue, 5) Potassium

Iodide, 6) Toluidine Blue, 7) Ruthenium Red, 8) Potassium Ferrocyanide and 9) PAS. See Appendix C for a summary of the uses and methods of preparation of these stains. Whole cells were fixed with glutaraldehyde, washed with buffer and stained in a 13 X 100 mm test tube. Loricas were isolated from culture vessels (see section on analytical microscopy for details) and stained after adhesion to slides with poly-D-lysine (Appendix C). Stained cells or loricas were photographed with Kodak Ektachrome tungsten film (ASA 160).

Enzyme Treatments

Cells were treated with two enzymes, trypsin and protease, to examine their effects on lorica structure. The digestion procedure is a modification of that described by Oliveira et al. (1980).

Cells cultured in MVM or SW were concentrated by gentle centrifugation and washed with 0.05 M tris-HCl buffer, pH 7.6. The cells were then incubated in one of the following: 1) 0.1 mg trypsin (Type III, Sigma) per ml of 0.05 M tris-HCl buffer, pH 7.6, for 45-60 minutes at 37°C, 2) 0.1 mg protease per ml 0.05 M tris-HCl buffer, pH 7.6, for 15-30 minutes at 37°C or 3) 0.05 M tris-HCl buffer at 37°C for the appropriate time (control treatment). After enzyme digestion, cells were washed with tris buffer and then processed for electron microscopy by conventional methods. Alcian Blue was added to the fixative to enhance staining of acid mucopolysaccharides. Alternatively, some cells were fixed prior to enzyme digestion and post-fixed after the digestion.

Isolation of Extracellular Mucilage (ECM)

Extracellular mucilage was usually isolated by an ethanol precipitation technique. This procedure is a modification of that described by Lewin (1956) and Evans et al. (1974).

Cells were harvested from the culture by centrifugation. The supernatant was filtered first with Millipore RA filters (mean pore size: 1.2 μm) and then re-filtered with Millipore HA filters (mean pore size: 0.45 μm). Occasionally, the supernatant was then dialyzed against distilled water for 24-48 hours; however, this step was usually omitted. The cell-free supernatant was concentrated and added to 3 volumes of cold ethanol. The resulting precipitated ECM was recovered by centrifugation, dried in a vacuum desiccator and stored over Drierite. See Appendix D for complete isolation protocol.

Mucilage was also recovered by the cetylpyridinium chloride (CPC) precipitation method (Scott, 1960; Ramus, 1972) (Appendix D). This technique was not routinely used, however, since hydrolysates of this material did not chromatograph as well as those of the ethanol precipitates.

Hydrolysis Procedures

Several methods of hydrolysis were routinely employed in this study. The choice of a particular method depended upon the hydrolysate product desired. For sugar analyses, 3-10 mg of ECM or isolated loricas were hydrolyzed in 1 N H_2SO_4 for one hour at 100°C, in 2 N trifluoroacetic acid (TFA) for one hour at 100°C or in 2 N TFA for one hour at 121°C (in an autoclave). For amino acid analyses, 10 mg of ECM

or loricas were hydrolyzed in a sealed tube with 6 N HCl at 138°C for 6 hours. For sulfate analyses, 20 mg of ECM or isolated loricas were hydrolyzed in 6 N HCl for 3 hours at 100°C. The resulting hydrolysate was deionized with Dowex 50 ion exchange resin before use. For complete details of hydrolysis procedures, see Appendix E.

Thin-Layer Chromatography (TLC)

Neutral sugars. Neutral sugars in the extracellular mucilage and lorica hydrolysates were separated and identified on layers of MN cellulose (thickness: 0.1 mm). Plates were developed with one of the following solvent systems: 1) System I--butanol:pyridine:water (6:4:3, by volume), 2) System II--ethyl acetate:pyridine:water (125:25:20, by volume, and 3) System III--isopropanol:water (80:20, by volume). See Appendix F for information on individual solvent systems. Sugar standards were run parallel to the hydrolysates to confirm the identities of unknown sugars. After development, plates were removed from the tank and air-dried in a hood. Sugars were detected with several spray reagents: anthrone (Kodak Technical Bulletin JJ-3), aniline phosphate (Kirchner, 1978) and p-anisidine hydrochloride (Kodak Technical Bulletin JJ-3). For use and preparation of spray reagents, see Appendix F.

Uronic acids. Hydrolysates of ECM were chromatographed on MN cellulose for separation of uronic acids. A solvent system of ethyl acetate:pyridine:water:acetic acid (5:5:3:1, by volume) (Solvent System IV, Appendix F) was effective in separating uronic acids from neutral

sugars. Uronic acids were visualized with either ethyl acetate-aniline-trichloroacetic acid or basic lead acetate (Gee and McCready, 1957) (Appendix F).

Amino acids. Two dimensional thin-layer chromatography of HCl-hydrolyzed material was used to separate and identify amino acids. Hydrolysates were chromatographed on MN cellulose plates. Development in the first dimension was with Solvent System V (chloroform:methanol:acetone:butanone:NH₄OH--80:80:20:10:30) (Appendix F) and in the second dimension with Solvent System VI (butanol:acetic acid:water--4:1:1) (Appendix F). Amino acids were detected after development with ninhydrin (Jones, 1962).

Gas-Liquid Chromatography (GLC)

Portions (0.5-1.0 ml) of dried, hydrolyzed ECM or loricas were derivitized to their trimethylsilyl (TMS) ethers with a silylating agent, TRI-SIL (Pierce Chemical Co.). See Appendix G for details of procedure. The samples were examined with a Bendix gas liquid chromatograph (Model GC-2600) equipped with a flame ionization detector. The stainless steel column (6' X 1/4") was packed with 3% SE-52 on 40-100 mesh Chromosorb W-AW. All samples were run at an oven temperature of 160°C, a detector temperature of 240°C and an inlet temperature of 250°C. The gas flow rates of all runs were: hydrogen, 80-100 cc/min.; helium, 50 cc/min. and air, 800 cc/min.

Combined Thick-Layer Chromatography-Gas Liquid Chromatography (THC-GLC)

Thick-layer chromatography was employed to separate larger amounts of hydrolysates into component sugars than could be separated on

thin layer plates. Pre-coated 500 μ m and 1000 μ m MN cellulose or Avicel plates were used for chromatography of ECM hydrolysates. Solvent System I (Appendix F) was used to develop the plates, and one lane was visualized with anisidine hydrochloride to note positions of separated sugars. Sugar bands from the other unsprayed lanes were then scraped from the plates and eluted from the adsorbant with methanol. The methanol was evaporated from the samples; they were subsequently derivitized and analyzed by gas liquid chromatography.

Colorimetric Analyses

Carbohydrates in the culture supernatant were assayed by either the anthrone method (Yemm and Willis, 1954) or the phenol-sulfuric acid method (Dubois et al., 1956) with galactose and/or arabinose as standards. Proteins were assayed with the Bio-Rad Protein Assay (Bio-Rad Laboratories), using BSA as a standard. Uronic acids were determined by the method of Ahmed and Labavitch (1977) with galacturonic acid as a standard. Hydroxyproline content was estimated by the method of Leech (1960), using 6 N HCl hydrolysates. Sulfate content was determined by the method of Lloyd (1959) using deionized hydrolysates and K_2SO_4 as a standard. See Appendix H for a complete description of all procedures.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten mg of isolated extracellular mucilage were suspended in 5 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate (SDS) and 1% β -mercaptoethanol. The mucilage was digested by heating at 37°C for 2 hours or at 100°C for 2 minutes, followed by gentle

centrifugation for 5 minutes. The supernatant was used as the sample for electrophoresis.

SDS-polyacrylamide gels were prepared according to procedures outlined by Weber and Osborn (1969), Davies (1972) and Lang and Chrispeels (1976) (see Appendix I). Gels were cast into 13-cm glass tubes with an inner diameter of 5 mm. The 7.5% gels were run without stacking gels at 5-8 mA/gel for 2-5 hours. After electrophoresis, protein bands were located by staining with 0.25% Coomassie Blue in 7.5% acetic acid for at least one hour, followed by destaining with 7.5% acetic acid. The presence of carbohydrates was confirmed by the PAS procedure (Zacharius et al., 1969; Holden et al., 1971). Gels were washed well with 7.5% acetic acid for 30 minutes followed by a distilled water rinse. After oxidation of the gels for 50-60 minutes with 1% periodic acid in 7.5% acetic acid, the gels were washed 6 times for 10 minutes each in distilled water. They were subsequently stained with Schiff's reagent in the dark for 50 minutes and washed with 3 changes of 0.5% metabisulfite for 10 minutes each. The gels were then washed with distilled water until all excess stain was removed. All gels were stored in screw-cap test tubes in 7.5% acetic acid.

Densitometer scans of the gels were made in a Beckman DU spectrophotometer with a Gilford gel scanner at 665 nm for Coomassie Blue-stained proteins and at 550 nm for PAS-stained carbohydrates.

Amino Acid Analysis

Samples were prepared for amino acid analysis by hydrolysis in 6 N HCl for 24 hours at 110°C in an evacuated, sealed hydrolysis tube.

Automated analysis was performed on a Beckman 116 Amino Acid Analyzer with a single column system.

Ultrastructural Cytochemistry

Two ultrastructural cytochemical staining procedures, periodic acid-silver hexamine (PASH) (Pickett-Heaps, 1967; Rambourg, 1967; Triemer, 1980) and periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) (Thiery, 1967) were used to localize carbohydrates in the cytoplasm, in the lorica and in the extracellular mucilage.

Periodic Acid-Silver Methenamine (PASH). Light gold-to-silver thin sections (90-120 nm) of glutaraldehyde- OsO_4 -fixed cells were floated onto 1% aqueous periodic acid in a staining dish by means of small plastic rings (Pickett-Heaps, 1967) and were oxidized for 30 minutes at room temperature. All subsequent transfers were done with the plastic rings. Sections were rinsed three times with distilled water and transferred to the staining solution, which consisted of 3% methenamine, 15 ml; 2% sodium borate, 25 ml; 1% silver nitrate, 5 ml; distilled water, 5 ml. Staining was accomplished at 60°C in the dark for 45-60 minutes or until the sections turned light brown. At that time, they were removed from the staining solution and washed with distilled water. Sections were then treated with 5% sodium thiosulfate for 1-2 hours to remove unreduced silver ions, followed by a distilled water wash. For controls periodic acid was omitted (Triemer, 1980) or dimedone, sodium bisulfite or iodoacetate blocking of aldehyde groups was employed (Pickett-Heaps, 1967). Following treatment, the sections were floated away from the plastic rings, mounted on 400-mesh

copper grids and examined by transmission electron microscopy without uranium or lead post-stains.

Periodic Acid-Thiocarbohydrazide-Silver Proteinate (PA-TCH-SP).

Light gold-to-silver thin sections (90-120 nm) of glutaraldehyde-OsO₄-fixed cells were floated onto 1% periodic acid for 30 minutes (see PASH section for technique) followed by a distilled water wash.

Sections were transferred to a solution of 0.2% thiocarbohydrazide in 20% acetic acid for 1, 2 or 24 hours. Sections were washed with 10% and then 5% acetic acid over a period of 30 minutes and then finally washed three times with distilled water. They were subsequently floated on aqueous 1% silver proteinate for 30-40 minutes in the dark, followed by a final rinse in distilled water. For controls, periodic acid, TCH or silver proteinate was omitted from the procedure (Courtoy and Simar, 1974). Sections were mounted and examined as for the PASH technique.

CHAPTER III

RESULTS

1. EFFECTS OF CULTURE CONDITIONS ON GROWTH AND CELLULAR AND LORICA MORPHOLOGY

General Cellular Morphology

The cell of Phacotus lenticularis is ovoid to spherical in shape, ca. 10 μm long and 8 μm wide, with two isokont flagella that are often twice as long as the protoplast itself (Plate I, Figures 1 and 2). With light microscopy, a centrally located nucleus and a single cup-shaped chloroplast with one to six pyrenoids, many starch grains and a latero-anterior stigma are visible.

Cells of P. lenticularis are surrounded by a non-living, bivalved wall-like structure called a lorica, which is ovoid to spherical in shape and may be laterally compressed. The lorica is usually separated from the protoplast but the distance between the two is highly variable. Loricas may be colorless or brown and are highly variable with respect to size, shape and color (Plate II, Figures 3-6). Because of the shape of the lorica, cells usually exhibit a twisting or spiral swimming movement.

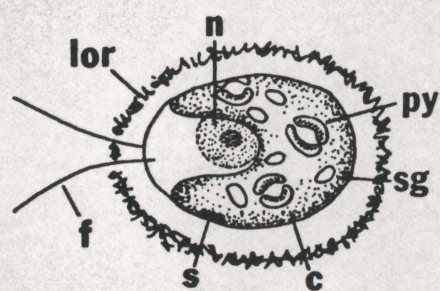
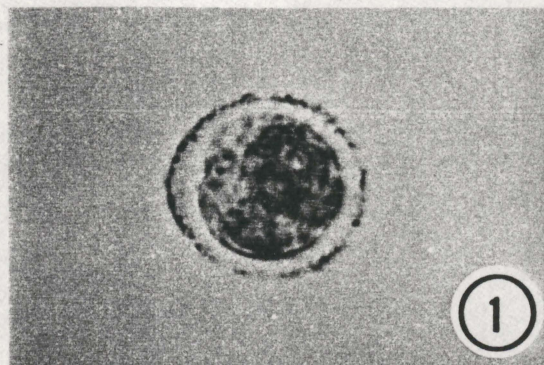
Cells Cultured in Soil-Water Medium

Loricas from cells cultured in soil-water medium have a variety of forms (Plate III, Figures 7-11). Cells grown in soil-water medium do not attain as high a density in culture as do cells grown in some

Plate I. General Cellular Morphology of Phacotus lenticularis.

Figure 1. Typical cell isolated from soil-water culture. Blue color of lorica is due to methylene blue stain. LM. Bright-field illumination. X 2100.

Figure 2. Diagrammatic drawing of the cell of Phacotus lenticularis. f, flagellum; n, nucleus; lor, lorica; sg, starch grain; py, pyrenoid; s, stigma; c, chloroplast.



2

Plate II. Variability of Lorica Morphology.

Figure 3. Colorless loricas which are considerably larger than the protoplast. Loricas are laterally compressed and have a prominent rim where the valves join. LM. Bright-field illumination. X 2100.

Figure 4. Dark loricas isolated from soil-water medium. LM. Bright-field illumination. X 800.

Figure 5. Cell with an ornamented brown lorica. Lorica is closely appressed to the cell. LM. Bright-field illumination. X 2100.

Figure 6. Empty, colorless loricas which have no obvious surface ornamentation. LM. Bright-field illumination. X 800.

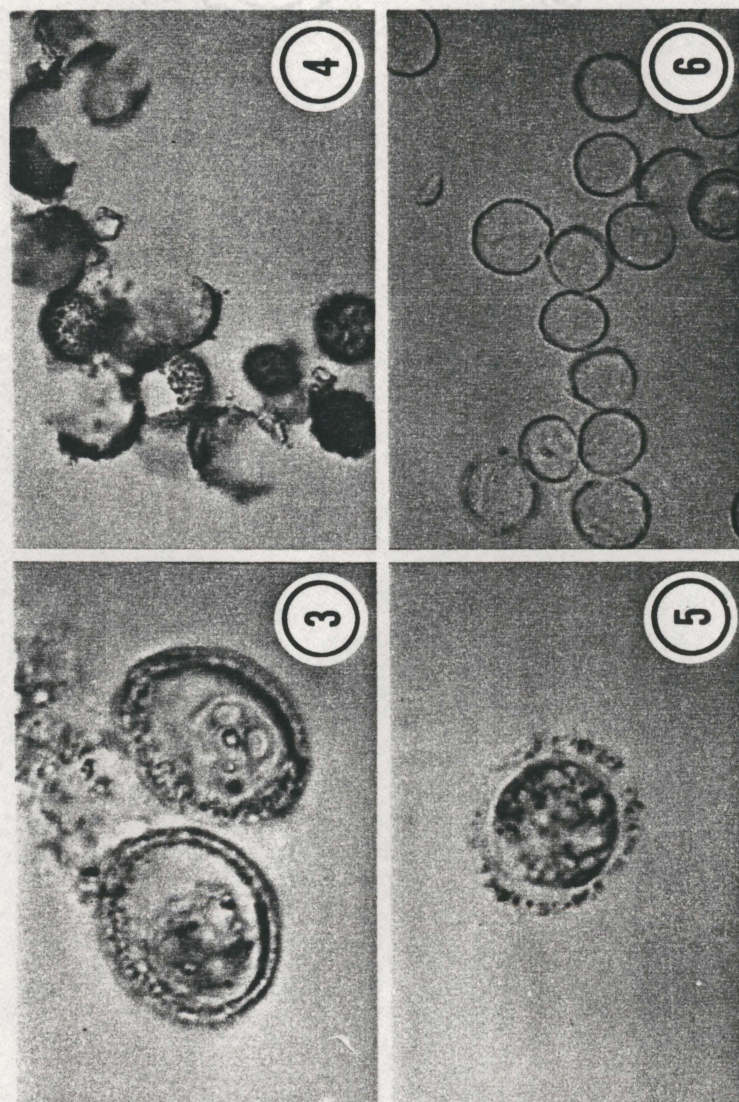


Plate III. Morphology of Loricas from Soil-Water Cultures.

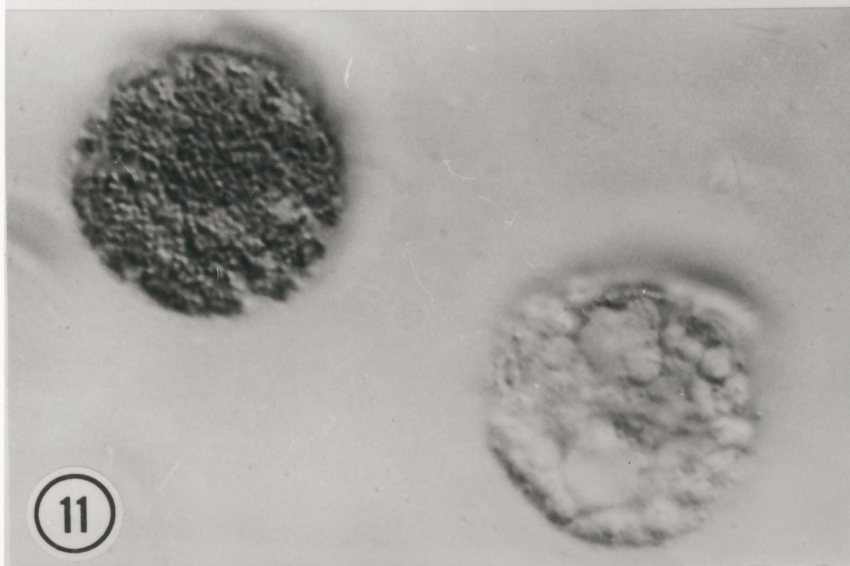
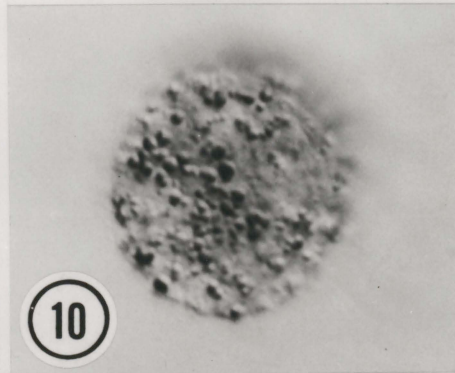
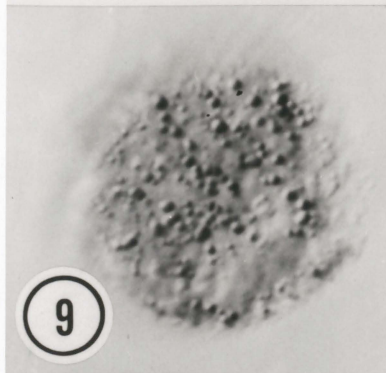
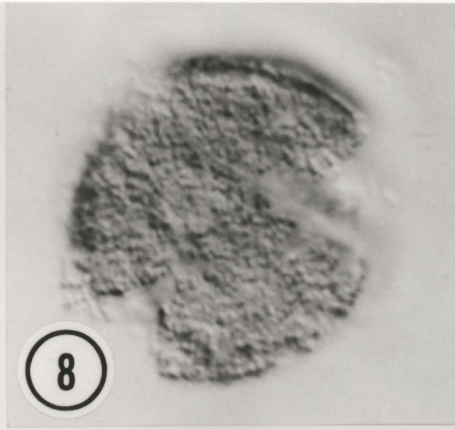
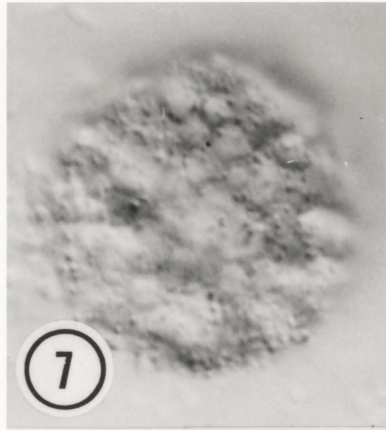
Figure 7. A thin, colorless lorica with scattered blunt protrusions on the surface. Starch grains visible in the underlying cell. LM. Oblique illumination. X 2800.

Figure 8. Fragmented, dark lorica with even distribution of blunt surface projections. LM. Oblique illumination. X 2800.

Figure 9. Dark loricas with many large globular surface projections. LM. Oblique illumination. X 2800.

Figure 10. Surface detail of dark lorica, showing blunt projections. LM. Oblique illumination. X 2800.

Figure 11. Comparison of dark lorica (left) and thin, colorless lorica (right). Dark lorica is highly ornamented and obscures all underlying cellular detail; intracellular starch grains visible through the colorless lorica. LM. Oblique illumination. X 2800.



defined media and tend to accumulate large amounts of starch. These cells may be maintained in a stationary phase for up to one year and for longer periods if the distilled water overlay is replenished.

Loricas of cells cultured in soil-water medium are highly variable. Young cultures bear loricas which are usually colorless and closely appressed to the protoplast. These loricas are thin and pliable; cellular detail is obvious. Young loricas are sparsely ornamented with blunt surface projections which are scattered across the surface (Plate III, Figures 7 and 11). The color of the loricas changes to a dark brown, after several months in culture. Color change is rapid, as there are few or no intermediate color types (such as golden or light brown) present in the cultures. Dark loricas are brittle and have more ornamentation than colorless loricas (Plate III, Figures 8-10). These loricas are generally more remote from the protoplast than those found on younger cells, but this is not always so.

Cells Cultured in Defined Medium

In an attempt to find a suitable medium which would later facilitate analytical investigations, several defined media were tested. Of all media selected for trial, two gave consistently good growth: Chu #10 and a modified Volvox medium (MVM). MVM was chosen for all subsequent work since lag phase was shorter in MVM than in Chu #10 (Figure 12).

Cells grown in MVM have a different morphology from those grown in soil-water medium (Plate IV, Figure 13). MVM cells never accumulate starch and they become non-motile as they age. After 3-4 weeks, the

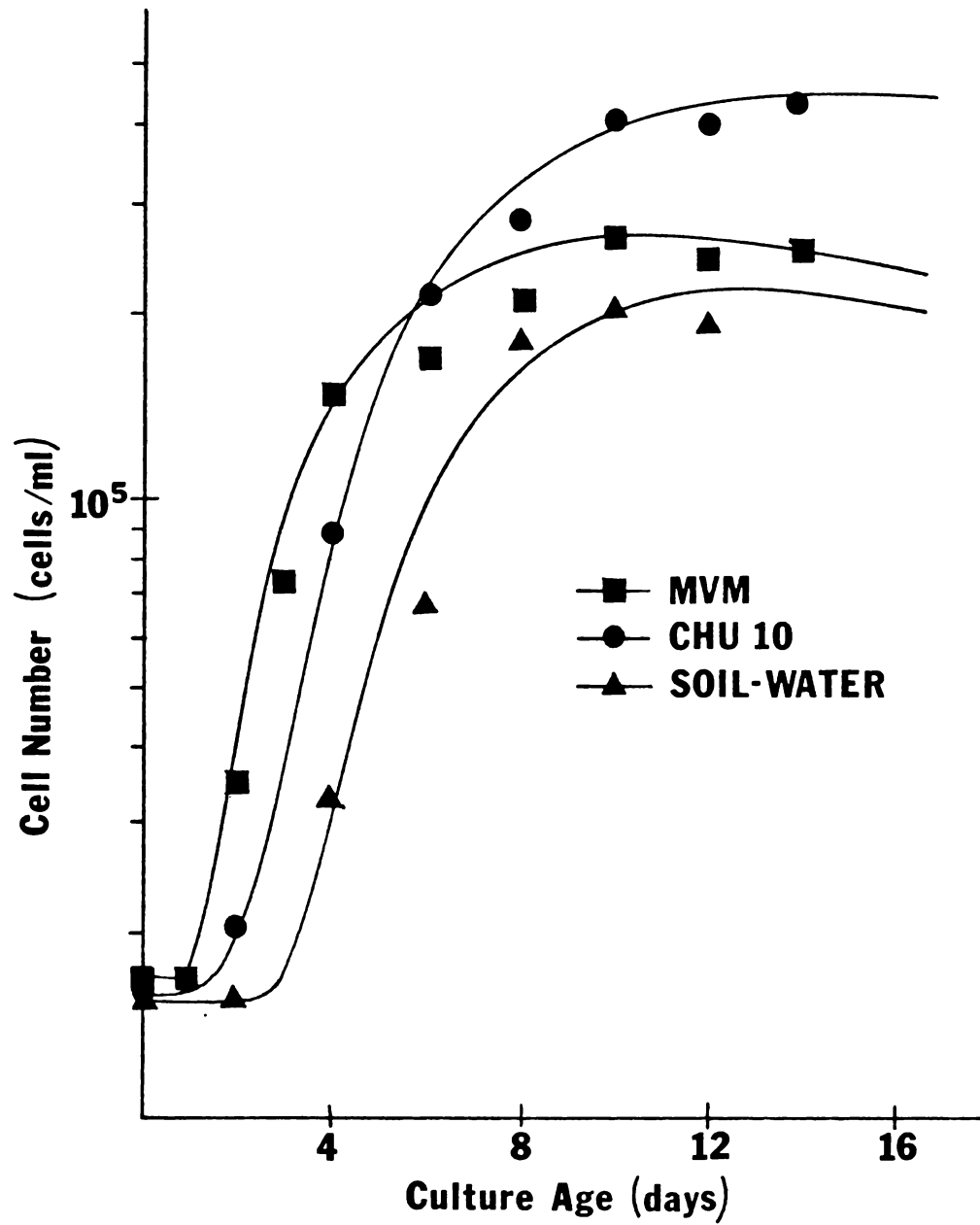
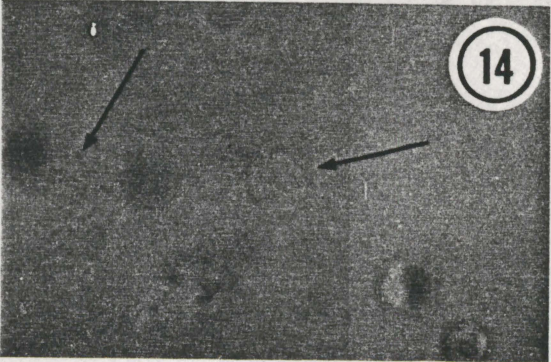
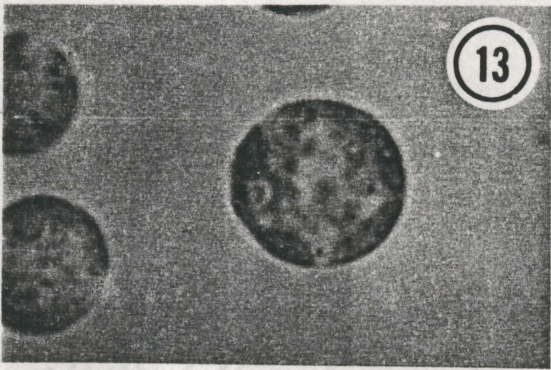


Figure 12. Growth curves of *Phacotus lenticularis* in defined and soil-water media. Cell numbers are greatest in defined media.

Plate IV. Appearance of Cells Grown in Defined Medium.

Figure 13. Cell grown in MVM medium. No lorica is obvious at the light-microscopic level. LM. Bright-field illumination. X 2100.

Figure 14. Thin, colorless loricas (arrows) found at the bottom of MVM culture flasks. LM. Bright-field illumination. X 800.



number of cells begins to decline and the remaining cells are large and highly vacuolate. No lorica is obvious at the light microscope level, although thin, colorless loricas are occasionally found at the bottom of culture vessels (Plate IV, Figure 14).

Cells Cultured in Defined Medium/Soil-Water

In an attempt to increase growth in soil-water cultures, the distilled water overlay was replaced with MVM. The resulting cells were identical to those grown in MVM alone; no cells resembling those typically found in soil-water cultures were seen.

Combinations of various defined media and soil-water extract (SWE) were examined for their effects on cell growth and morphology. Data compiled from these experiments are shown in Table IV and indicate that certain media effect mass shedding of loricas; these media were later used when large amounts of loricas were needed for chemical analyses.

Cells Cultured in Mineral-Supplemented Soil-Water

Mineral supplements were added to soil-water medium to test the effects of particular nutrient additions on cell growth, starch accumulation and lorica morphology. In preliminary experiments, mineral supplements were in the form of individual nutrient stocks used in MVM medium. It was hoped that by using single MVM components some clue could be found as to why cells behaved differently in soil-water and MVM media.

Results of these experiments are shown in Table V. All mineral supplements produced cells resembling those grown in soil-water alone

TABLE IV
EFFECT OF COMBINATIONS OF SOIL-WATER EXTRACT/DEFINED
MEDIUM ON LORICA MORPHOLOGY

Medium	Lorica Morphology
MVM/SWE	
9:1	Colorless, well-defined loricas; many empty loricas on bottom of test tube.
1:1	Thin, colorless loricas.
1:9	Thin, colorless loricas.
Chu/SWE	
9:1	Some well-defined loricas visible; most thin and colorless.
1:1	Colorless, well-defined loricas; many empty loricas on bottom of test tubes.
1:9	Colorless, well-defined loricas; many empty loricas on bottom of test tubes.

TABLE V
CHARACTERISTICS OF MINERAL-SUPPLEMENTED SOIL-WATER CULTURES

Mineral Supplement	Growth ¹	Starch Accumulation	Loricas Visible
None	+	+	+
Complete MVM	++++	-	-
Ca(NO ₃) ₂	++++	-	-
MgSO ₄	+	+	+
Sodium Glycero-phosphate	+	+	+
KCl	+	+	+
Vitamins	+	+	+
Trace Elements	+	+	+

¹See Appendix J for growth criteria.

except those cultures containing a $\text{Ca}(\text{NO}_3)_2$ supplement. These cells were identical to those grown in MVM, with rapid growth and no visible lorica or starch accumulation.

Cells grown in mineral-supplemented medium which resembled the cells cultured in soil-water were also examined for lorica color. These data are summarized in Table VI. Various supplements effect a range of dark:light ratios. Only dark loricas are found in cultures with no supplement, whereas in cultures supplemented with complete MVM, all cells have colorless loricas.

Cells cultured in soil-water were further tested with specific nutrient supplements which gave promising results in preliminary experiments. The addition of individual nitrogen and calcium supplements was indicated by the fact that the addition of $\text{Ca}(\text{NO}_3)_2$ to soil-water caused a radical change in cell morphology. The results of these experiments are presented in Table VII. These data indicate that it is the nitrogen, not calcium, which causes the cells to grow differently from those in soil-water.

Cells Cultured in Nitrogen-Supplemented Medium

Since preliminary experiments indicated that the appearance of the lorica was influenced by the presence of nitrogen, various nitrogen sources were tested as supplements to both soil-water and defined media. These cultures were monitored in 2-, 3- and 4-week intervals for growth, starch accumulation and lorica appearance.

Data from MVM/N-supplemented cultures are presented in Table VIII. Cells cultured in soil-water medium inoculated into these media

TABLE VI
RELATIVE ABUNDANCE OF LIGHT AND DARK LORICAS IN
MINERAL-SUPPLEMENTED SOIL-WATER CULTURES

Mineral Supplement	% Dark Loricas	% Light Loricas	Ratio, Dk:Lt Loricas
None	100	0	all dark
MVM Vitamins	12	88	1:7
MVM Trace	44	56	1:1
KCl	27	73	1:3
Sodium Glycero-phosphate	20	80	1:4
MgSO ₄	13	87	1:7
Ca(NO ₃) ₂	0	100	all light

TABLE VII
CHARACTERISTICS OF NITROGEN- AND CALCIUM-SUPPLEMENTED
SOIL-WATER CULTURES

Mineral Supplement	Growth ¹	Starch Accumulation	Loricas Visible
None	+	+	+
Ca(NO ₃) ₂	++++	-	-
CaCl ₂	+	+	+
NaNO ₃	++++	-	-

¹See Appendix J for growth criteria.

TABLE VIII
CHARACTERISTICS OF NITROGEN-SUPPLEMENTED MVM CULTURES

Supplement	Starch Accumulation			Growth ¹			Lorica Morphology ²		
	2 Wk	3 Wk	4 Wk	2 Wk	3 Wk	4 Wk	2 Wk	3 Wk	4 Wk
-N	-	-	-	+	0	0	T,C	T,C	T,C
Ca(NO ₃) ₂	-	-	-	++++	++++	++++	T,C	T,C	T,C
NaNO ₃	-	-	-	++++	++++	++++	T,C	T,C	T,C
KNO ₃	-	-	-	++++	++++	++++	T,C	T,C	T,C
NaNO ₂	-	-	-	++	+++	+++	T,C	T,C	T,C
(NH ₄) ₂ SO ₄									
NH ₄ NO ₃	-	-	-	+++	++	++	T,C	T,C	T,C
(NH ₄) ₂ HPO ₄	-	-	-	+++	+++	+++	T,C	T,C	T,C
CONH ₂	-	-	-	+++	++	+	T,C	T,C	D,C
Casein Hydrolysate	-	-	-	0	0	0	-	-	-

¹See Appendix J for growth criteria.

²T,C = Thin, Colorless.

D,C = Well-defined, Colorless.

retained visible loricas only in the urea-supplemented cultures. None accumulated starch during the course of the four-week period. Growth varied in the different nitrogen sources, with little or no growth in NaNO_2 and casein hydrolysate. The most luxuriant growth was achieved in $\text{Ca}(\text{NO}_3)_2$, NaNO_3 and KNO_3 .

Results from SW/N-supplemented cultures are presented in Table IX. At the end of four weeks, loricas were visible in control cultures as well as in $(\text{NH}_4)_2\text{SO}_4$ and urea-supplemented cultures. A few cells with fragmentary loricas were recorded from the four-week-old $\text{Ca}(\text{NO}_3)_2$ and NaNO_3 -supplemented cultures. Starch was seen only in control cells.

Effect of Bacteria on Growth of Cultures

The growth rate of axenic cultures of Phacotus lenticularis is much different from that seen in bacterially contaminated cultures (Figure 15). In addition to greater cell numbers, lag phase is greatly reduced in cultures with bacteria. Doubling time in cultures with bacteria is approximately 24 hours, compared with 84 hours in axenic culture cultures. Removal of bacteria from the cultures slows the growth rate and reduces cell numbers.

2. DESCRIPTIVE RESULTS

General Cellular Organization

Transmission electron microscopy (TEM) also shows the typically chlamydomonad internal organization of Phacotus lenticularis (Plate V, Figure 16). A cup-shaped chloroplast, which occupies the peripheral portion of the cell, contains several pyrenoids, each surrounded by two

TABLE IX
CHARACTERISTICS OF NITROGEN-SUPPLEMENTED SW CULTURES

Supplement	Starch Accumulation			Growth ¹			Lorica Morphology ²		
	2 Wk	3 Wk	4 Wk	2 Wk	3 Wk	4 Wk	2 Wk	3 Wk	4 Wk
-N	-	+	+	+	+	++	T,C	D,C	D,B
Ca(NO ₃) ₂	-	-	-	+++	+++	+++	T,C	T,C	D,C
NaNO ₃	-	-	-	+++	+++	+++	T,C	T,C	D,C
KNO ₃	-	-	-	+++	+++	+++	T,C	T,C	T,C
NaNO ₂	-	-	-	0	0	0	-	-	-
(NH ₄) ₂ SO ₄	-	-	-	+++	+++	+++	T,C	D,C	D,B
NH ₄ NO ₃	-	-	-	++++	++++	+++	T,C	T,C	T,C
(NH ₄) ₂ HPO ₄	-	-	-	+	+	+	D,C	D,C	D,C
CONH ₂	-	-	-	+++	+++	+++	T,C	D,C	D,C
Casein Hydrolysate	-	-	-	0	0	0	-	-	-

¹See Appendix J for growth criteria.

²T,C = Thin, Colorless.

D,C = Well-defined, Colorless.

D,B = Well-defined, Brown.

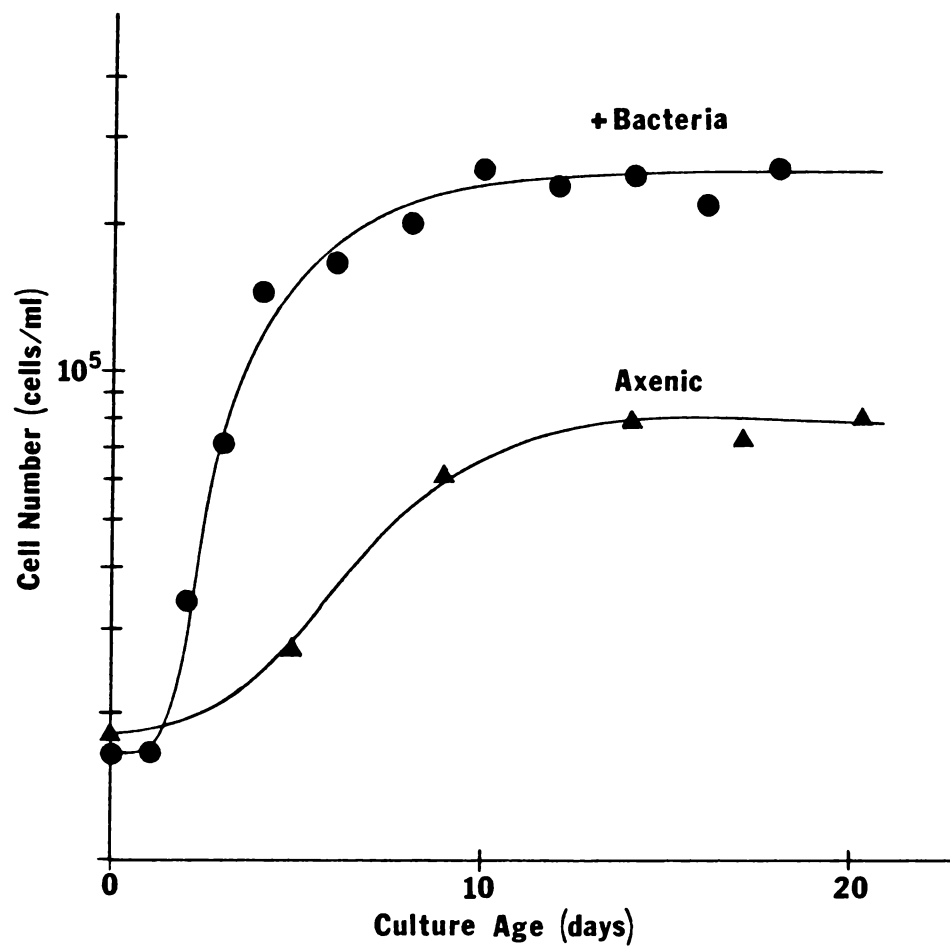
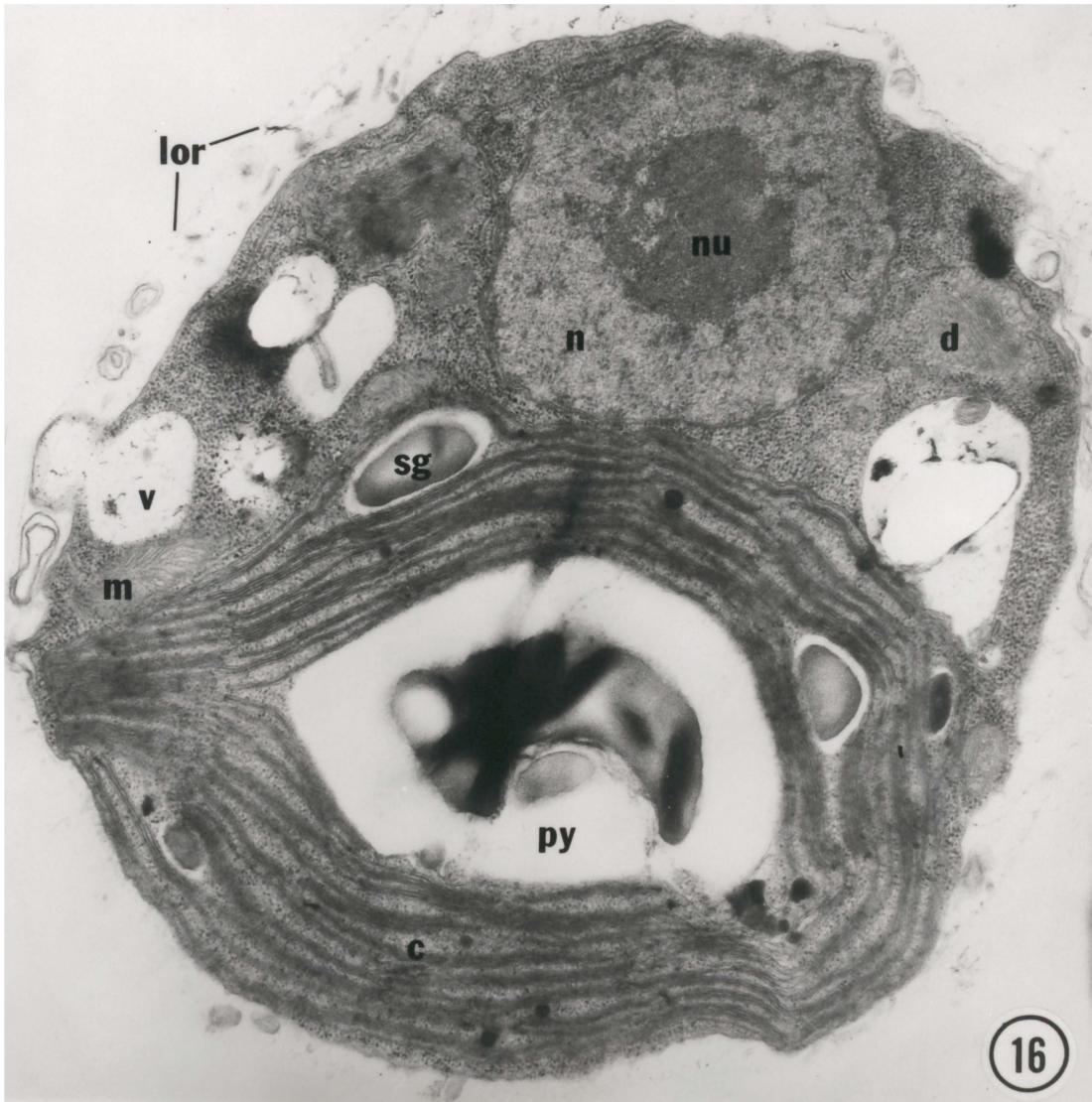


Figure 15. Growth curves of *Phacotus lenticularis* in the presence and absence of bacteria in the culture medium. The medium used in this study was MVM.

Plate V. General Ultrastructure of the Cell.

Figure 16. Oblique section through posterior end of vegetative cell shows typical organelles and barely visible lorica. TEM thin section. X 19,000.



large starch plates. The nucleus is located in a central to slightly anterior position, while mitochondria, dictyosomes and endoplasmic reticulum profiles are seen scattered in the cytoplasm. The cell is surrounded by a non-living covering, the lorica.

Cellular Fine Structure

Nucleus and endoplasmic reticulum. The nucleus is variable in shape, from round to elongate and lobed. The interphase nucleus contains a prominent, darkly staining nucleolus (Plate VI, Figure 17) and is bounded by a nuclear membrane which is perforated by many nuclear pores and studded with ribosomes on the cytoplasmic surface of the outer membrane (Plate VI, Figure 19).

Chromatin condenses during cell division into discrete, darkly staining chromosomes (Plate VI, Figures 18 and 20). The nuclear membrane presumably persists throughout mitosis.

The nuclear membrane is often continuous at one or more places with a profile of endoplasmic reticulum (ER). ER is also frequently found in association with the forming face of dictyosomes or in close proximity to the plasma membrane (Plate VII, Figures 21 and 22). The association of the nuclear membrane, ER and Golgi apparatus is commonly seen in the Volvocales and is termed an amplexus (Lang, 1965).

Dictyosomes. Dictyosome profiles are common throughout the cell but are often seen near the nucleus and in conjunction with secretory activity (Plate VII, Figure 23). Dictyosomes consist of eight to twenty cisternae, which are often curved toward the maturing face and are sometimes swollen and associated with small secretory vesicles.

Plate VI. Nucleus.

Figure 17. Typical interphase nucleus with darkly stained nucleolus. Note associated ER and dictyosomes. TEM thin section. X 25,800.

Figure 18. Prophase nucleus with areas of condensed chromatin. TEM thin section. X 25,800.

Figure 19. Section through portion of nuclear membrane showing two nuclear pores. TEM thin section. X 73,500.

Figure 20. Metaphase nucleus with seven chromosomes visible in the center of the nucleus and intact nuclear membrane. TEM thin section. X 28,700.

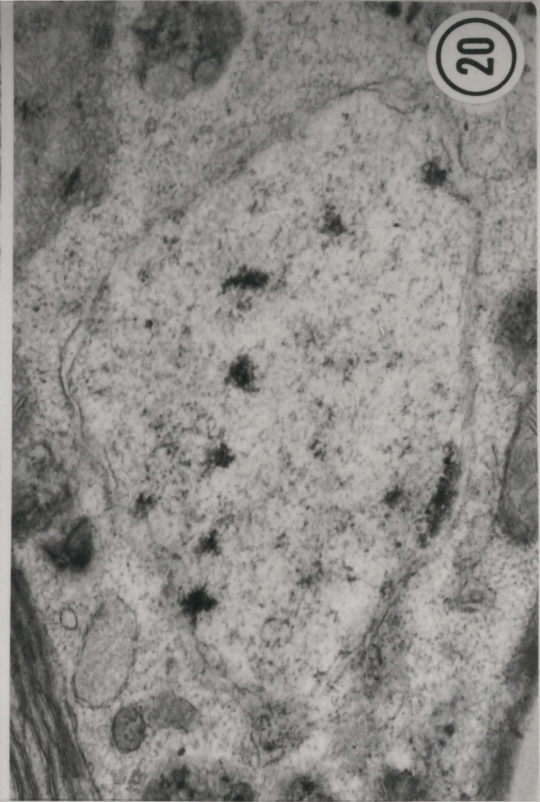
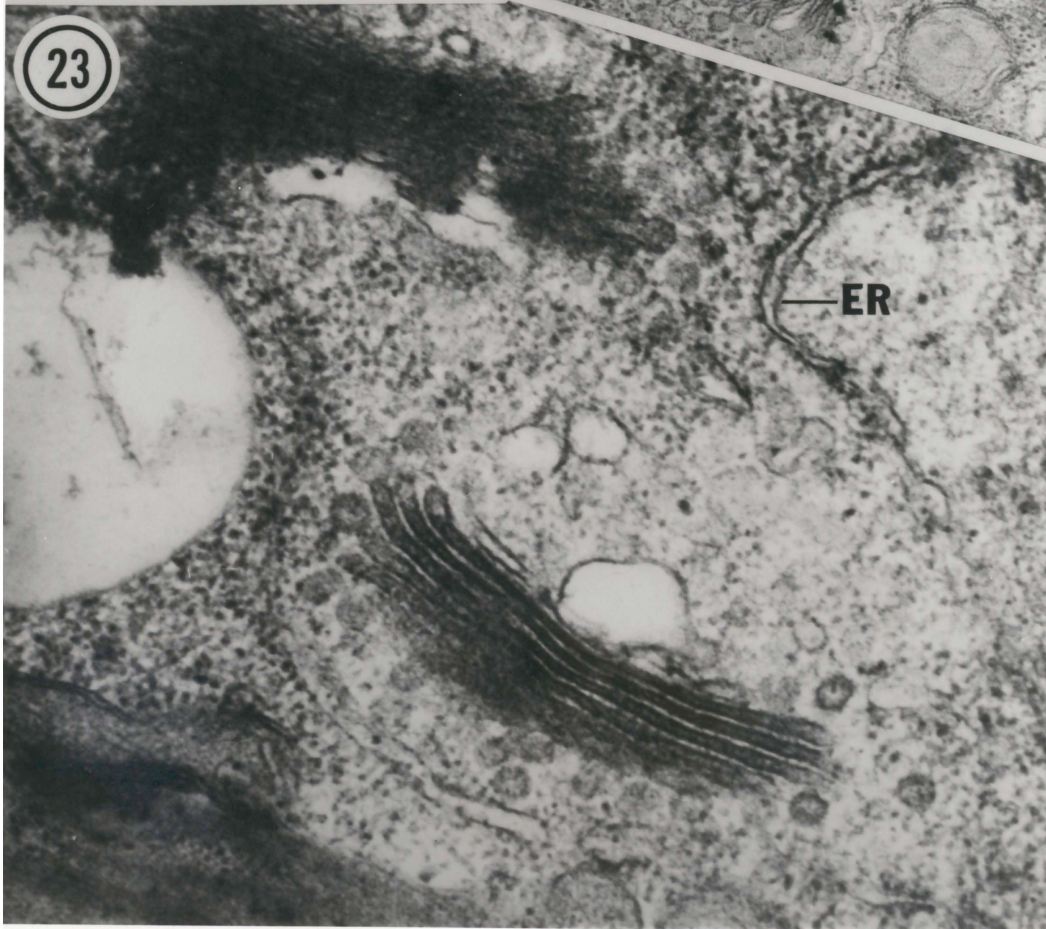
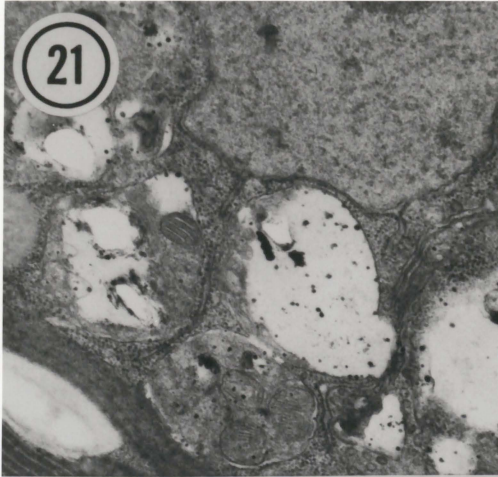


Plate VII. Endoplasmic Reticulum and Dictyosomes.

Figure 21. Profile of rough ER continuous with the nuclear membrane. TEM thin section. X 25,000.

Figure 22. Dictyosome with swollen cisternal margins and nearby profile of transitional ER. Vesicles are obvious between the ER and forming face of the dictyosome and also near the cisternal margins at the maturing face. TEM thin section. X 32,000.

Figure 23. Dictyosomes with associated vesicles and RER. TEM thin section. X 61,000.



Mitochondria. The mitochondria are tremendously varied in shape and appearance. They range from long and narrow (Plate VIII, Figure 24) to highly branched (Plate VIII, Figure 25). Cristae are numerous and highly visible in most sections in both face view and longitudinal section. Mitochondria are often found near the periphery of the cell or in close association with the chloroplast (Plate VIII, Figure 25). Several small, round-to-oblong mitochondria are located at the base of the flagella (Plate VIII, Figure 26), presumably functioning as an energy source during flagellar beating and cellular locomotion.

Chloroplast, pyrenoids and starch grains. The chloroplast contains numerous thylakoids, which occur in stacks of 2-10 membranes (Plate IX, Figure 27). Chloroplast microtubules, frequently seen in the stromal spaces between thylakoids, are present singly or in groups (Plate IX, Figures 27 and 29). Osmophilic granules are also evident in many chloroplast sections (Plate IX, Figures 27, 29 and 31).

Pyrenoids are intrachloroplastidic and as many as eight pyrenoids are visible in some cells. Two starch plates surround the pyrenoid matrix, which is traversed by a single thylakoid membrane (Plate IX, Figures 28 and 31). Starch grains (Plate IX, Figure 30) become more numerous after nutrient depletion or as the cells age. In some cells, starch accumulates to such an extent that it obscures all other cellular detail (Plate IX, Figure 32).

Chloroplast lamellar lattice -- In the chloroplasts of many cells there is a "quasi-crystalline lamellar lattice" (McLean and Pessoney, 1970) which consists of regular, folded arrays of chloroplast lamellae

Plate VIII. Mitochondria.

Figure 24. Longitudinal section of long, slender unbranched mitochondrion situated among many cytoplasmic vesicles. Many cristae are visible; most do not extend past the middle of the organelle. TEM thin section. X 30,000.

Figure 25. Large, highly branched mitochondrion near the surface of the cell and between the lobes of the chloroplast. Cristae are seen in lateral and face views. TEM thin section. X 32,300.

Figure 26. Section through anterior portion of cell shows two flagella and associated microtubules. Several round to oblong mitochondria are located below the flagellar apparatus. TEM thin section. X 25,800.

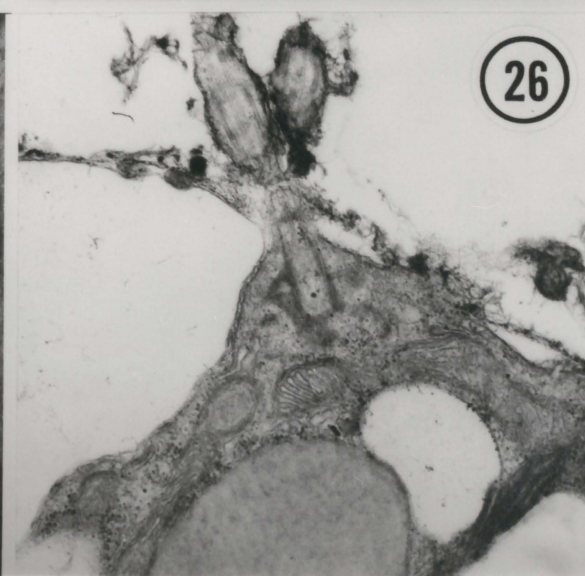
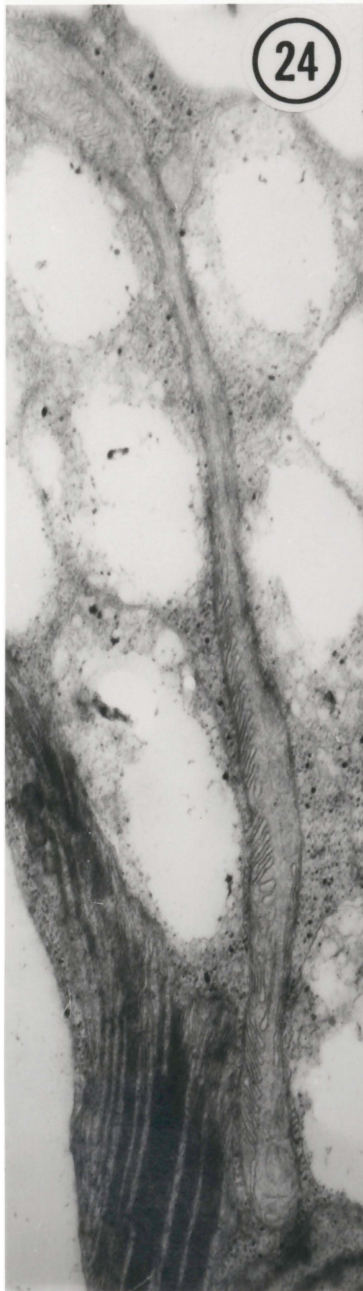


Plate IX. Chloroplast, Pyrenoids and Starch Grains.

Figure 27. Portion of chloroplast with pyrenoid at left and several chloroplast microtubules (arrows) in chloroplast stroma. The number of stacked thylakoids is highly variable. TEM thin section. X 30,100.

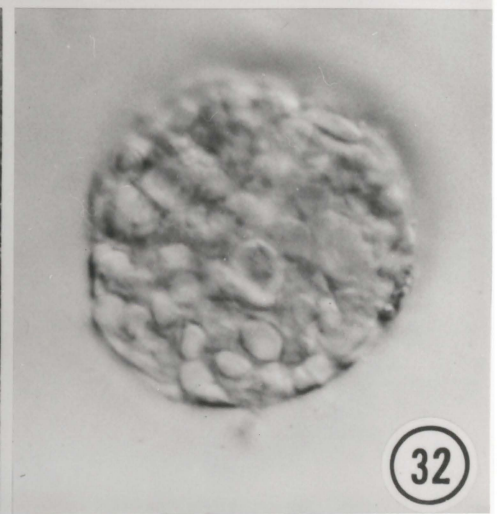
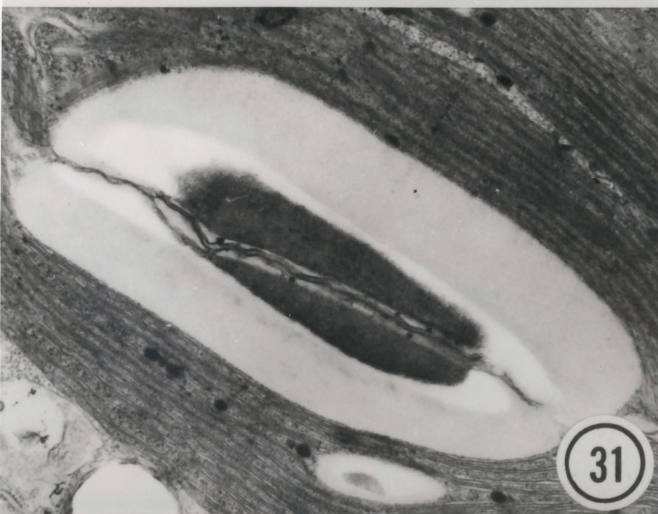
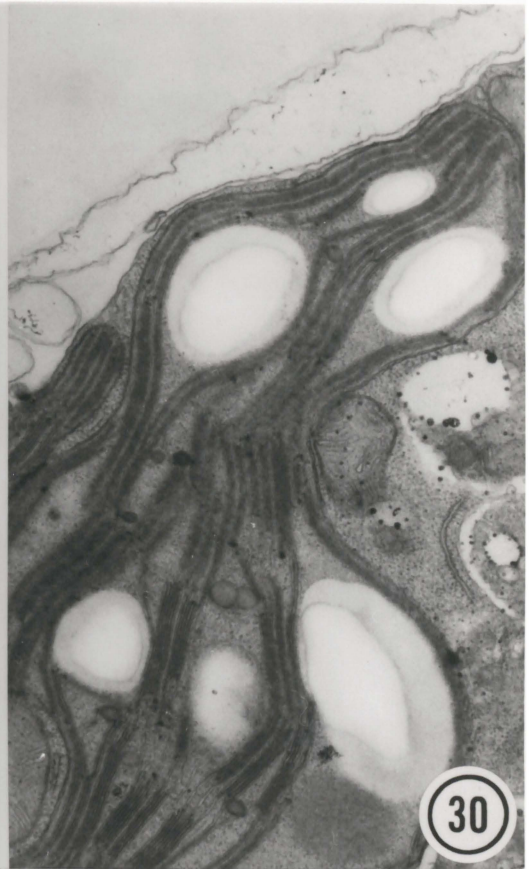
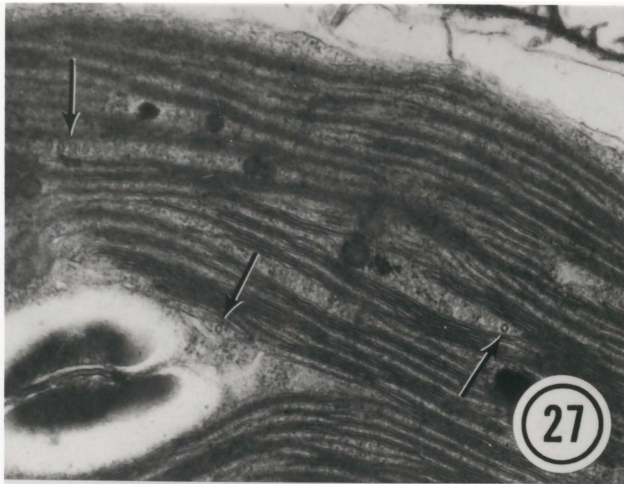
Figure 28. Cell with a chloroplast and two pyrenoids evident. The two starch plates characteristic of the pyrenoid of this organism are visible (arrows). LM. Oblique illumination. X 2,800.

Figure 29. Four chloroplast microtubules (arrow) in the chloroplast stroma; lipid droplets are obvious both above and below the microtubules. TEM thin section. X 23,700.

Figure 30. Chloroplast with starch grains between thylakoids, which are commonly associated in groups of three. TEM thin section. X 28,700.

Figure 31. Transverse section of large pyrenoid with paired starch plates and chloroplast lamella which traverses the pyrenoid matrix. TEM thin section. X 20,900.

Figure 32. Aged cell with accumulation of many starch grains within the chloroplast. LM. Oblique illumination. X 2,900.



(Plate X, Figures 33-36). The lattice structures may often occupy most of the chloroplast volume and are surrounded by typical stacked thylakoids that are continuous with them (Plate X, Figures 33-35). A lattice is formed when the chloroplast thylakoids coil and slide between one another. In order to accommodate this disturbance in the stacked architecture of typical thylakoids, a three-dimensional lattice is formed to permit an orderly arrangement of the membranes. Thylakoids which are destined for a lattice arrangement often have distended intrathylakoidal spaces that become the electron-lucent matrix of the lattice after its formation (Plate X, Figures 34 and 35). Stromal material, similar to that seen between non-lattice thylakoids, is evident in the lattice, presumably trapped during the coiling of the lattice membranes (Plate X, Figures 34 and 35). The lamellar lattice is seen in cells of all ages, from those which have just divided to those which are in stationary phase culture.

Stigma -- The stigma is also intrachloroplastidic and readily apparent even at the light microscopic level (Plate XI, Figure 37). It is composed of several rows of closely packed osmophilic granules. Transverse sections through the stigma reveal as many as four rows of stigma granules (Plate XI, Figures 38-42), with each row separated from the next by a single thylakoid membrane (Plate XI, Figures 39 and 40). The stigma is often seen as a bulge on the surface of the cell and is closely appressed to the chloroplast membrane. Oblique sections through the stigma region reveal that the stigma is very large and associated with arrays of microtubules (Plate XI, Figures 38 and 42).

Plate X. Chloroplast Lamellar Lattice.

Figure 33. Oblique section through a lamellar lattice and surrounding chloroplast thylakoids. Note the regular arrays of curved tubules which originate from swollen chloroplast thylakoids (arrow). TEM thin section. X 25,800.

Figure 34. Section through a lamellar lattice and adjacent thylakoids. Many distended thylakoids are seen in the area surrounding the lattice, while stromal material is enclosed by the lattice membranes. TEM thin section. X 30,600.

Figure 35. Large lattice occupies a major portion of the chloroplast. Several lattice units can be seen in transverse section (arrows). Many lipid droplets are present in both the lattice region and among the thylakoids. TEM thin section. X 28,700.

Figure 36. Lamellar lattice in a young cell. Note distended intrathylakoidal spaces and the coiling of the membranes. Initial organization of the lattice membranes is evident immediately below the stigma (arrow). TEM thin section. X 28,700.

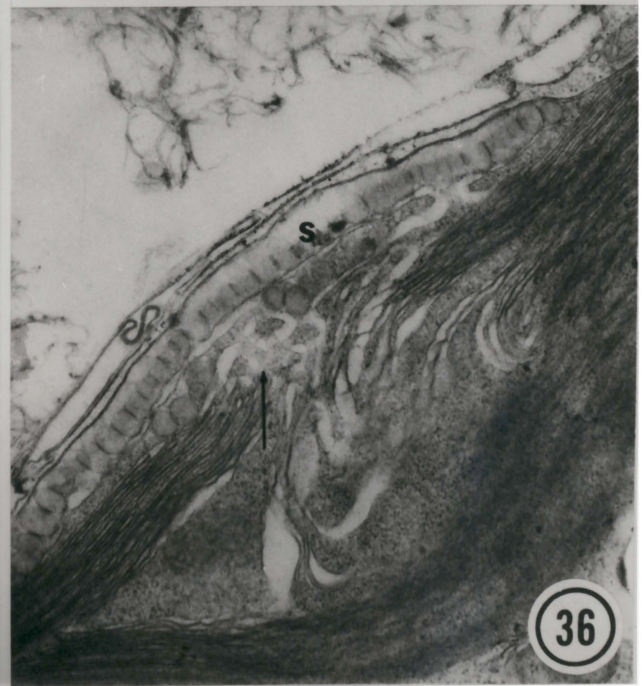
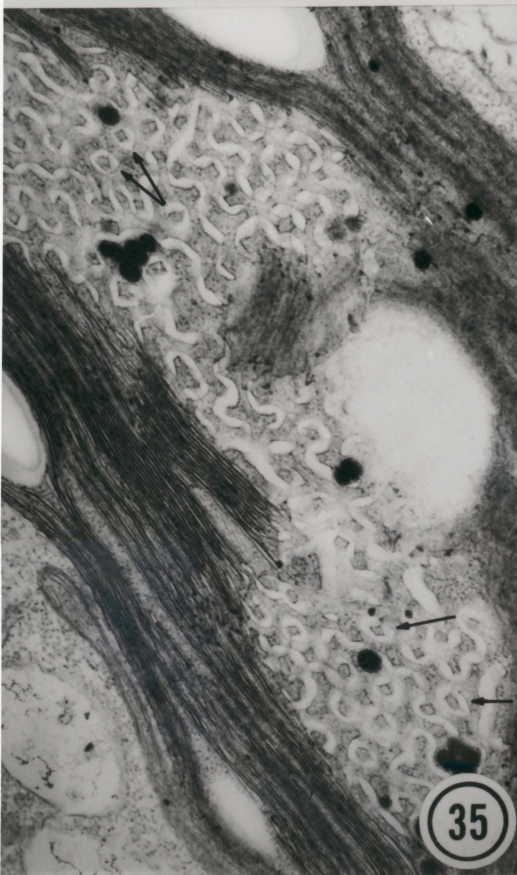


Plate XI. Stigma.

Figure 37. Cell in which the stigma is clearly discerned (arrow). Note the individual granules which make up the structure. Also evident are six pyrenoids and several starch grains. LM. Oblique illumination. X 2,500.

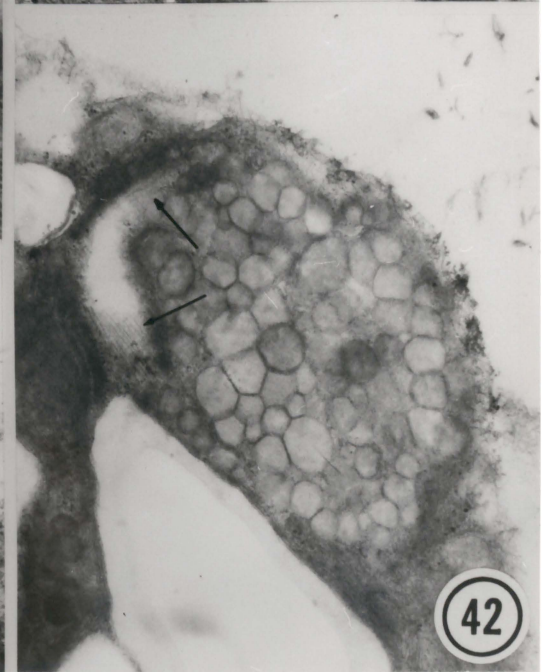
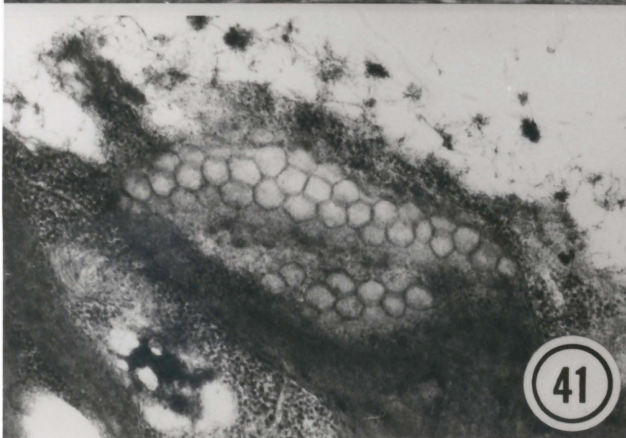
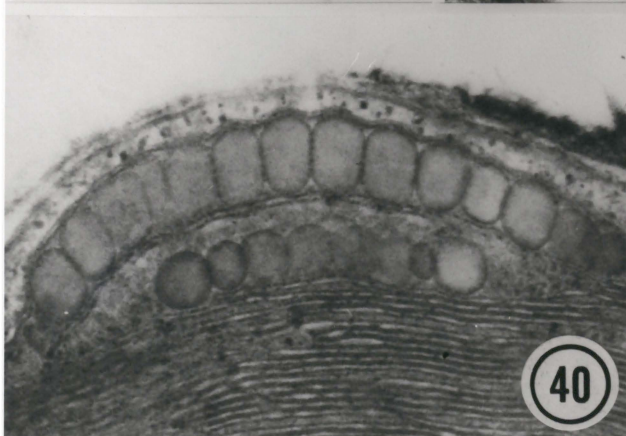
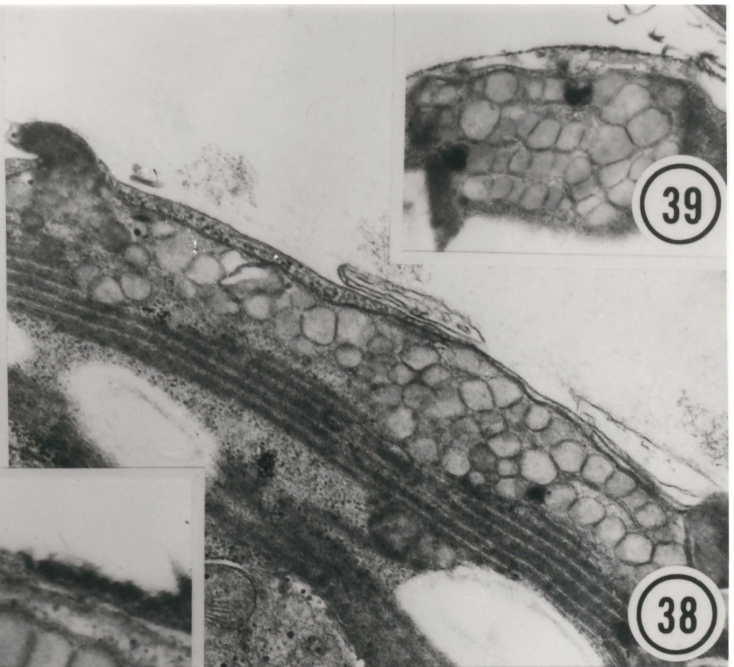
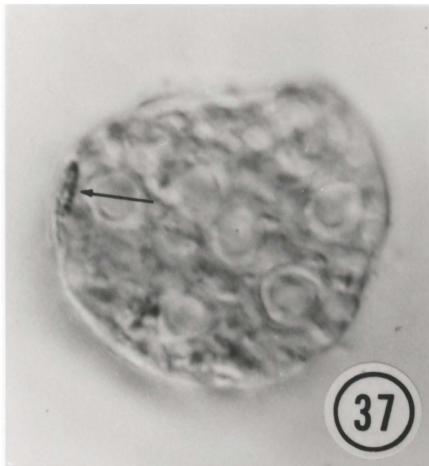
Figure 38. Oblique section through a large stigma. Thylakoids are almost all arranged in triplets in this portion of the chloroplast. TEM thin section. X 38,700.

Figure 39. Transverse section through a stigma with four layers of stigma granules. TEM thin section. X 30,100.

Figure 40. Transverse section through a portion of the stigma where only two layers of stigma granules are evident. A bulge in the cell membrane is a result of the convex nature of the underlying stigma. TEM thin section. X 82,700.

Figure 41. Glancing section through the stigma region shows close packing of granules resulting in a polygonal configuration of granules. TEM thin section. X 37,300.

Figure 42. Glancing section through the stigma region of the chloroplast shows microtubules in close proximity to the stigma (arrows). TEM thin section. X 30,100.



Glancing sections through the stigma show the tight packing of the stigma granules, resulting in a polygonal shape (Plate XI, Figure 41).

Flagellar apparatus and cytoplasmic microtubules. The isokont flagella are located at the anterior end of the cell and measure approximately 2 μm by 17 μm . In cross section, a typical 9 + 2 axoneme is evident (Plate XII, Figure 43). The flagellar membrane extends into long hair-like structures which are ca. 0.17 μm wide and may be up to 5 μm long (Plate XII, Figures 43 and 46). The surface of each flagellum is covered with a finely fibrillar mucilaginous material, which reacts positively with both Alcian Blue and PA-TCH-SP staining procedure (Plate XII, Figures 44, 45, 47 and 48), results indicating that it is polysaccharide and probably contains some negatively charged moieties.

The flagella exit through the lorica via separate flagellar pores (Plate XIII, Figures 49-51) each surrounded by a thickened collar region (Plate XIII, Figure 53). The flagella are inserted obliquely into the cytoplasm with respect to the long axis of the cell and at a 25-30° angle with respect to one another (Plate XIII, Figure 52).

The basal bodies usually lie in a V-configuration with respect to one another and in this area many microtubules are evident (Plate XIV, Figures 54 and 55). Basal bodies are connected to one another at their distal ends by a striated fiber, which appears in thin section as a dense region crossed with dark bands (Plate XIV, Figure 55). Four microtubular roots are seen in fortuitous sections, radiating from the basal body region, beneath the distal striated fiber (Plate XIV,

Plate XII. Flagellar Structure.

Figure 43. Transverse section through a flagellum with a typical 9 + 2 axoneme. Note the flagellar hair which is an extension of the flagellar membrane (arrow) and the covering of fine mucilage fibrils around the entire flagellum. TEM thin section. X 114,100.

Figure 44. Longitudinal section of flagellum emerging from the lorica shows heavy Alcian Blue precipitate on the surface of the flagellum. The transition region and basal body of the flagellum are evident. TEM thin section. X 25,800.

Figure 45. Silver proteinate stain of this flagellum shows a precipitate (arrow) of silver grains on the surface of the flagellum and on flagellar hairs, a result indicating a polysaccharide component in the mucilage which covers the flagella. TEM thin section. X 55,100.

Figure 46. This longitudinal section of a flagellum shows two flagellar hairs (arrows), axonemal microtubules and the basal body region of the cell. TEM thin section. X 30,100.

Figure 47. Transverse section of a flagellum showing the 9 + 2 axoneme and Alcian Blue stain precipitate on the flagellar membrane (arrow). TEM thin section. X 96,400.

Figure 48. Longitudinal section shows a heavy Alcian Blue stain precipitate along the entire length of the flagellum. TEM thin section. X 27,900.

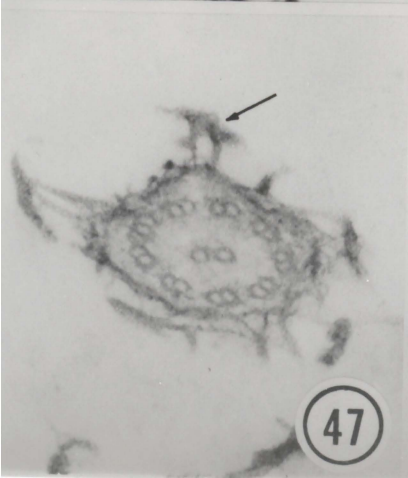
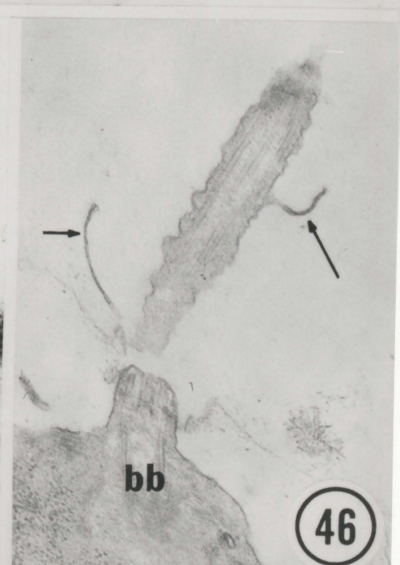
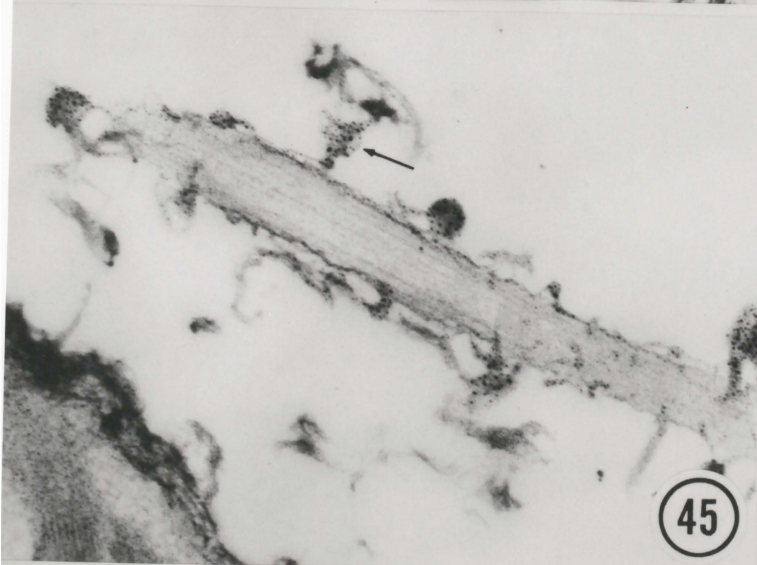
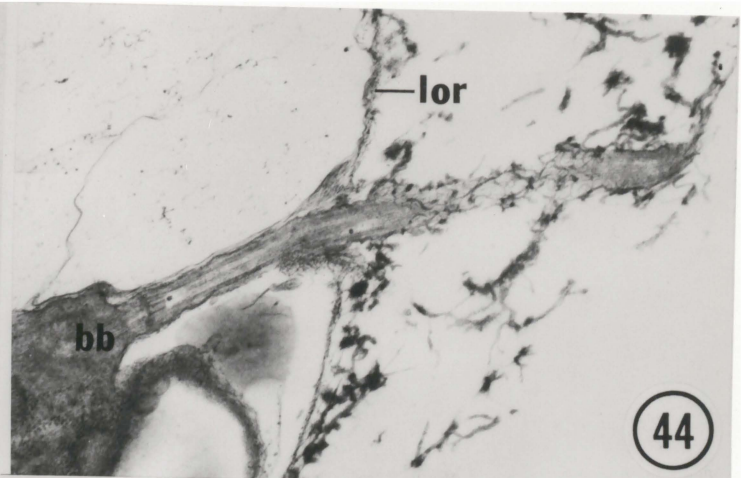


Plate XIII. Flagella-Lorica Relationship.

Figure 49. Emergent flagellum constricted at point of contact with lorica. The wavy nature of the flagellar membrane is also evident. AB/TEM thin section. X 34,400.

Figure 50. Oblique section through lorica and two flagella. Each flagellum exits the lorica through a separate opening. AB/TEM thin section. X 35,500.

Figure 51. Oblique section through the flagellar region of the cell and lorica, showing only one flagellum per flagellar pore. AB/TEM thin section. X 34,400.

Figure 52. In this section, the lorica is closely appressed to the cell and to the emerging flagella. AB/TEM thin section. X 55,100.

Figure 53. Near transverse section through a flagellar pore in lorica. The opening is surrounded by a narrow rim (arrow) and associated fibrillar material (double arrow). Cytoplasmic microtubules underlie the plasma membrane. AB/TEM thin section. X 68,900.

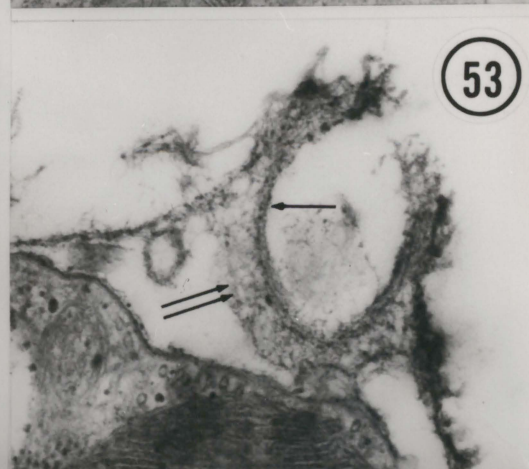
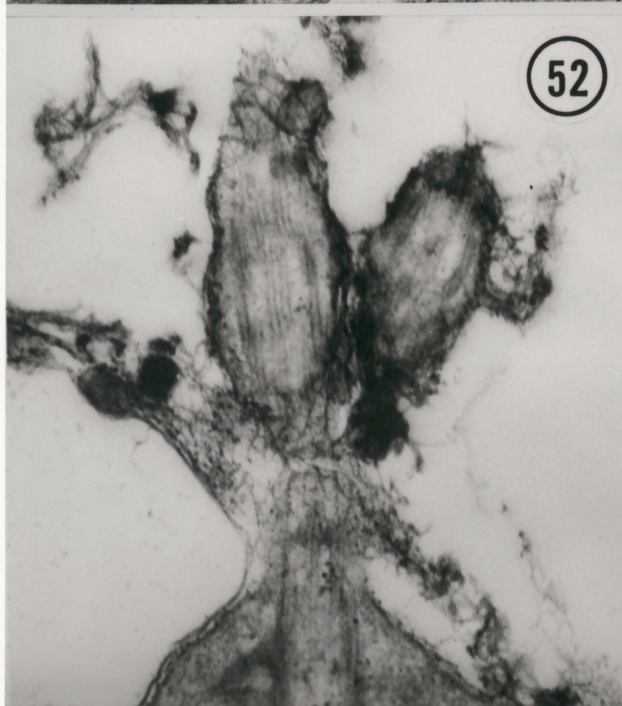
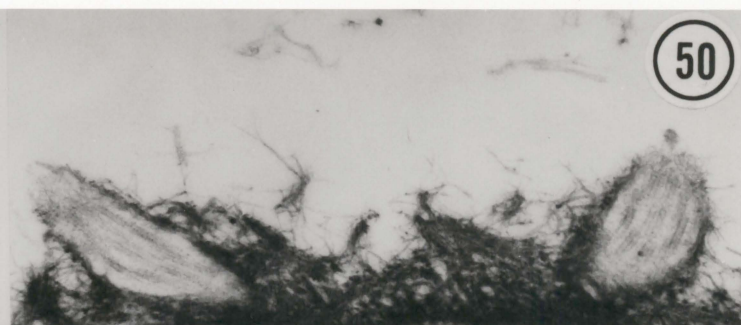
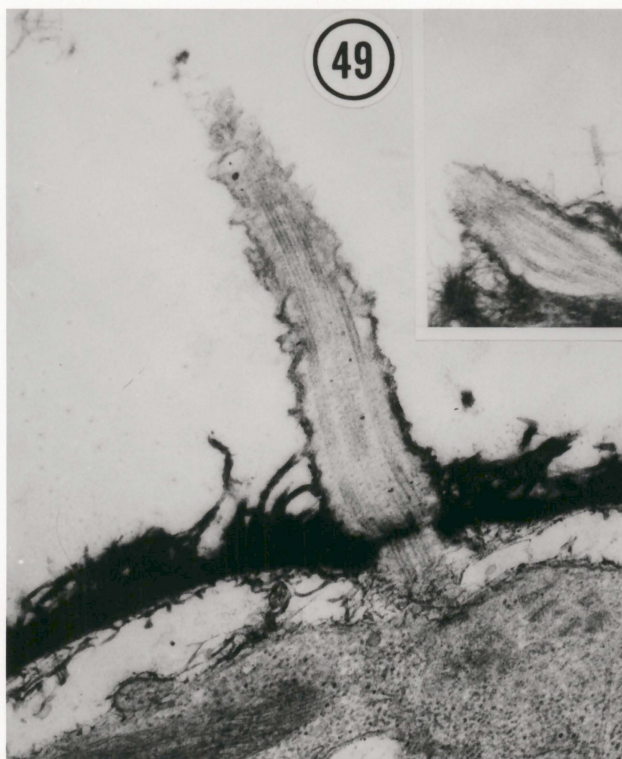


Plate XIV. Basal Bodies and Flagellar Root System.

Figure 54. Oblique section through two flagella shows basal body and four radiating bands of microtubules (numbered). Other scattered microtubules are in this area. TEM thin section. X 82,700.

Figure 55. Oblique section through three flagellar bases. A third basal body, possibly an indication of impending cell division, is indicated by arrow. Three microtubular roots (numbered) and a striated fiber are visible. Inset shows transverse section of flagellar transitional region. TEM thin section. X 89,500.

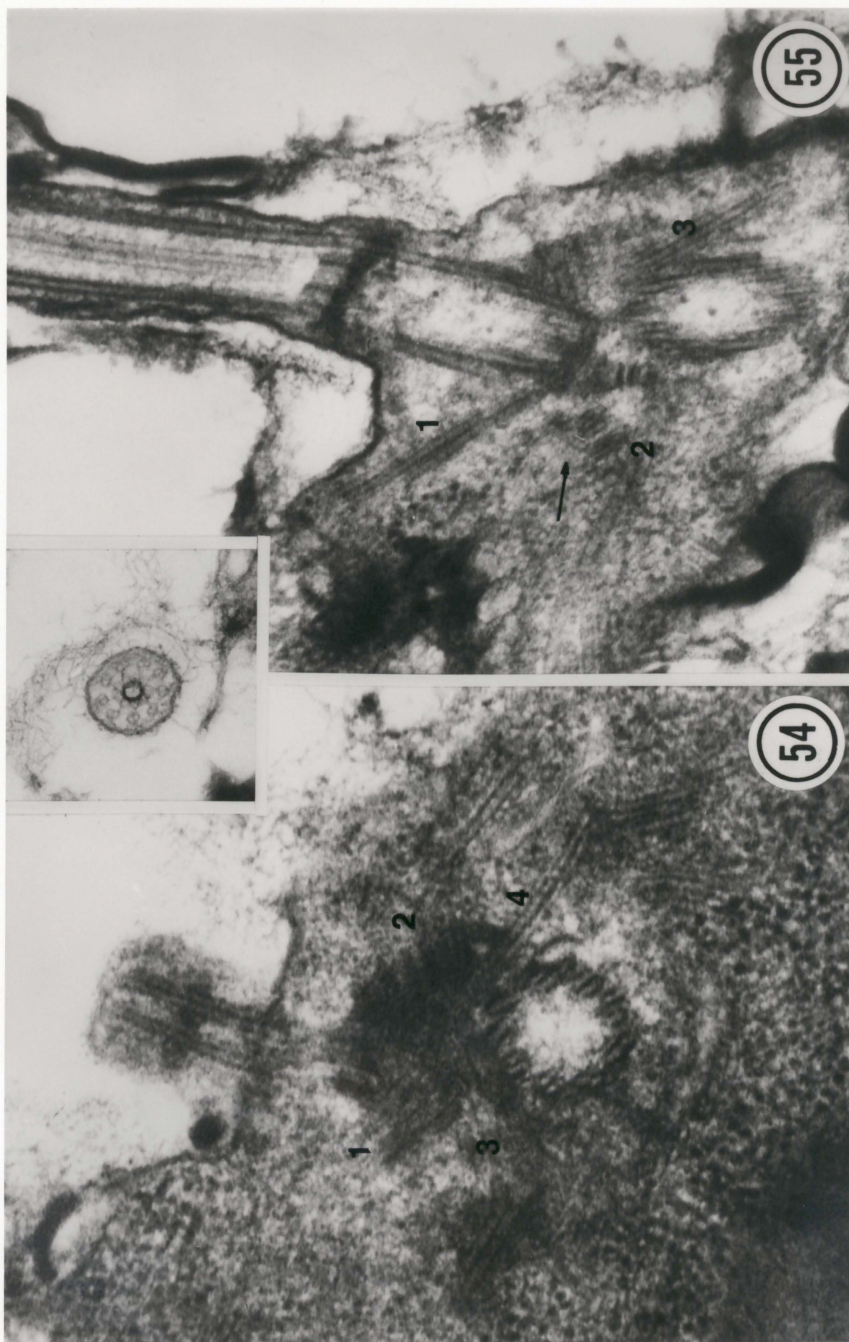


Figure 54). Each root consists of from 2-4 microtubules, depending on the plane of section.

Cytoplasmic microtubules are found in close association with the plasma membrane, often between it and a profile of endoplasmic reticulum (Plate XV, Figures 56 and 57). Microtubules may occur singly (Plate XV, Figure 59), but are often found in pairs or triplets (Plate XV, Figures 57, 58 and 61). A three-over-one arrangement is occasionally seen (Plate XV, Figure 60).

Lorica Morphology

Lorica surface morphology. Scanning electron microscopy (SEM) reveals that the lorica has a rough, ornamented surface (Plate XVI, Figures 62 and 63). The surface may be only slightly rugose or may be covered with many blunt projections. The surface configuration can be obscured by a heavy covering of mucilage. Young loricas collapse easily when air-dried and are perforated by many pores, whereas older loricas are rigid and thickened and the pores may become obscured by mucilage as cells age (Plate XVI, Figures 62 and 65). Additionally, the bivalved nature of the lorica is often not readily apparent because of a heavy coating of mucilage (Plate XVI, Figure 67).

The shape of the valves as well as the nature of the surface ornamentation is easily manipulated by changing the culture medium. Loricas from cells grown in defined medium tend to have a smooth surface appearance and lack any defined ridge where the valves meet (Plate XVI, Figure 64). Loricas from soil-water cultures have a variety of morphological shapes and a wide range of surface ornamentations and valve

Plate XV. Cytoplasmic Microtubules.

Figure 56. Microtubules near the plasma membrane (arrows), either singly or in pairs. An association of ER, microtubules and the plasma membrane is obvious. TEM thin section. X 26,600.

Figure 57. Microtubules near the plasma membrane, arranged in groups of two or three. TEM thin section. X 82,700.

Figure 58. Cytoskeletal microtubules in groups of three and singly, close to the plasma membrane. TEM thin section. X 82,700.

Figure 59. Higher magnification of cytoplasmic microtubules. A single microtubule (right) located near a group of microtubules, is seen in glancing section. TEM thin section. X 82,700.

Figure 60. Section through the anterior end of the cell reveals a three-over-one arrangement of microtubules. TEM thin section. X 40,100.

Figure 61. High magnification of cytoskeletal microtubules near the plasma membrane. TEM thin section. X 82,700.

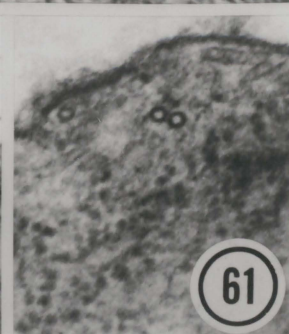
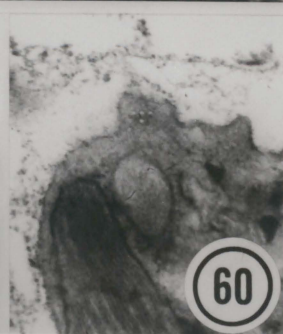
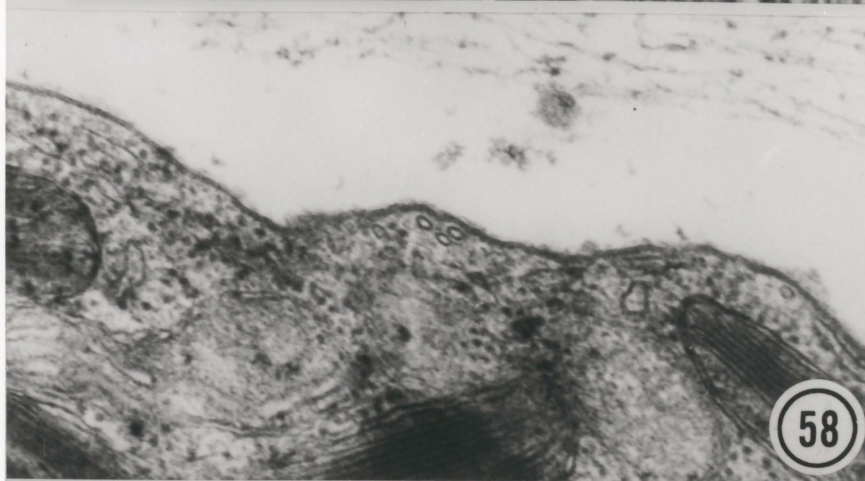
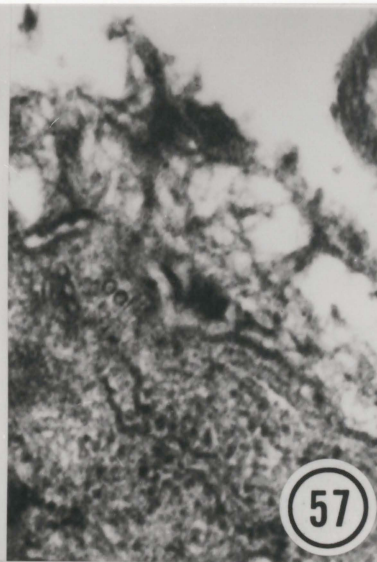
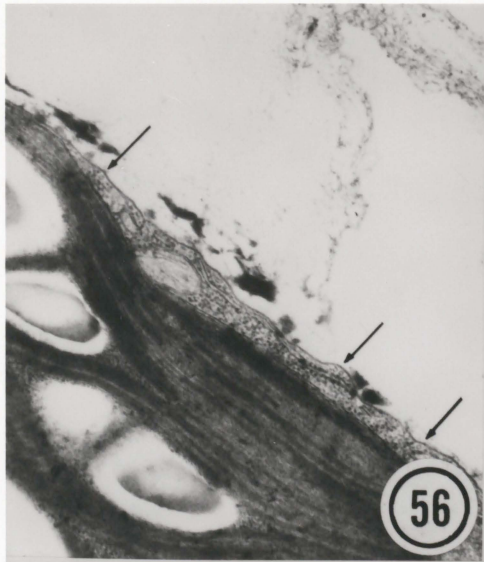


Plate XVI. SEM of the Lorica.

Figure 62. Immature cell from SW culture has flexible, porous lorica with pebbled surface. Starch grains (arrows) released when cell collapsed during air-drying. Au/Pd coating. X 2,800.

Figure 63. Loricas isolated from SW cultures display varying degrees of rigidity during air-drying. No pores are visible in the loricas; however, the surface is highly textured. Au/Pd coating. X 2,800.

Figure 64. Cells isolated from defined medium have a smooth lorica consisting of a reticulum with no obvious surface projections. Two lorica halves are seen on several cells; a flagellar pore is also obvious (arrow). CPD. Au/Pd coating. X 2,800.

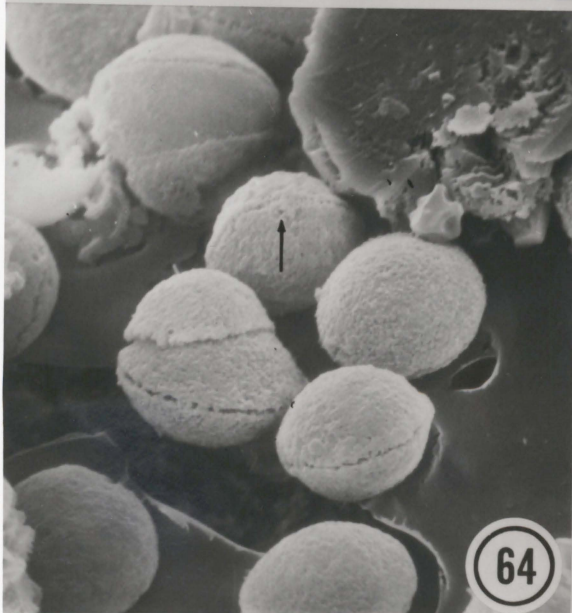
Figure 65. This fractured cell illustrates the sturdy, non-porous nature of a mature lorica. Air-dried. Au/Pd coating. X 5,600.

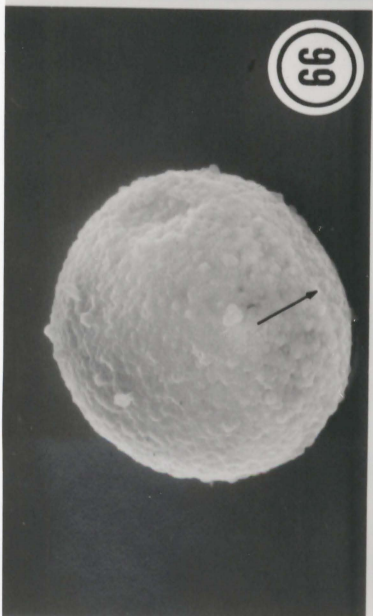
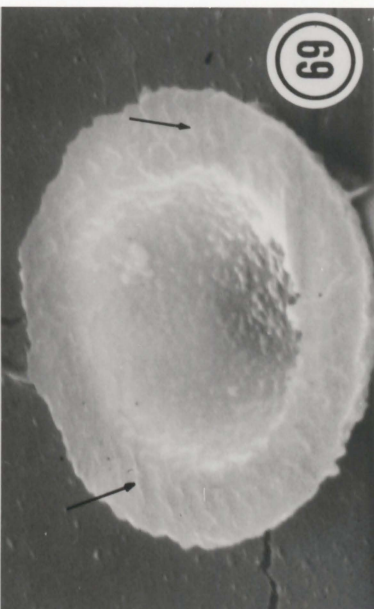
Figure 66. Immature lorica isolated from SW has few surface projections and a barely discernible suture (arrow) where the two halves join. Air-dried. Au/Pd coating. X 2,800.

Figure 67. Mature lorica isolated from SW culture has no visible pores and a pebbled surface. CPD. Au/Pd coating. X 5,600.

Figure 68. Mature lorica isolated from defined medium shows a prominent rim where the two lorica halves join. Air-dried. Au/Pd coating. X 5,000.

Figure 69. One half of a mature lorica reveals a large rim around the valve edge. The spiral pattern in the rim area (arrows) is a species-specific characteristic. Air-dried. Au/Pd coating. X 4,900.





configurations (Plate XVI, Figures 62, 66 and 67). They also manifest some variation in rim structure. The rim of some loricas has a spiral pattern on its flat, internal surface, visible only when the two valves are separated (Plate XVI, Figure 69). This is noteworthy, since the spiral rim pattern is supposedly characteristic of the species Phacotus lendneri.

Lorica microarchitecture: unstained cells. Cells which are fixed for TEM by conventional methods do not have a prominent lorica. Transverse sections of such loricas reveal a wispy granulo-fibrillar layer which is associated with large electron-dense fibrillar masses (Plate XVII, Figures 70-72). Loricas from cells grown in soil-water have more electron-dense structures than from those grown in defined medium. The space between the lorica and the protoplast is often filled with many small fibrillar components (Plate XVII, Figure 72).

Lorica microarchitecture: stained cells. Cytochemical staining of the lorica (see Part 3 of Results) indicates the presence of negatively charged moieties, such as sulfate groups and uronic acids. Stains selective for these anionic groups, Ruthenium Red and Alcian Blue, were therefore added to fixations to aid in the visualization of the lorica. Both stains are selective for acidic polysaccharides and deposit an electron-dense reaction product wherever negatively charged groups occur.

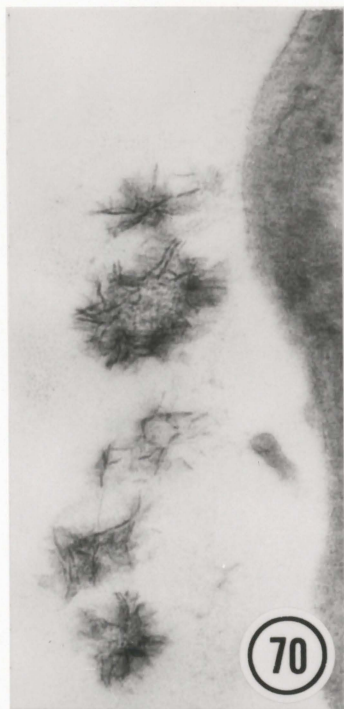
Ruthenium Red -- Axenic, actively growing cells were labelled with Ruthenium Red. Most cells have a heavily labelled lorica with a distinct pattern of deposition in which a continuous granular layer is

Plate XVII. Morphology of Loricas from Unstained Cells.

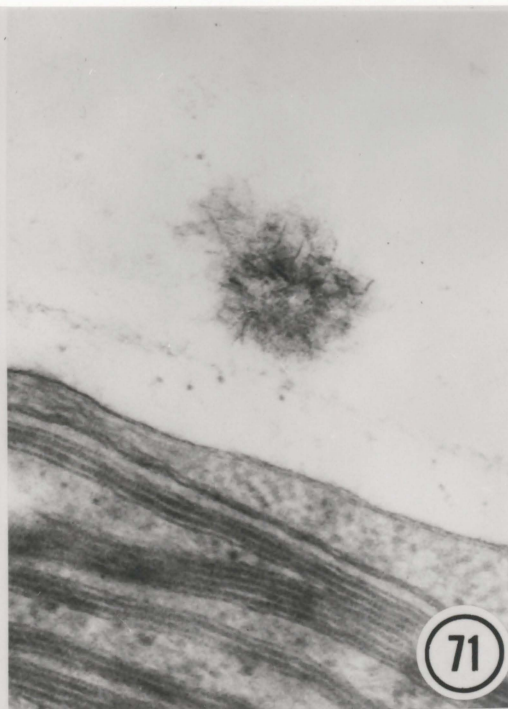
Figure 70. Transverse section of lorica reveals an association of dense fibrillar cores with a granulo-fibrillar layer. TEM thin section. X 68,900.

Figure 71. Cross section of a lorica illustrates a single fibrillar core with a granular layer below it. TEM thin section. X 73,500.

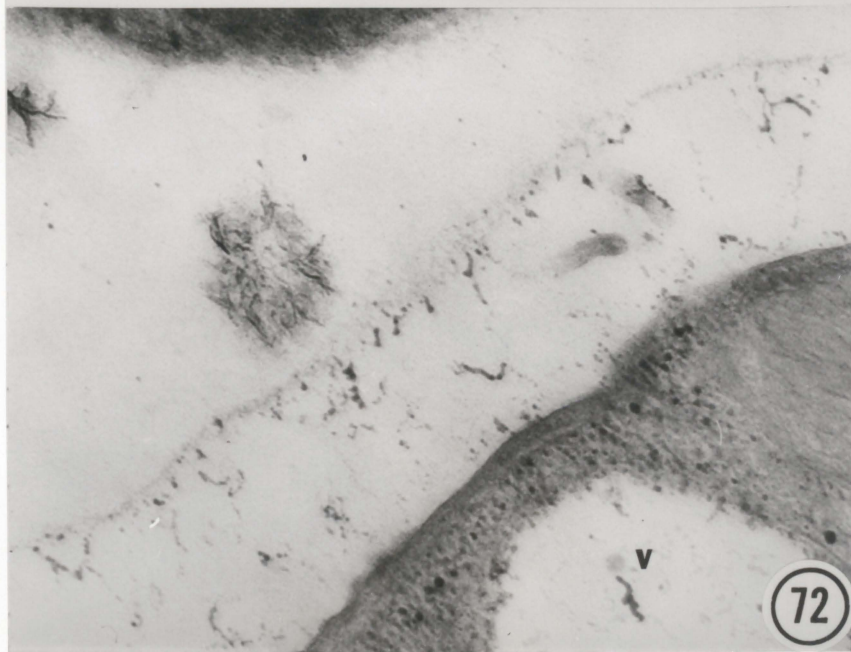
Figure 72. Space between lorica and cell is filled with small fibrils, as is vesicle near cell membrane. TEM thin section. X 82,700.



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studded with larger stellate cores (Plate XVIII, Figures 73 and 75). The granular layer is not as heavily stained as the stellate cores, which seem to have a fibrillar texture. The surfaces of the plasma membrane and flagella are also lightly stained with Ruthenium Red (Plate XVIII, Figures 74 and 75). Extracellular mucilage extruded from the cells also stains heavily with RR (Plate XVIII, Figure 76), a result indicating that an acidic polysaccharide moiety is part of the mucilage.

Alcian Blue -- Alcian Blue-stained cells have a prominent electron-dense lorica (Plate XIX, Figure 77) whose thickness depends in large part on the age of the cell. Mature loricas are up to 2.5 μm thick, and have a regular, ridged surface pattern with a periodicity of ca. 3.5 μm (Plate XX, Figures 78 and 79). Fibrillar projections at the surface may be numerous enough to obscure the ridged pattern.

The lorica consists of arrays of stellate fibrillar elements which are deposited on a granulo-fibrillar layer (Plate XX, Figure 80). The stellate components have a densely stained core region from which radiate wispy, fibrillar projections. These lorica components are best seen in young cells; older cells usually have thickened, dense loricas in which single components are difficult or impossible to discern.

Asexual Reproduction

Cells about to begin division slow their motile activity, eventually cease movement altogether, then begin to elongate and assume an oval shape (Plate XXI, Figures 81 and 84). Nuclear division occurs quickly, and the onset of cytokinesis is signalled by the pinching in of the plasma membrane (Plate XXI, Figures 82 and 83). Furrowing from

Plate XVIII. Ruthenium Red Staining for Acid Mucopolysaccharides.

Figure 73. Longitudinal section through cell and lorica. A granular RR-osmium precipitate is seen both on the surface of the cell and throughout the lorica. Note that the stain precipitate is localized in a thin continuous layer to which are attached larger stain aggregates. RR/TEM thin section. X 13,900.

Figure 74. Longitudinal section through anterior region of cell. Granular RR deposits are evident along the surface of the flagellar membrane and lorica. RR/TEM thin section. X 34,400.

Figure 75. Higher magnification of RR-stained lorica. Lorica is composed of a lighter-staining fibrillar network and large, darkly staining aggregates. RR-stain precipitate is also present along the plasma membrane. RR/TEM thin section. X 25,800.

Figure 76. Extracellular mucilage associated with the lorica. RR/TEM thin section. X 22,900.

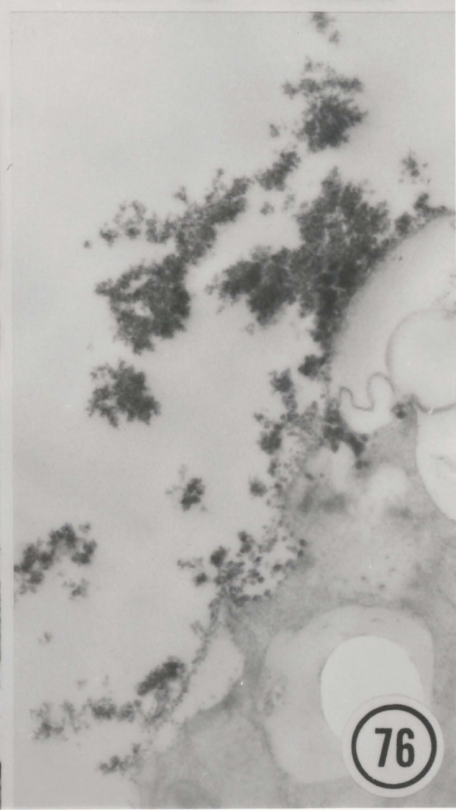
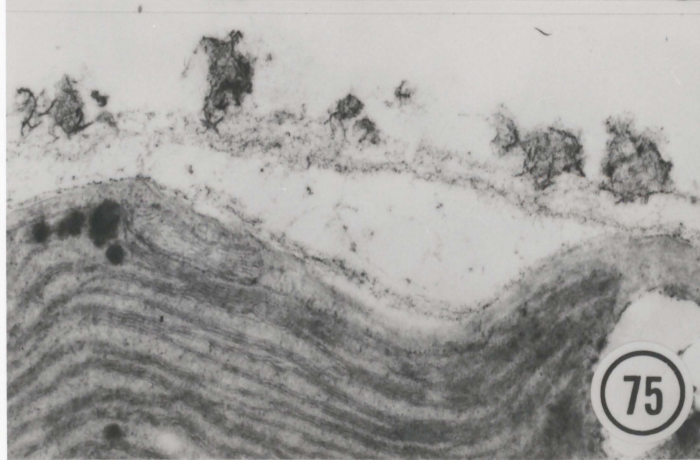
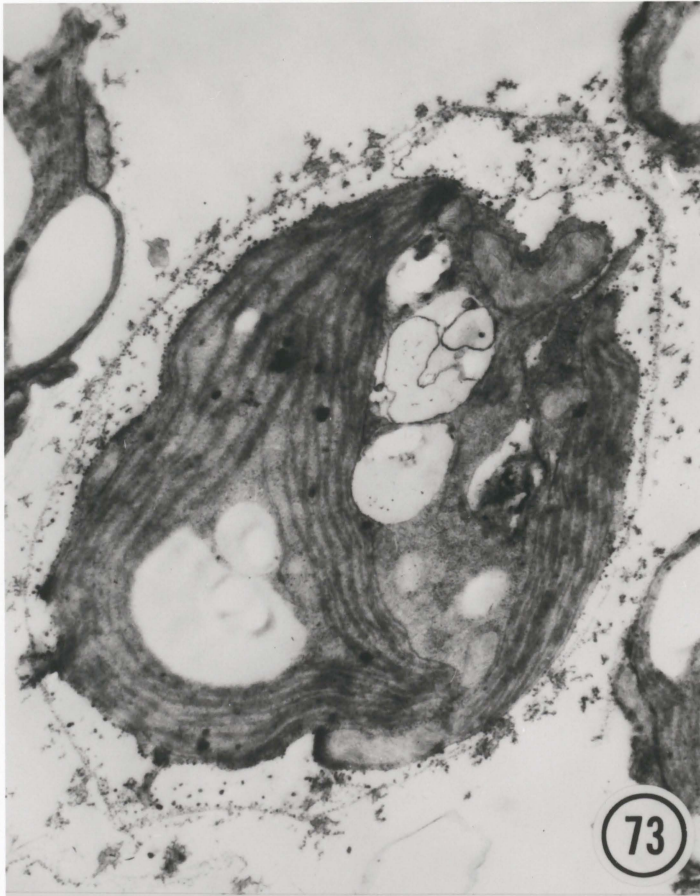


Plate XIX. Alcian Blue Staining for Acid Mucopolysaccharides.

Figure 77. This section through a vegetative cell illustrates the position of many major organelles. The ready visualization of the lorica and surrounding extracellular mucilage is a result of staining with Alcian Blue, a cationic stain for acid mucopolysaccharides. AB/TEM thin section. X 14,100.



Plate XX. Morphology of Loricas from Cells Stained with Alcian Blue.

Figure 78. Transverse section of mature lorica stained with AB. Ridged pattern is evident at the margin of the lorica matrix. AB/TEM thin section. X 72,300.

Figure 79. Transverse section of lorica closely appressed to the cell with no visible substructure. The ridged pattern is obscured by lorica components which protrude from the margin. AB/TEM thin section. X 74,300.

Figure 80. Transverse section of an immature lorica. This lorica is composed of a fibrillar basal layer upon which is deposited stellate matrix components. A close-up of one such component (upper inset) shows a dense central core with fine radiating fibrils. The bottom inset illustrates the spiny nature of the lorica components. AB/TEM thin section. X 64,300.

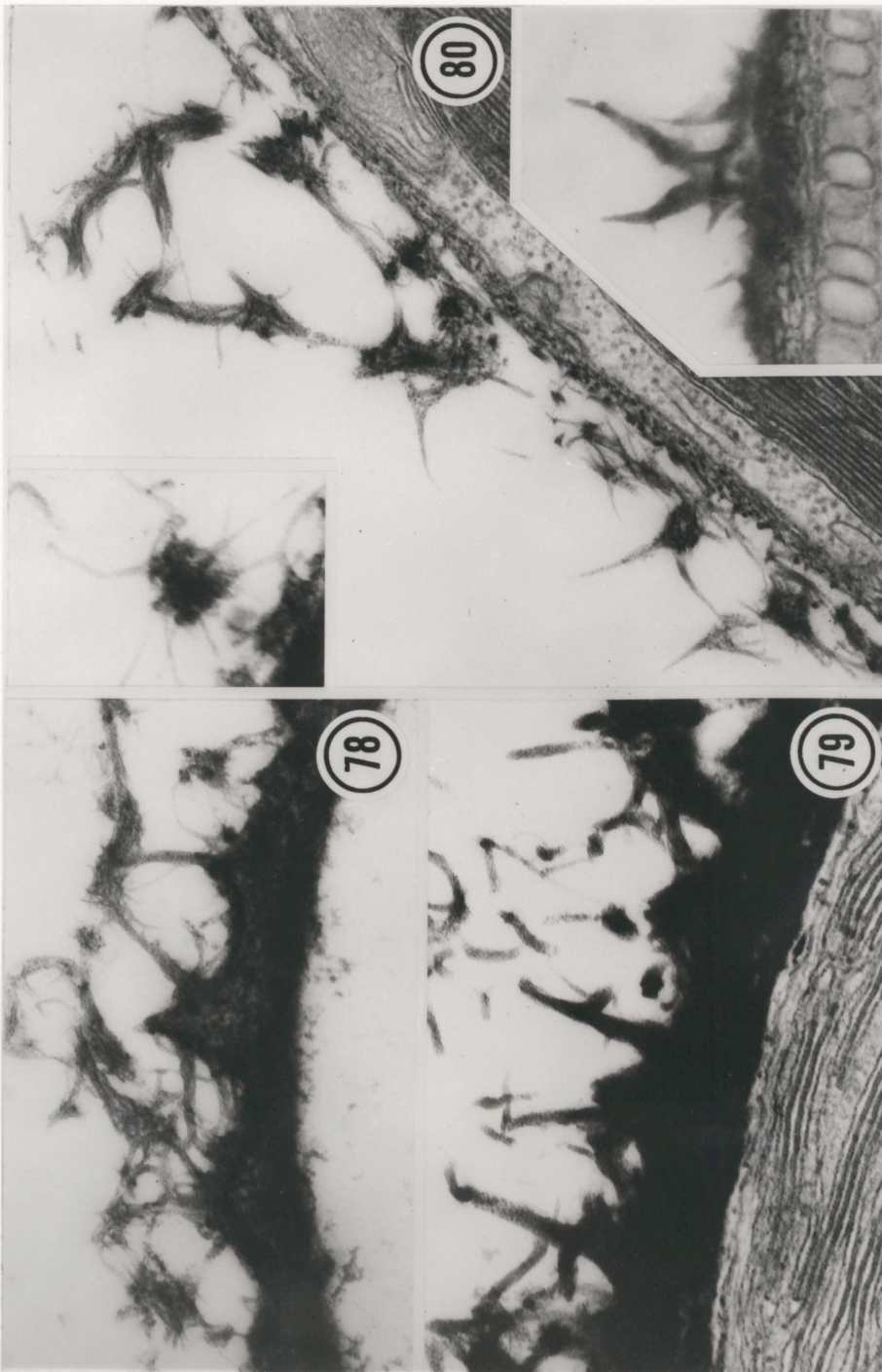


Plate XXI. Cell Division: Early Stages.

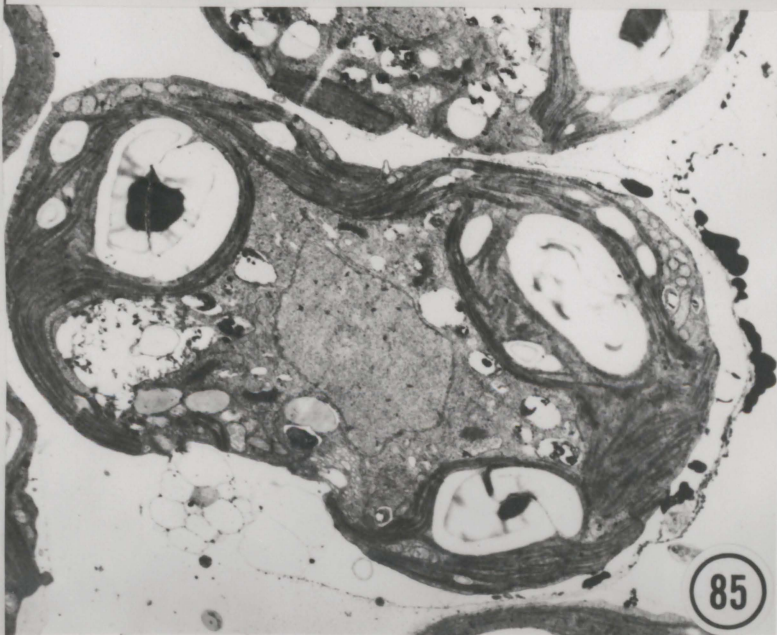
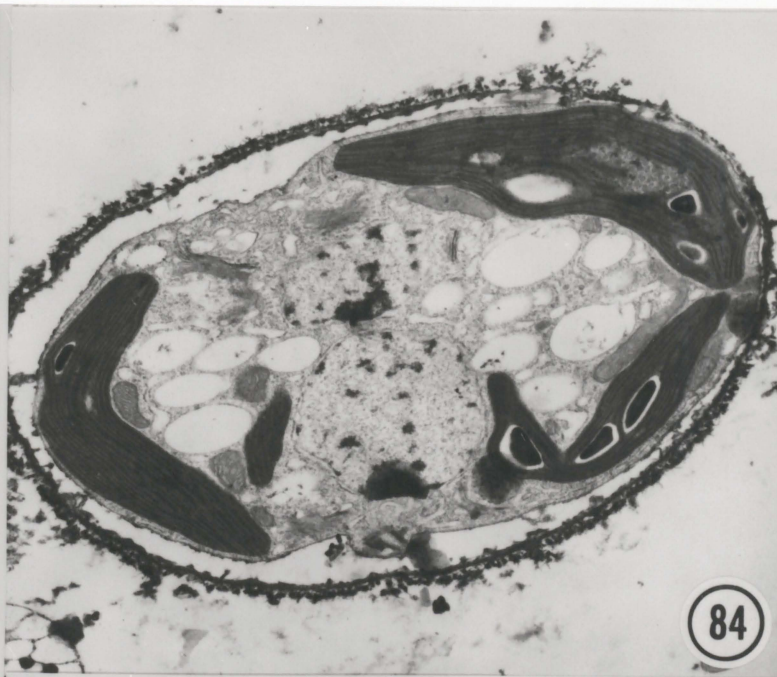
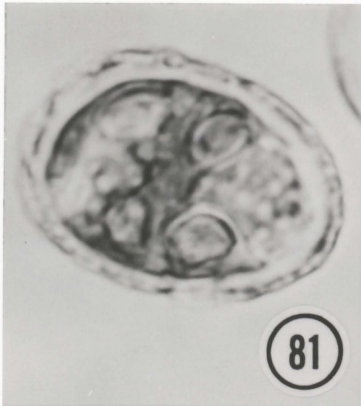
Figure 81. The oblong shape of this cell is indicative of impending cell division. Four pyrenoids are visible near the middle of the cell, where the nucleus lies. The lorica has expanded to fit the shape of the dividing cell. LM. X 2,400.

Figure 82. The cell is constricted at the midregion and lorica shape is distorted. LM. X 2,400.

Figure 83. Further constriction of the cell membrane (c.f. Figure 82) at the periphery of the cell. Lorica is visible but is not uniform in size or texture. LM. X 2,400.

Figure 84. Near median longitudinal section of cell in late telophase. Chloroplast and nucleus have divided although the chromosomes remain condensed and daughter nuclei lie very close together. Lorica is intact, but distorted in shape. AB/TEM thin section. X 12,900.

Figure 85. Cell with a constricted midregion. Chloroplast has not yet completely divided. There are many mitochondria at the periphery of the dividing cell between the chloroplast and cell membrane. AB/TEM thin section. X 16,500.



both sides occurs in most cells; however, furrowing from one side to the opposite is not uncommon. During the early stages of cytokinesis, division of the chloroplast is completed and all major organelles move to opposite ends of the cell (Plate XXI, Figure 85). The lorica elongates and becomes more flexible as cytokinesis progresses.

Daughter cells are formed as the furrow becomes complete, separating the two closely associated daughter nuclei (Plate XXII, Figures 86 and 87). In the region of the furrow many vesicles and dictyosomes are evident. Even after cytokinesis, the daughter nuclei remain close to the plasma membrane for some time, along the plane of division (Plate XXIII, Figure 88). Cytokinesis occurs via formation of a phycoplast, with microtubules abundant along the plane of the cell furrow (Plate XXIII, Figure 89). After division is complete, the new daughter cells separate and flagella become visible again (Plate XXIV, Figure 92). Daughter cells may remain associated for a short time within a thin membranous enclosure that is continuous with the mother lorica (Plate XXIV, Figure 93). The production of new loricas on daughter cells is apparent before their release from the mother lorica. Cell division may result in two, four or eight daughter cells within the mother lorica (Plate XXIV, Figures 90-93), depending upon the number of successive divisions.

Lorica Development and Disintegration

Production of a new lorica begins with a cell which has recently completed division. A very delicate granulo-fibrillar layer is deposited around the new daughter cell, usually in close proximity to

Plate XXII. Cell Division: Later Stages.

Figure 86. Late phase of cell constriction and division. Division furrow is almost complete. Many vesicles, rough endoplasmic reticulum and dictyosomes are visible in this area. Large arrows denote two basal bodies in the left daughter cell, while small arrows indicate microtubules along the plane of division. AB/TEM thin section. X 30,700. Inset: Light micrograph of cell at the same stage of cell division as is shown in the TEM micrograph.

Figure 87. Division furrow and two resulting daughter cells. Note the abundance of vesicles along the plane of division. A fluffy, delicate layer of new lorica material (arrow) is evident. AB/TEM thin section. X 30,100.

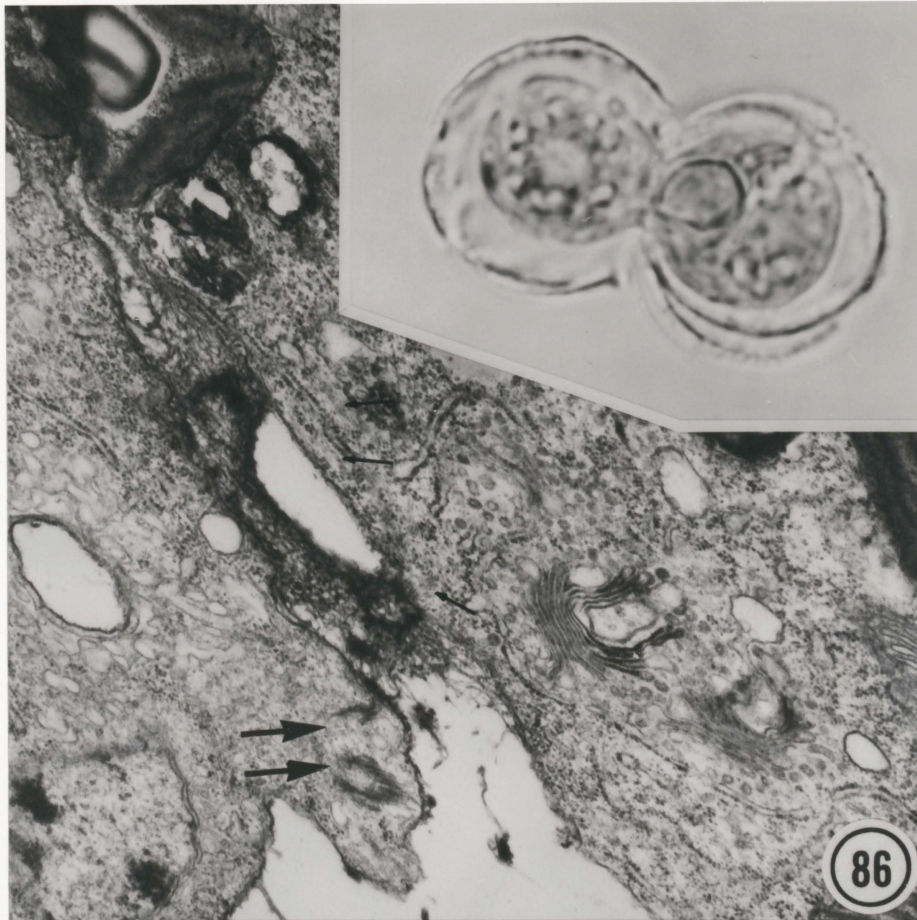


Plate XXIII. Cell Division: Cell Furrow.

Figure 88. Daughter nuclei are in close proximity to the cell furrow. Chromosomes are no longer visible in the daughter nuclei of new cells and the nucleolus has reorganized. TEM thin section. X 11,000.

Figure 89. Higher magnification of the furrow region, showing an abundance of dictyosomes and vesicles in this area. Arrows denote phycoplast microtubules along the plane of the cell furrow. TEM thin section. X 23,700.

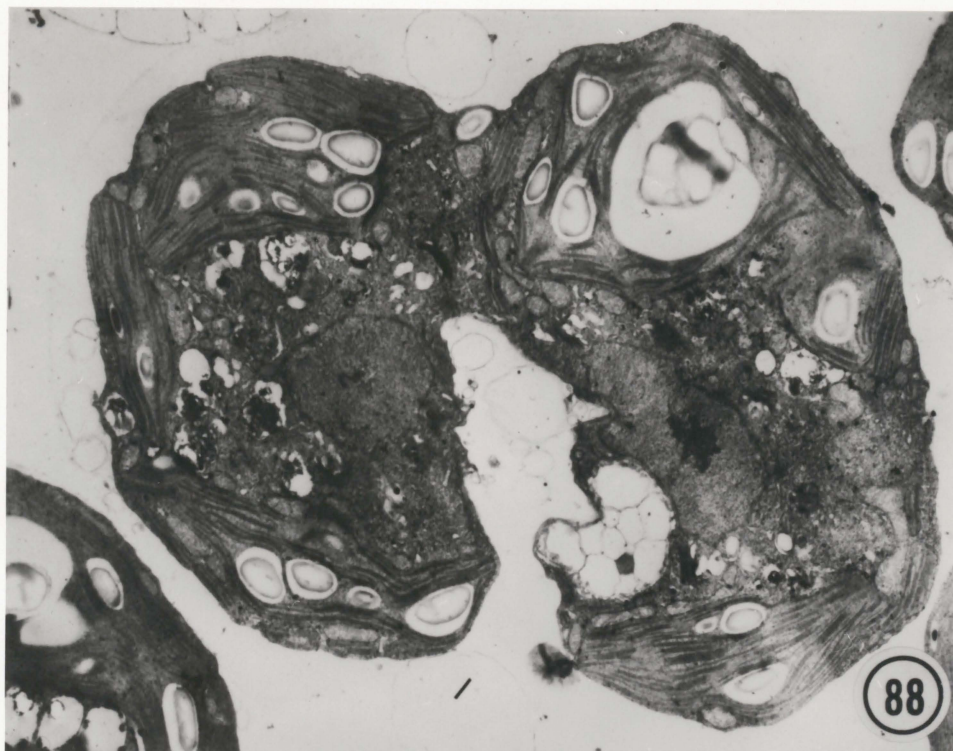


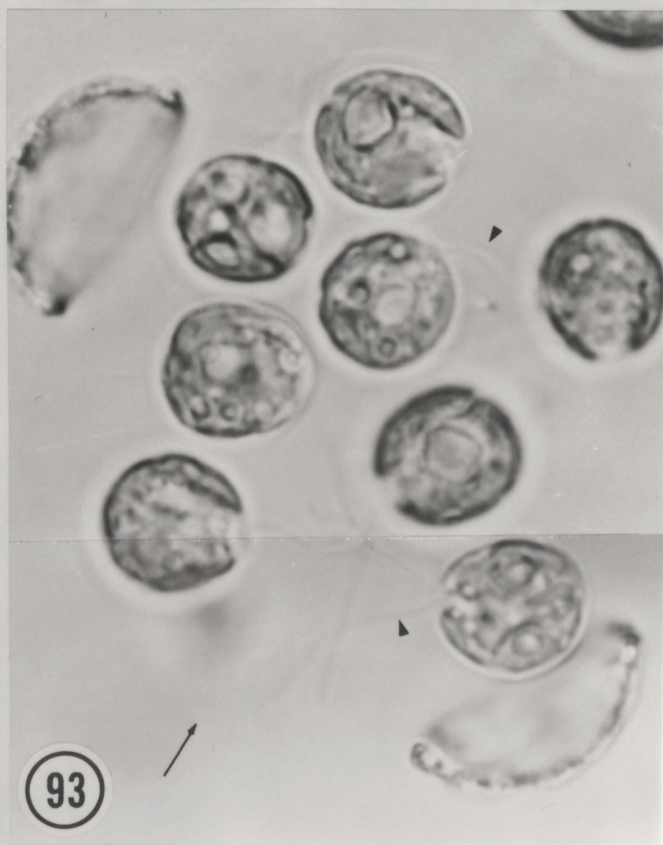
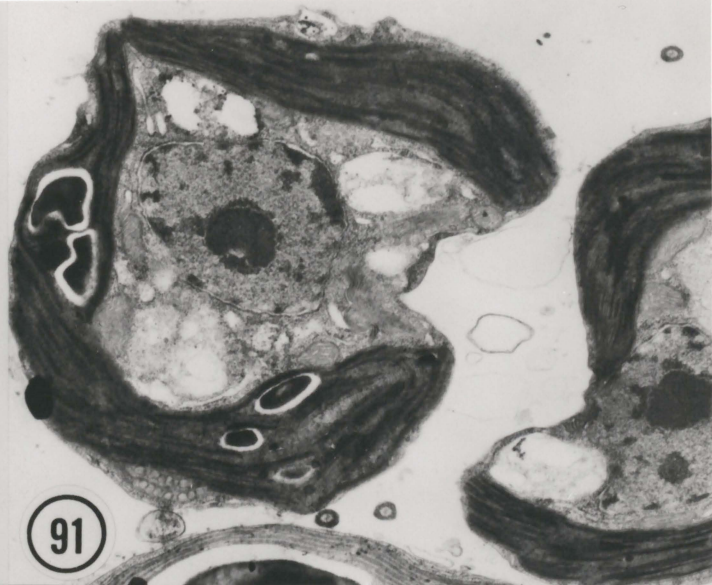
Plate XXIV. Cell Division: Daughter Cells.

Figure 90. One mitotic division produces two daughter cells. Mother lorica is visible at lower right; new loricas are visible around daughter cells (arrows). LM. Bright-field illumination. X 2,500.

Figure 91. Near median longitudinal section through two daughter cells. Most major organelles are visible in the cells. TEM thin section. X 12,100.

Figure 92. Two successive divisions produce four daughter cells. LM. Bright-field illumination. X 2,400.

Figure 93. Three successive divisions produce eight daughter cells. A barely discernible matrix surrounds the newly formed cells (arrow). Flagella (arrowheads) are visible on some cells. LM. Bright-field illumination. X 2,300.



the plasma membrane (Plate XXV, Figure 94). The primary layer becomes thicker and more visible by the addition of more material to it; however, it remains close to the cell surface (Plate XXV, Figures 95 and 96). Subsequently, fibrillar material is secreted and deposited along the granulo-fibrillar primary layer (Plate XXV, Figures 97-100). When viewed in a favorable plane of section, a regular arrangement is evident in the distribution of the fibrillar material, which has both a fine wispy component and a dense core region when viewed at high magnification (Plate XXVI, Figures 101 and 102). Fibrillar material is deposited until a continuous layer surrounds the cell. The lorica retains a wispy, fragile appearance until the late stages of fibril deposition, at which time the lorica becomes dense and rigid (Plate XXVI, Figures 103-105). The accumulation of more fibrillar material in some areas gives rise to the blunt projections visible in some thin sections and in SEM micrographs.

Accumulation of polysaccharide material destined for incorporation into the lorica begins during the process of cytokinesis. Polysaccharide material is seen in the furrow which develops as cell division progresses (Plate XXVII, Figures 106 and 107). A thin layer of polysaccharide surrounding the daughter cells (Plate XXVII, Figures 108 and 109) is found in the same position as the developing granulo-fibrillar layer in young cells, indicating that the developing lorica is largely polysaccharide.

Preceding division, the lorica is a rigid structure, however, during cytokinesis, lorica structure is distorted, with a concomitant loss of rigidity and well-defined shape. The new daughter cells are

Plate XXV. Lorica Development: Early Stages.

Figure 94. Recently divided cells with new loricas (arrows), which are delicate and barely visible. AB/TEM thin section. X 80,000.

Figure 95. The initial layer of lorica material is slightly thickened and has become more visible than that shown in Figure 94. AB/TEM thin section. X 96,400.

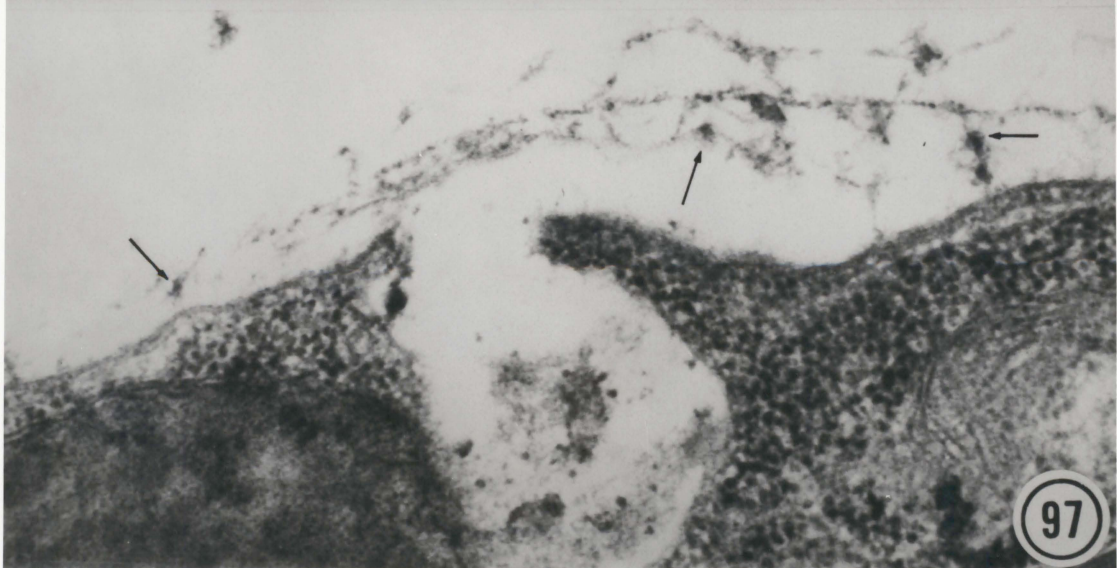
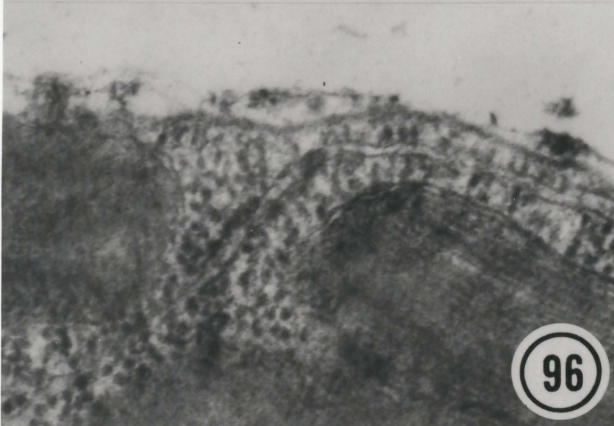
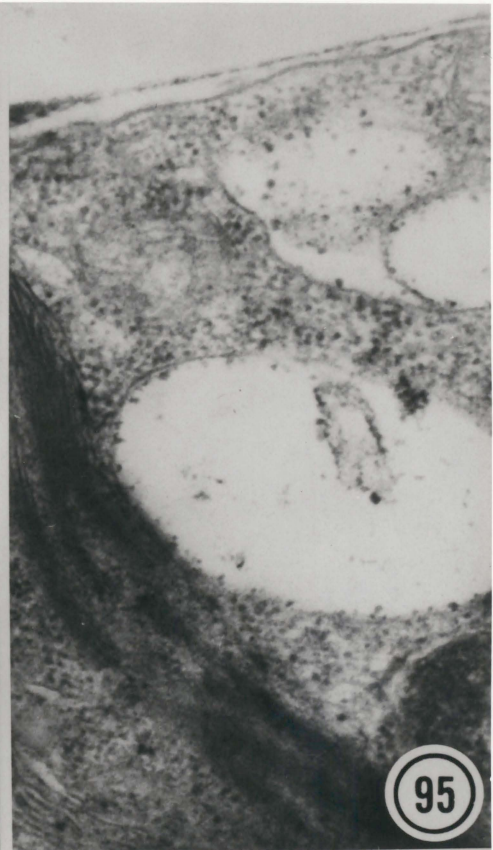
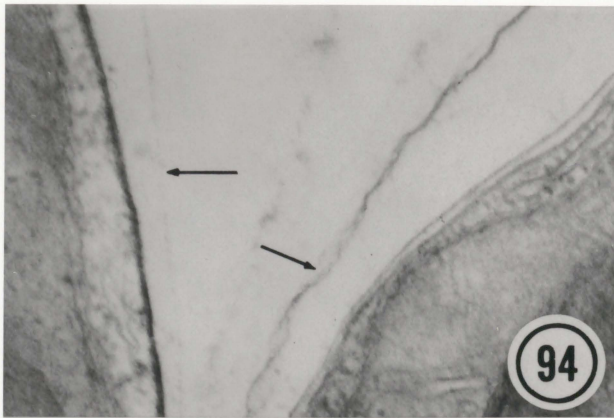
Figure 96. Small amounts of fibrillar material are deposited along the primary initial layer. Lorica lies close to the plasma membrane. AB/TEM thin section. X 82,700.

Figure 97. Further accumulation of fibrillar material (c.f. Figure 96) along the granulo-fibrillar initial layer. Some of the fibrillar material has a dense middle region (arrows). AB/TEM thin section. X 88,200.

Figure 98. Oblique section through fibrillar layer of young lorica, illustrating the delicate nature of the fibrils. AB/TEM thin section. X 75,800.

Figure 99. Distinction between fibrillar material and denser cores is evident. This cell is still enclosed within the mother lorica (arrow). AB/TEM thin section. X 23,700.

Figure 100. Several dark cores are prominent in this oblique section (arrows). Each core is associated with some finely fibrillar material. AB/TEM thin section. X 68,900.



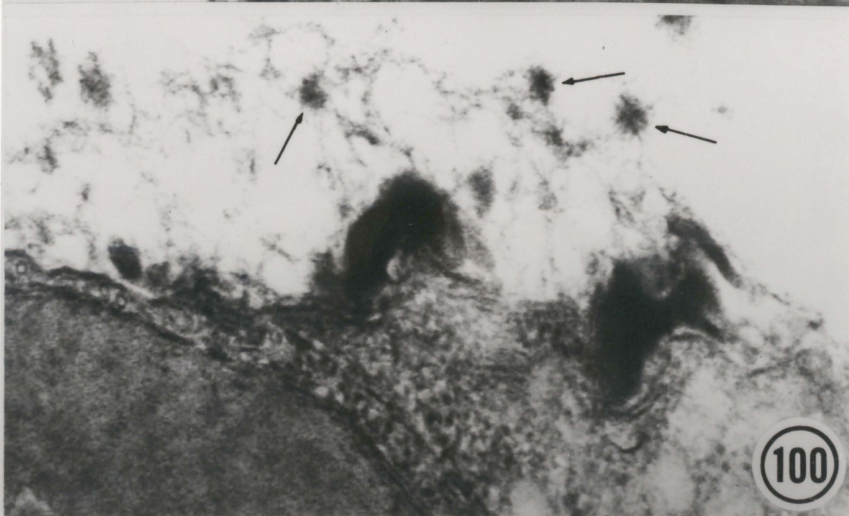
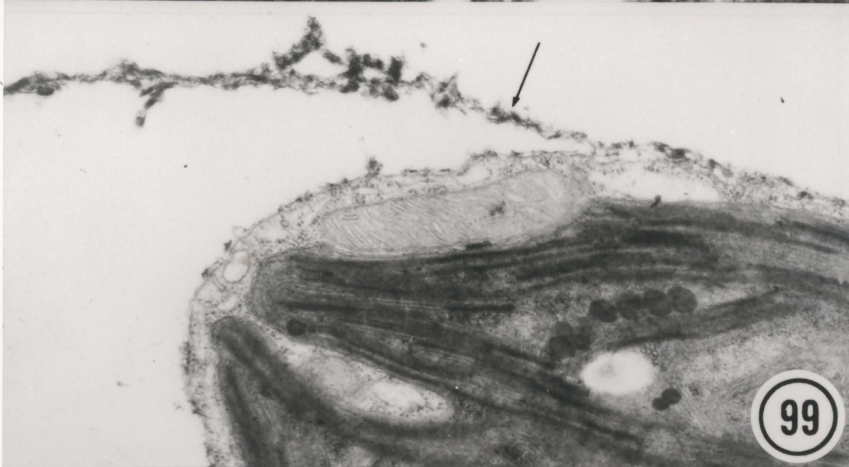
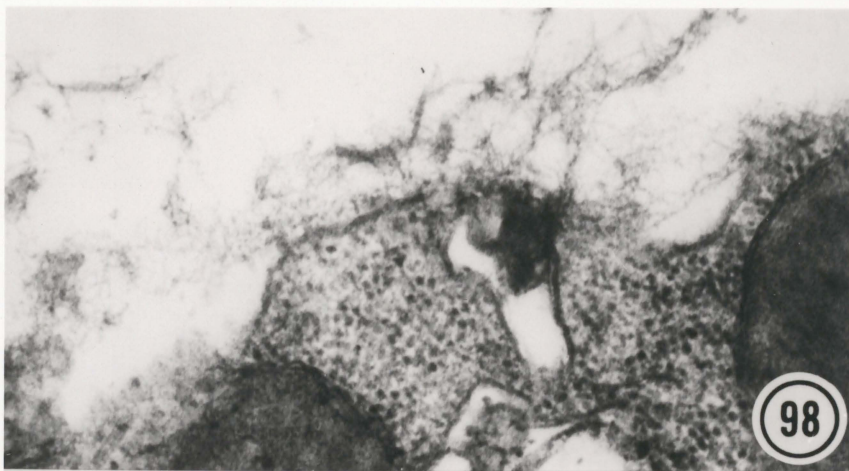


Plate XXVI. Lorica Development: Later Stages.

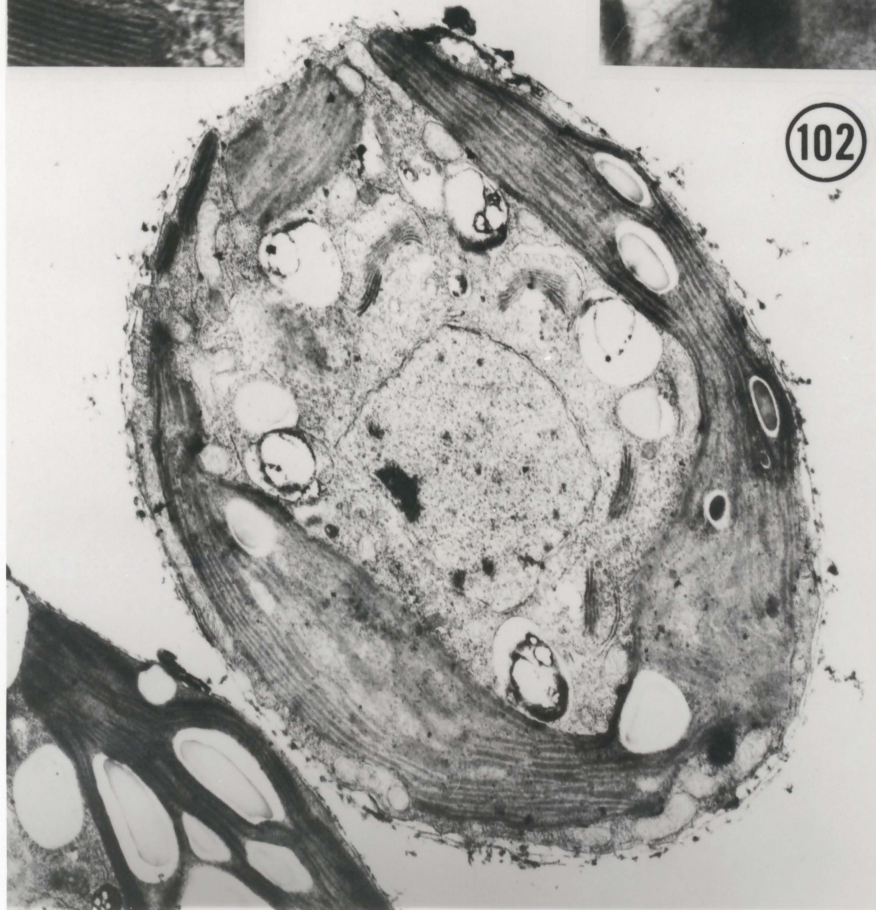
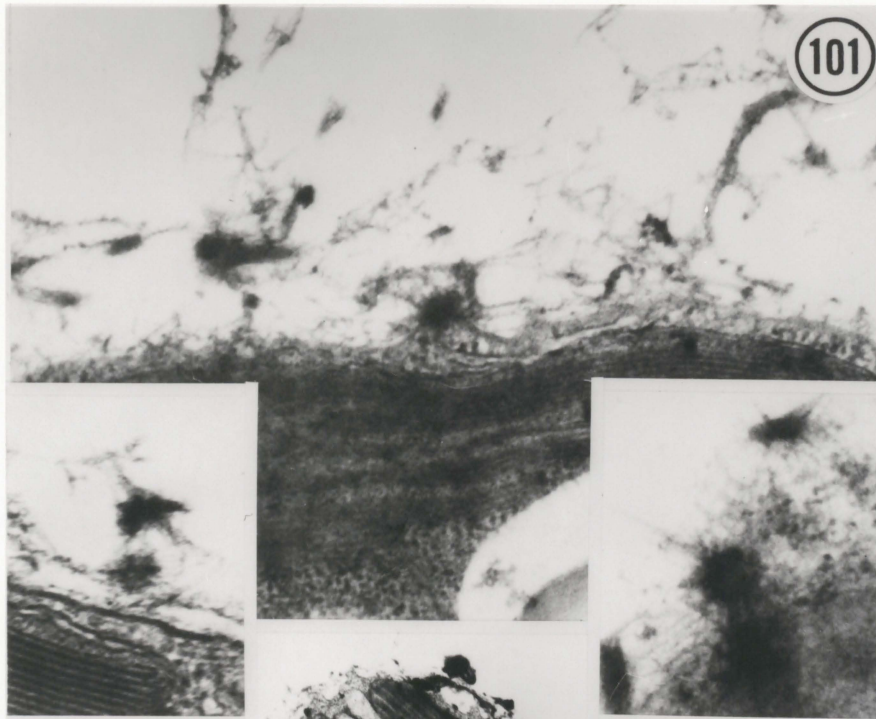
Figure 101. Dense cores associated with free fibrillar material. Left inset shows two dark cores associated with the initial layer of lorica material. Right inset shows three stellate cores. AB/TEM thin section. X 68,900.

Figure 102. Near median longitudinal section illustrates regular spacing of dark cores on the initial layer of lorica material. Several dictyosomes show cisternae with electron-dense contents. AB/TEM thin section. X 12,500.

Figure 103. Distinction between wispy and dense material is no longer obvious. Lorica is dense and continuous around the cell and individual components are no longer distinct. AB/TEM thin section. X 19,400.

Figure 104. Higher magnification of lorica in transverse section. Fibrillar material is visible at inner margin and some dense cores are still distinguishable (arrows). An accumulation of the lorica matrix material results in blunt projections, or spines, along the lorica margin. AB/TEM thin section. X 36,000.

Figure 105. Comparison of a mature lorica (bottom) and an immature lorica (top). Note difference in lorica texture and thickness. AB/TEM thin section. X 30,100.



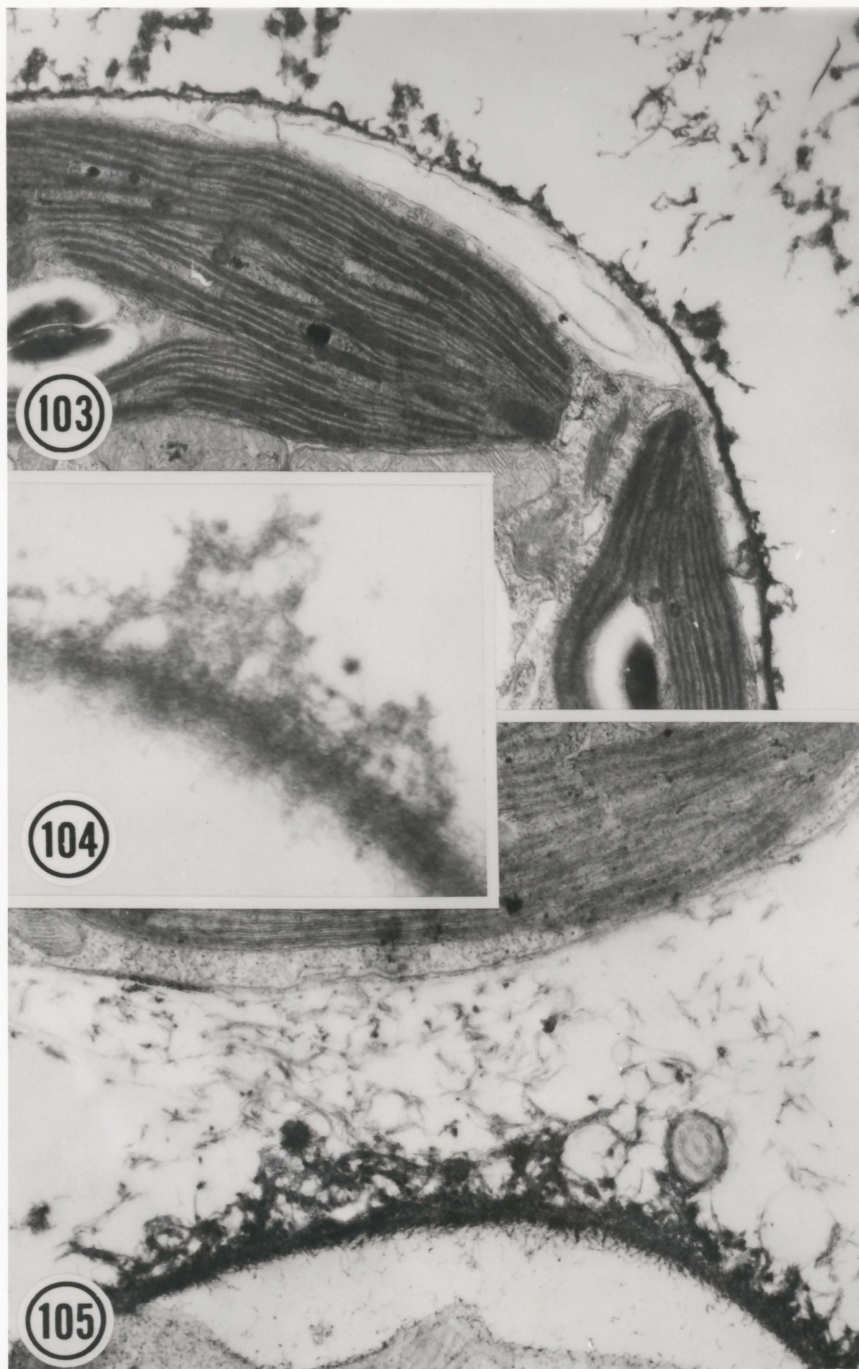


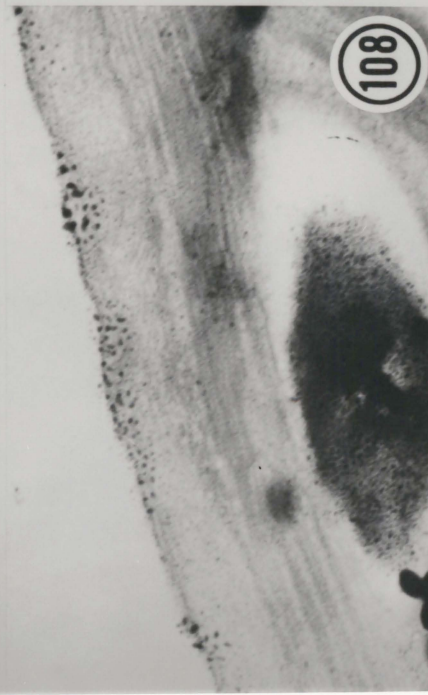
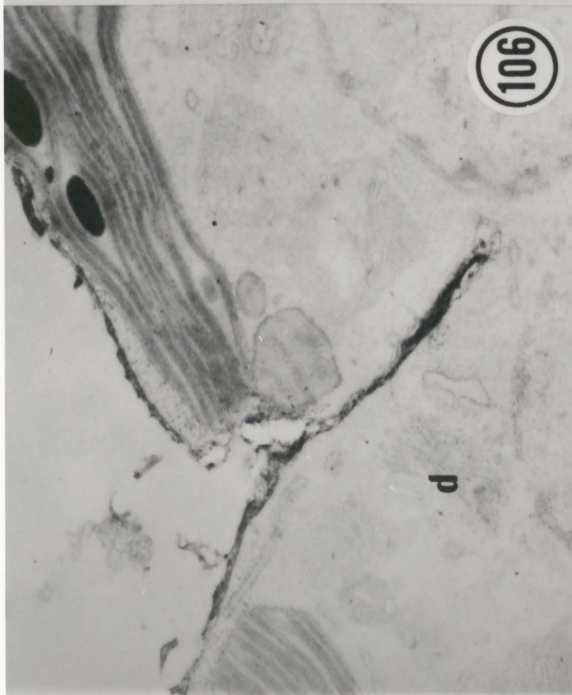
Plate XXVII. Distribution of Polysaccharide in Developing Lorica.

Figure 106. Deposition of polysaccharide material in the furrow between two forming daughter cells is shown by silver precipitate in a plane which will pass between the two daughter nuclei. A dictyosome is present to the left of this material. PA-TCH-SP stain. TEM thin section. X 20,600.

Figure 107. Higher magnification of polysaccharide material between two newly formed cells. PA-TCH-SP stain. TEM thin section. X 96,400.

Figure 108. Inception of lorica formation in a young cell. Thin layer of silver precipitate is evident on the cell surface, indicating the initiation of carbohydrate deposition. PA-TCH-SP stain. X 66,100.

Figure 109. An increase (c.f. Figure 108) in polysaccharide deposition on the surface of the cell is evident in this section. The amount of silver precipitate is more evenly distributed than that seen in Figure 108. PA-TCH-SP stain. TEM thin section. X 68,900.



accommodated by an expansion of the mother lorica, which involves its structural disintegration into fibrillar and core components (Plate XXVIII, Figures 110-117). As expansion proceeds, the mother lorica further distends by means of a dissociation of dense cores from the fibrillar layer (Plate XXVIII, Figures 114-117). The dense cores separate and disperse until only a fibrillar layer remains. This remaining layer is highly plastic and itself disintegrates into loosely associated fibrous components which disperse into the surrounding medium (Plate XXIX, Figures 118 and 119). By this autolytic process, the lorica is expanded and disintegrated thus accommodating the division and release of the new daughter cells.

Transport of Lorica and/or ECM Precursor Material

Two types of precursor materials are commonly seen in cytoplasmic vesicles and dictyosome cisternae. One is a finely fibrillar material that may be visualized in vesicles near the plasma membrane and in the space between the lorica and plasma membrane (Plate XXX, Figures 120-123). This material is similar in appearance to that seen in the ECM and in the fibrillar component of the lorica. Since the fine fibrillar strands which make up the mucilage are so similar in appearance to those in the lorica, it is difficult to distinguish them from each other inside of the cell.

Many vesicles also contain dense cores of heavily staining fibrillar material, similar in appearance to the dense components of the lorica. The dense cores are seen both in dictyosome cisternae and Golgi-derived vesicles (Plate XXXI, Figures 124-127).

Plate XXVIII. Release of Daughter Cells.

Figure 110. Mother lorica surrounds newly formed daughter cells. A structural breakdown of the lorica into fibrillar and dense cores has begun. AB/TEM thin section. X 13,700.

Figure 111. Three newly formed daughter cells with immature loricas. Pieces of the mother lorica remain around the cells. AB/TEM thin section. X 8,400.

Figure 112. Higher magnification of immature lorica and disintegrating mother lorica. Large openings have appeared in the old lorica matrix. AB/TEM thin section. X 25,800.

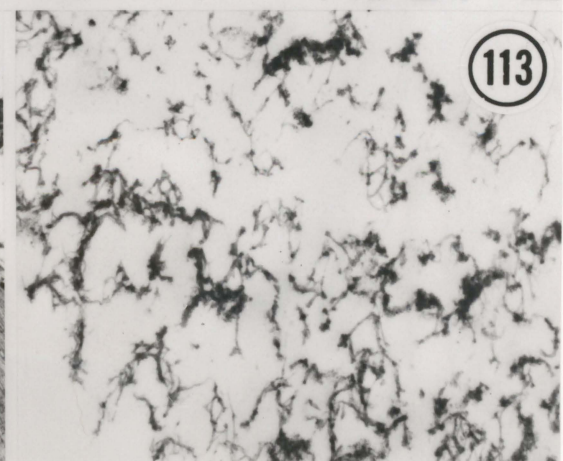
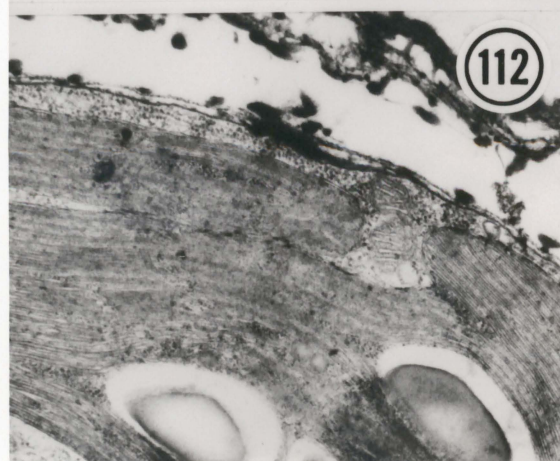
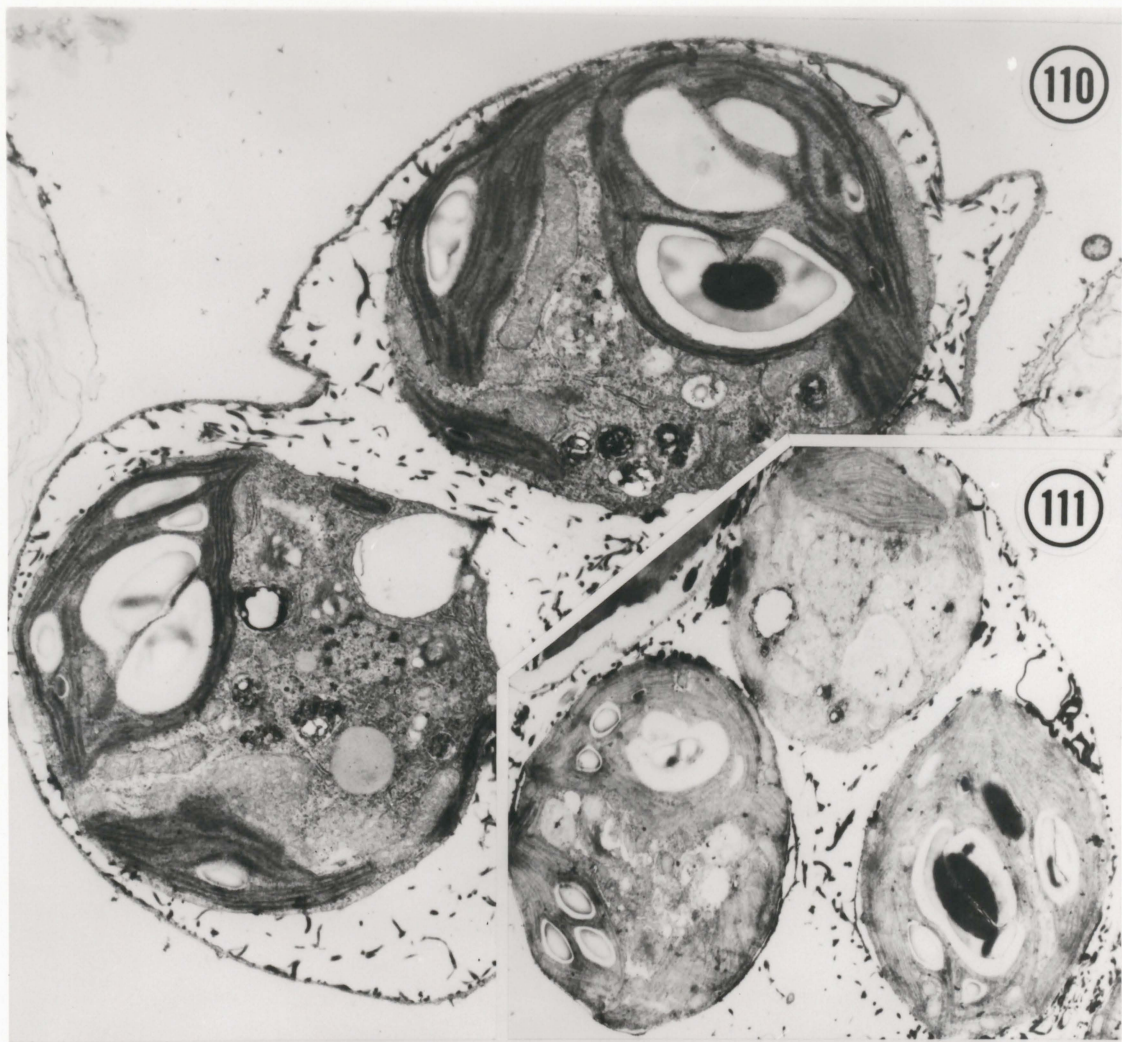
Figure 113. Higher magnification of dissociated lorica matrix. AB/TEM thin section. X 24,100.

Figure 114. Daughter cell with forming lorica is surrounded by part of the disintegrating mother lorica. Lorica is composed of an outer fibrillar layer with loosely attached dense cores on the inner side. AB/TEM thin section. X 20,100.

Figure 115. Glancing section through a newly formed cell and the remains of the mother lorica. Dense cores in mother lorica separate from the fibrillar layer at this stage. AB/TEM thin section. X 21,500.

Figure 116. Higher magnification of mother lorica. Dark cores are distinct from the fibrillar layer. AB/TEM thin section. X 57,600.

Figure 117. Integrity of mother lorica intact; however, openings in the lorica matrix are apparent. AB/TEM thin section. X 82,700.



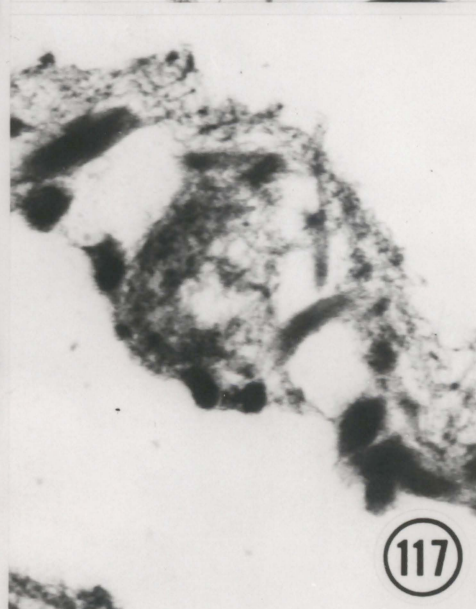
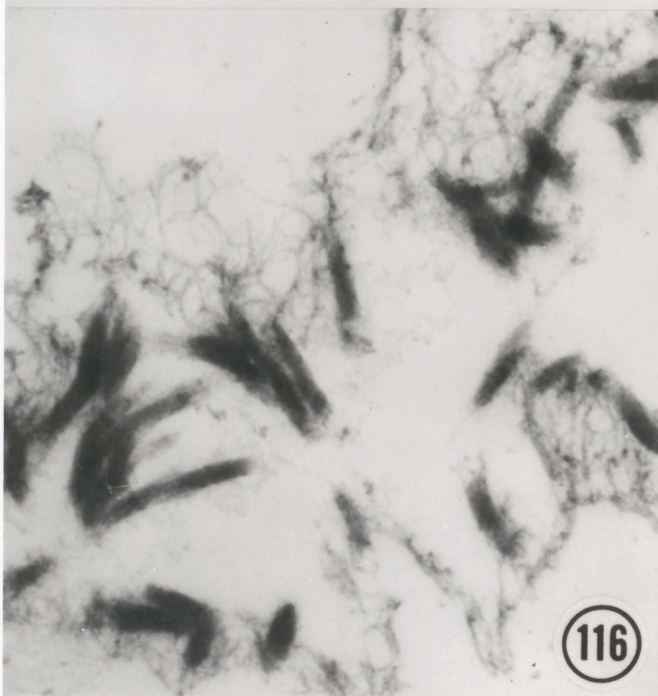
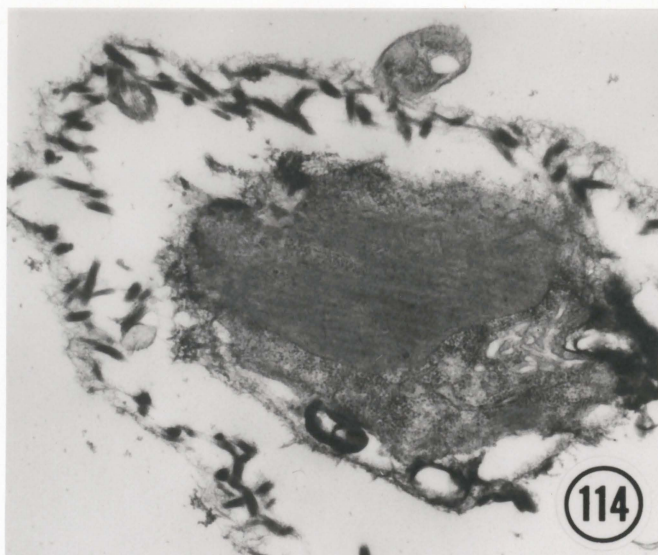


Plate XXIX. Disintegration of Lorica.

Figure 118. Glancing section through early stage of disintegrating lorica. Accumulation of fibrillar matrix and dispersal of dense cores is obvious. Stellate shape of dense cores is evident. AB/TEM thin section. X 31,000.

Figure 119. Late stage of disintegration. Separation of cores from fibrillar mats is complete. Dissociation of mats evident at top (arrow). AB/TEM thin section. X 27,500.

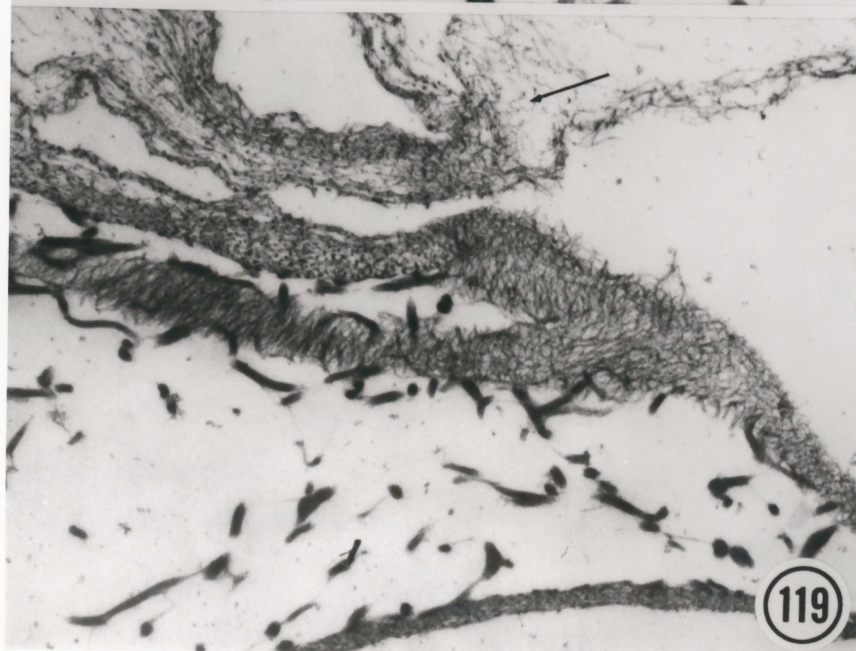
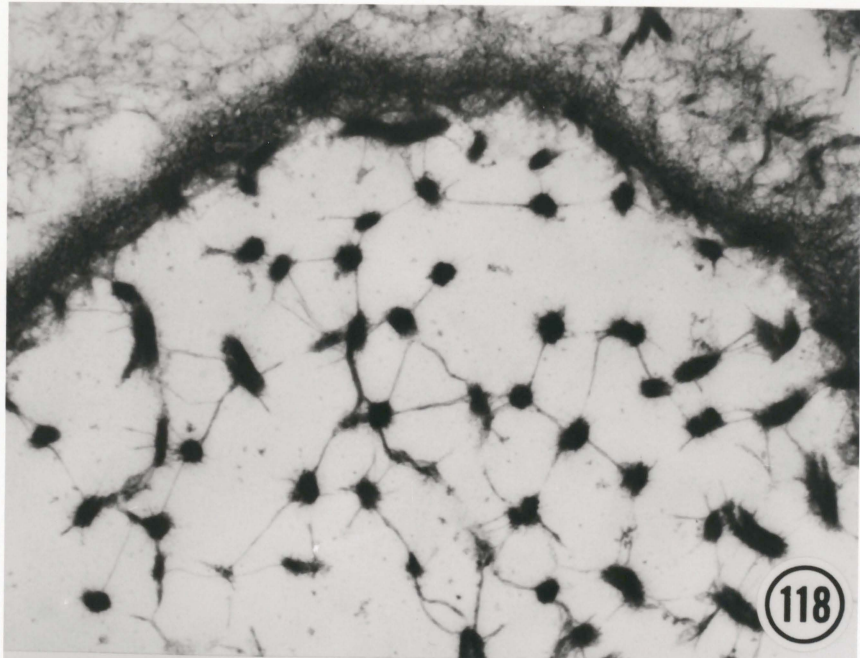


Plate XXX. Cellular Transport: Fibrillar Components.

Figure 120. Dictyosome and associated cytoplasmic vesicles. Vesicles contain wispy fibrillar material. AB/TEM thin section. X 17,200.

Figure 121. Cytoplasmic vesicles and two dictyosome cisternae in face view. Dictyosome cisternae contain wispy fibrillar material, while some adjacent vesicles also contain densely staining material. AB/TEM thin section. X 30,100.

Figure 122. Space between cell and lorica is filled with a wispy material that may be a lorica component or part of the ECM. AB/TEM thin section. X 29,100.

Figure 123. Large vesicles filled with small fibrils near the plasma membrane. AB/TEM thin section. X 23,300.

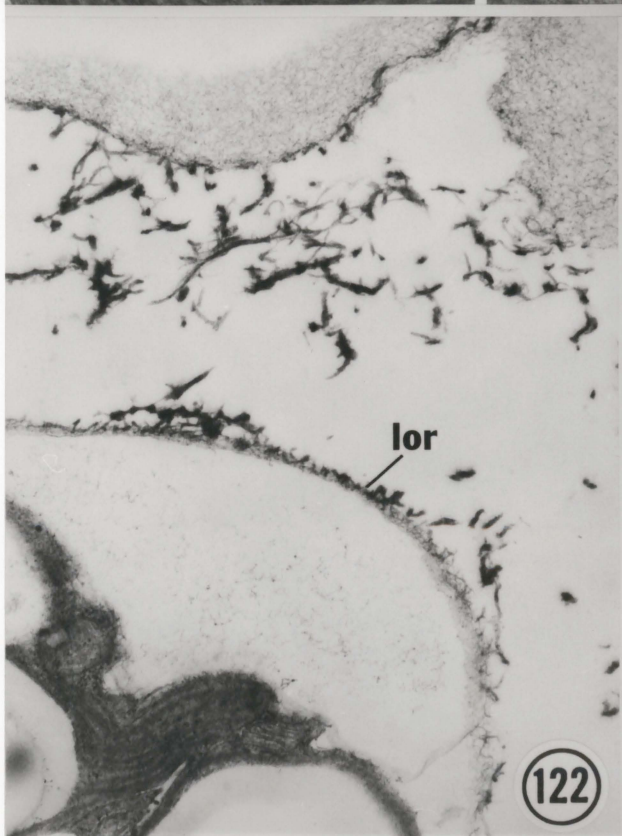
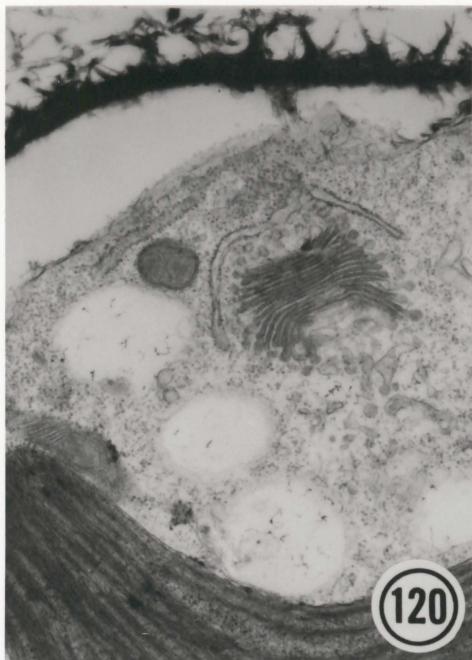


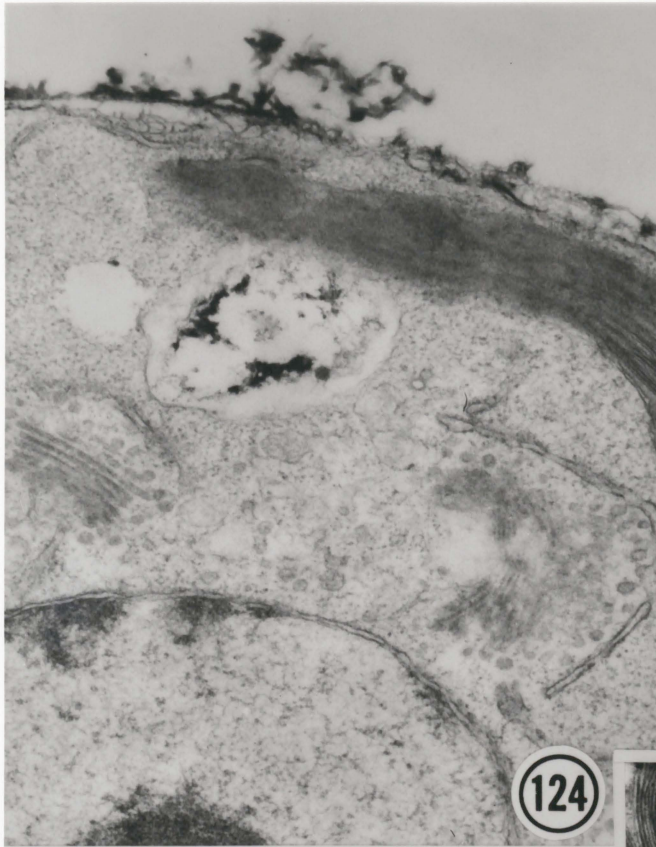
Plate XXXI. Cellular Transport: Dense Cores.

Figure 124. Cytoplasmic vesicles containing dark-staining dense cores, similar to those seen in lorica. AB/TEM thin section. X 28,400.

Figure 125. Several cytoplasmic vesicles containing both finely fibrillar and densely staining bodies. AB/TEM thin section. X 27,000.

Figure 126. Higher magnification of a vesicle filled with many dense cores, some stellate in shape (arrows). AB/TEM thin section. X 36,900.

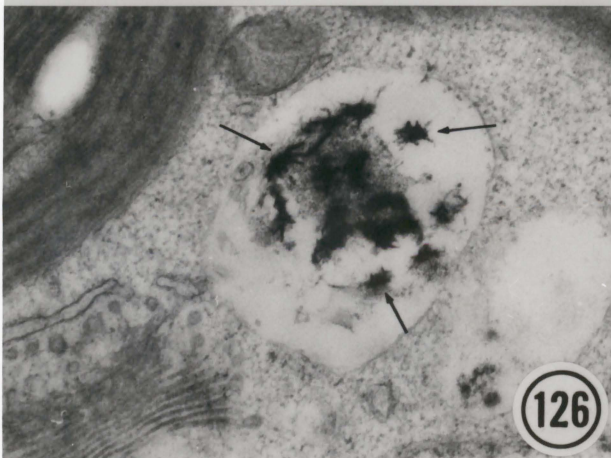
Figure 127. Two adjoining cytoplasmic vesicles. Most of the contents are wispy, fibrillar material; however, six dense cores are also present. AB/TEM thin section. X 28,000.



124



125



126



127

These two types of intracellular substances may sometimes occur in the same vesicle (Plate XXXI, Figures 125 and 127) but are most often seen in separate vesicles. They are presumably exported from the cell by reverse pinocytosis and are subsequently incorporated into the lorica and ECM.

Extracellular Mucilage

In thin section, the extracellular mucilage secreted from Phacotus lenticularis is visualized as an assemblage of very fine fibrils. These fibrils occur as large mats which are found either free in the culture medium (Plate XXXII, Figure 129) or attached to the cell surface (Plate XXXII, Figure 128). The density of the mucilage varies; it may be tightly compacted (Plate XXXII, Figure 132) or loosely associated (Plate XXXII, Figures 130 and 131). The mucilage is not visible in thin section unless it has been previously stained with a cationic dye, such as Alcian Blue, suggesting a high anionic charge density in the mucilage.

Bacterial Associations

In cultures that are not axenic, coccoid and rod-shaped bacteria form specific associations with organic material found in the medium. Large mucilage fields often contain many embedded bacteria; these bacteria are seldom seen free in the medium (Plate XXXIII, Figure 133). Bacteria are also frequently surrounded by organic material which bears a strong resemblance to dissociated lorica components (Plate XXXIII, Figure 134). In both cases, the bacteria are in close physical contact

Plate XXXII. Extracellular Mucilage.

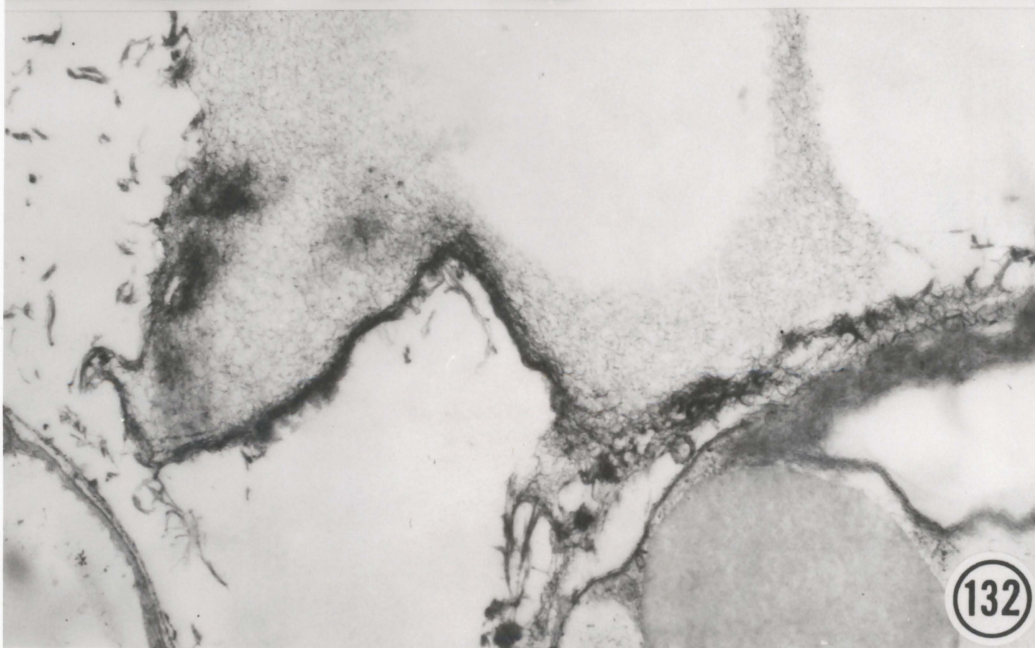
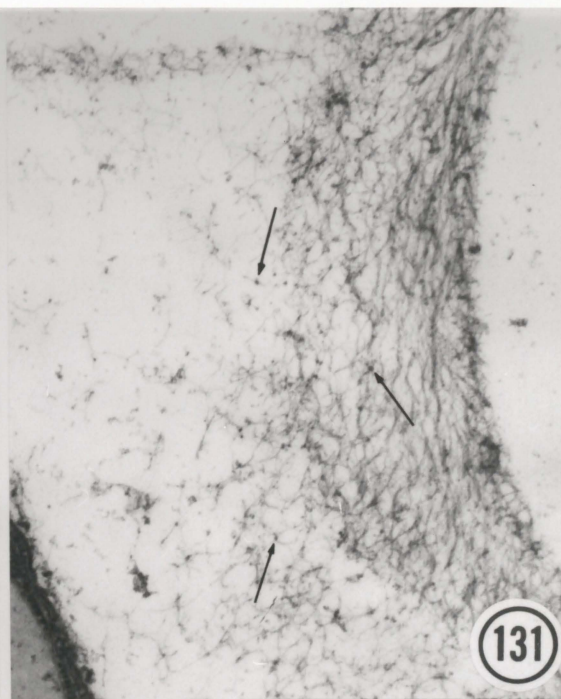
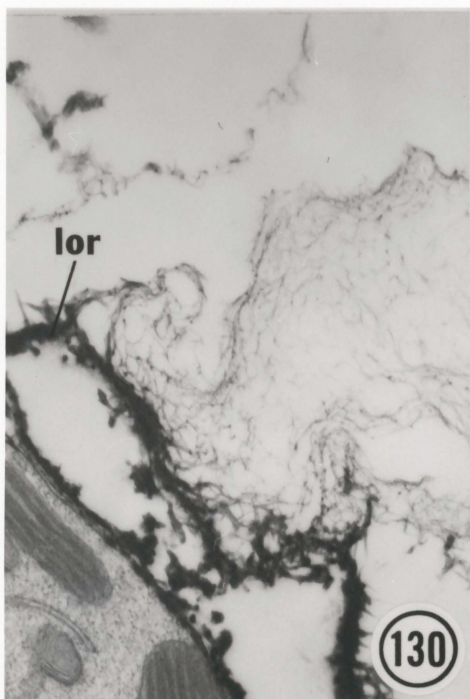
Figure 128. Extracellular mucilage which has been extruded from the cell and remains attached to the cell surface. A substructure of fine mucilage strands is obvious. AB/TEM thin section. X 25,800.

Figure 129. Extruded mucilage free in the culture medium shows same type of fibrillar substructure as that seen in Figure 128. AB/TEM thin section. X 32,500.

Figure 130. Extracellular mucilage associated with a disintegrating lorica. The fibrils of this mucilage are not tightly packed, revealing their threadlike nature. AB/TEM thin section. X 21,500.

Figure 131. Higher magnification of a field of extracellular mucilage. Fibrils consist of fine strands with occasional thickened portions (arrows). AB/TEM thin section. X 38,700.

Figure 132. Dense accumulation of mucilage on the surface of a young cell. AB/TEM thin section. X 32,700.



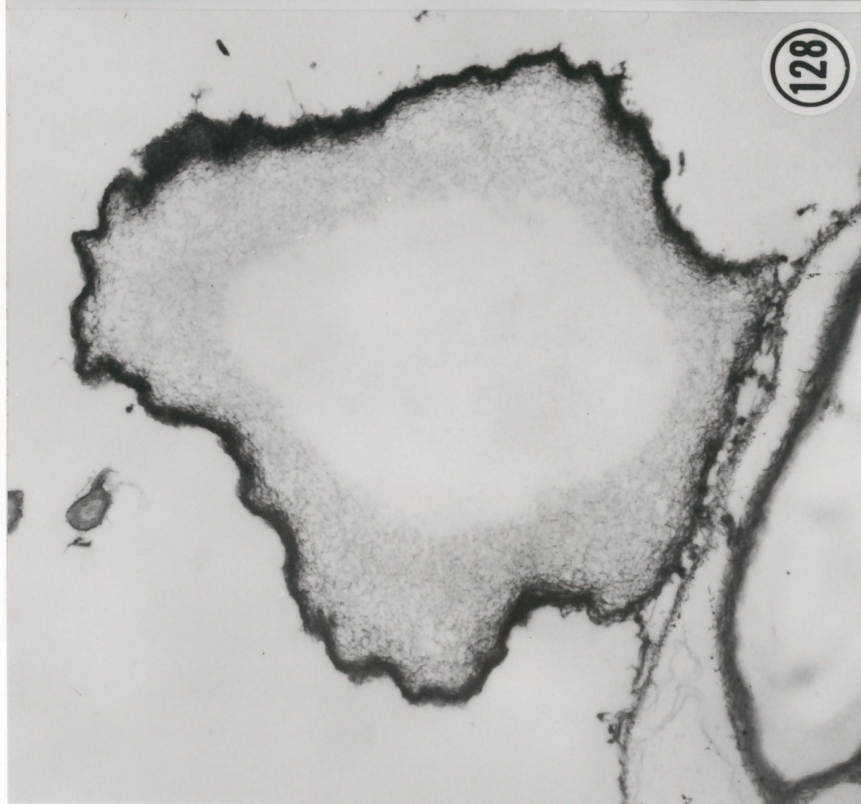
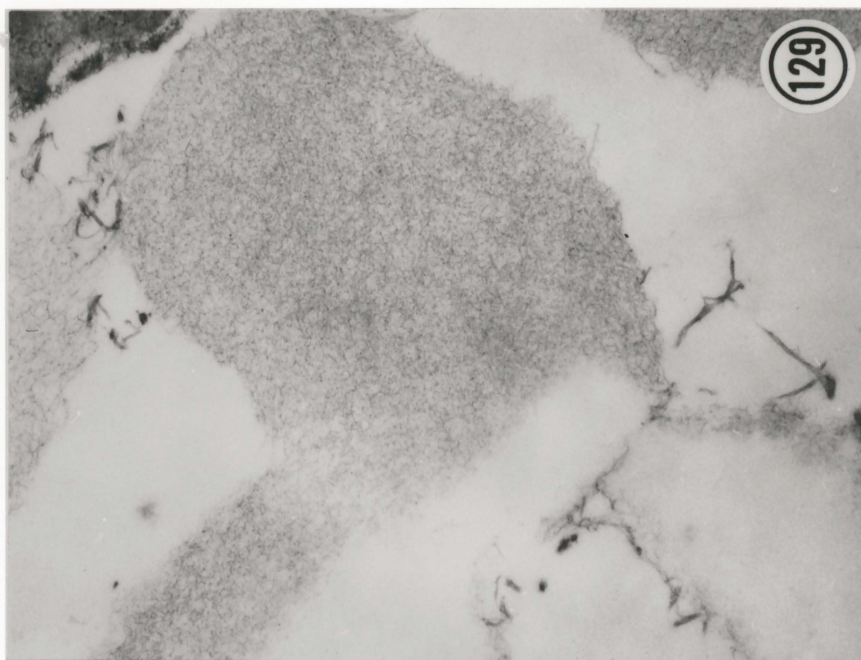
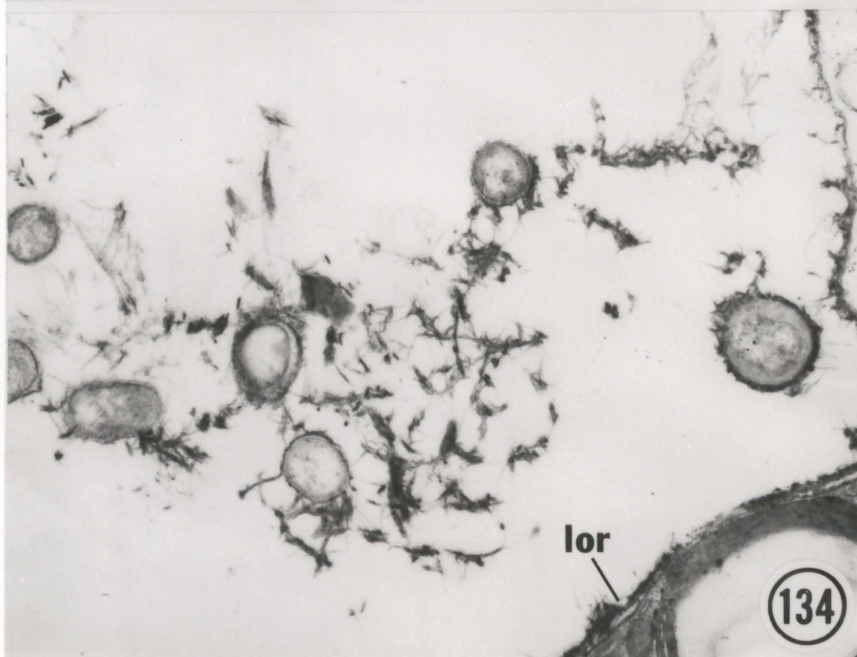
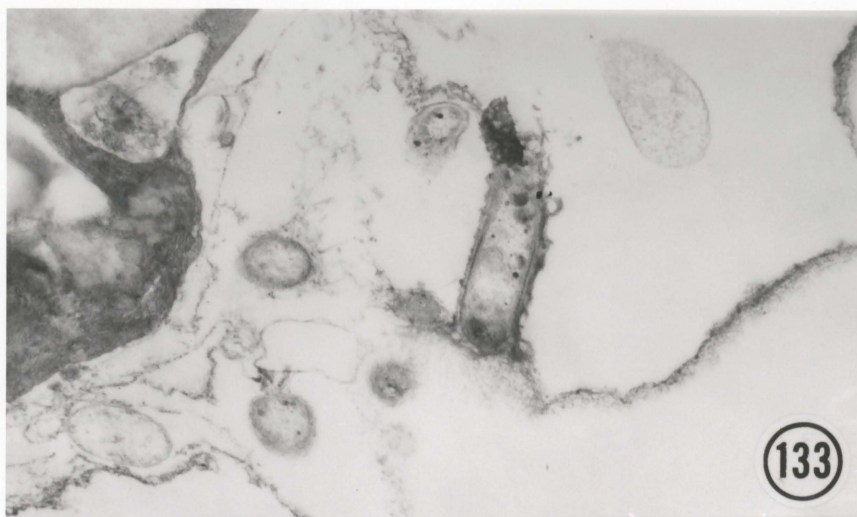


Plate XXXIII. Bacterial Associations.

Figure 133. Bacteria embedded in extracellular mucilage found near surface of a nearby cell. AB/TEM thin section. X 25,800.

Figure 134. Bacteria in close association with fibrils that resemble dissociated lorica components (c.f. fibrils and lorica in the lower right corner). AB/TEM thin section. X 27,500.



with the organic matter, suggesting that the organics may serve as a nutrient source for the bacteria.

3. ANALYTICAL RESULTS

Elemental Analysis of Isolated Loricas

Dark loricas. Cell-free dark loricas from 2-month-old soil-water cultures were harvested, washed 5 times with distilled water and subsequently analyzed with SEM-EDS for elemental content. A typical spectrum together with an SEM micrograph of the corresponding dark lorica are presented in Plate XXXIV, Figures 135 and 136.

Manganese is the major peak recorded for all dark loricas, with calcium as the next most common element. Aluminum, silicon, sulfur, potassium, chlorine and phosphorus are also found in small-to-trace quantities in all loricas examined. Barium and arsenic appear frequently but are not persistent components. Elements reported in Figure 136 are those most frequently recorded in dark loricas.

Colorless loricas. Cell-free colorless loricas were harvested from 2-month-old MVM-SWE and SW cultures, washed and analyzed with SEM-EDS for elemental composition. Representative spectra and accompanying micrographs appear in Plate XXXV, Figures 137-140.

In colorless loricas, no manganese is recorded for any part of the lorica. Instead, calcium is the predominant element, with much the same assortment of minor and trace elements as in dark loricas.

Plate XXXIV. SEM-EDS Spectrum of Individual Dark Lorica.

Figure 135. Uncoated dark lorica with numerous blunt surface projections. Area of analysis enclosed by the square. X 5,600.

Figure 136. Spectrum derived from area indicated in Figure 135. Manganese is the predominant element, with calcium as the second most commonly occurring element.

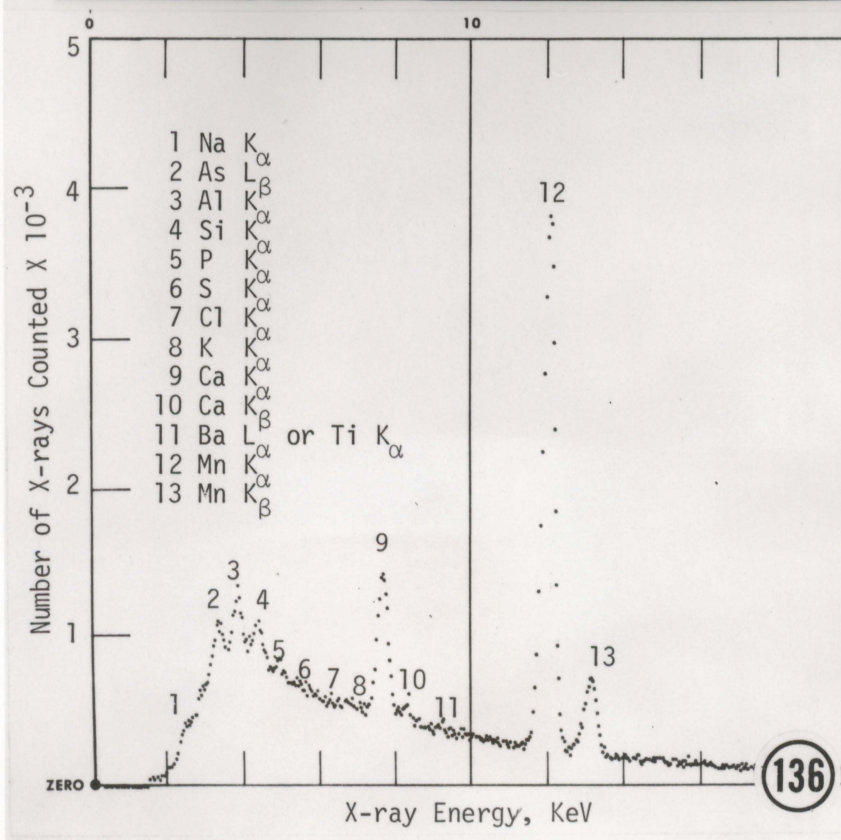
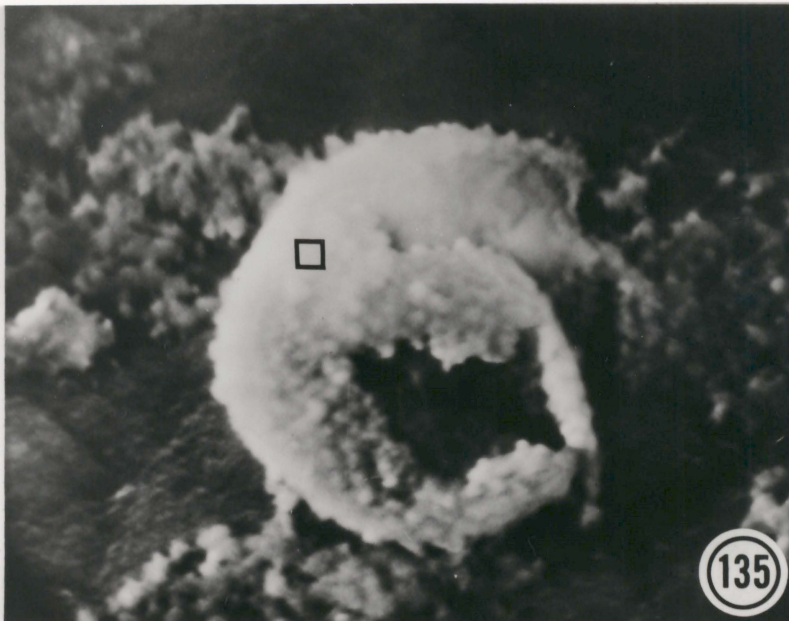


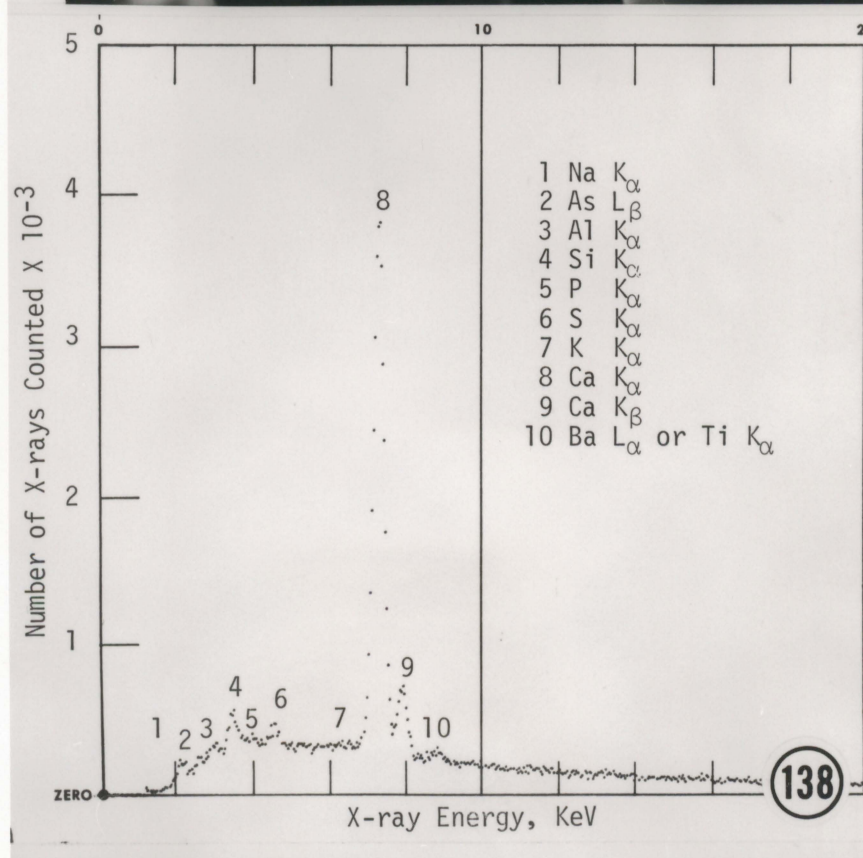
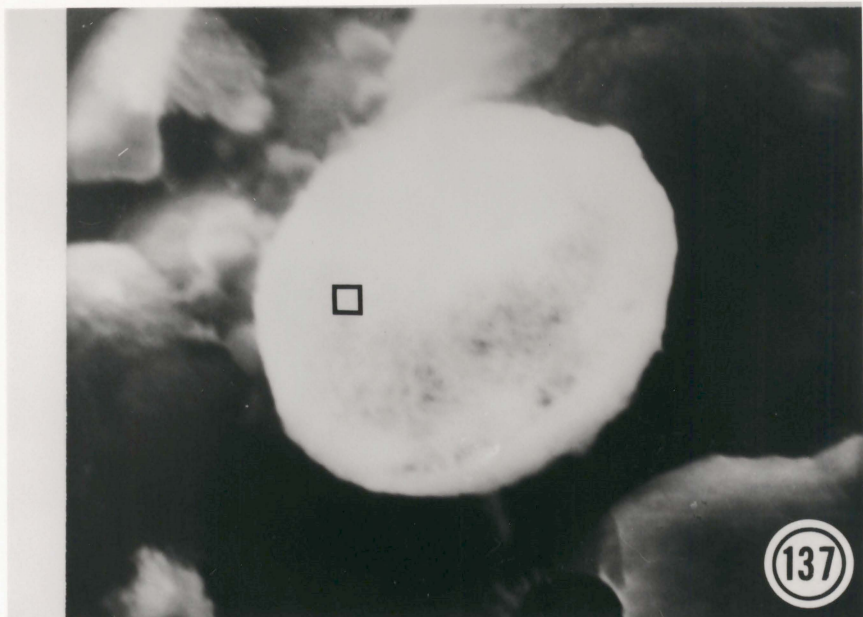
Plate XXXV. SEM-EDS Spectrum of an Individual Colorless Lorica.

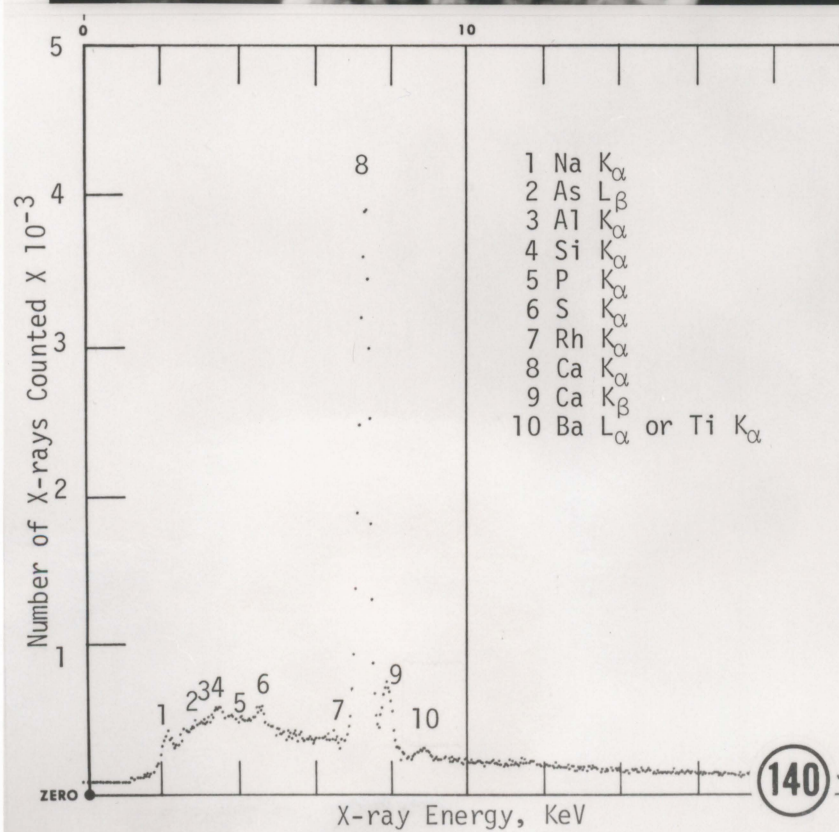
Figure 137. Exterior of an uncoated colorless lorica with no obvious surface ornamentation. Area of analysis enclosed by square. X 5,600.

Figure 138. Spectrum derived from area indicated in Figure 137. Calcium is the predominant element. Note lack of manganese (c.f. Figures 135 and 136).

Figure 139. Interior of an empty, colorless lorica which has separated into two halves. Surface ornamentation is not apparent. Area of analysis enclosed by square. X 5,600.

Figure 140. Spectrum derived from area indicated in Figure 139. This spectrum is very similar to that shown in Figure 138, with calcium as the predominant element.





Comparative spectra of colorless and dark loricas are presented in Plate XXXVI, Figures 141-143. These data indicate that the color of the lorica is a direct function of the presence of manganese.

Manganese-Supplemented Cultures

Neither the addition of manganese chloride nor manganese acetate to the culture medium promoted any detectable changes in lorica structure or composition. Cell growth and lorica development in supplemented cultures were indistinguishable from cultures with no manganese supplement.

Chelating Experiments

A chelating agent such as EDTA or EGTA will extract the brown color and certain metal components of the lorica. Since MVM medium contains Na_2EDTA as a component, the EDTA may prevent the loricas from darkening in defined medium. Therefore, EDTA was omitted from MVM medium to test the effects of the absence of a metal chelator on lorica structure and composition.

Cell density in such a medium was less than that of control cells. Cell morphology was identical to control cells, with no darkening of the loricas. It is possible that inhibition of growth was caused by the toxicity of certain metals in the absence of EDTA.

EDTA treatment. Isolated dark loricas from 2-month-old soil-water cultures were treated with 5 mM EDTA for 0-30 minutes. With light microscopy, a gradual loss of color is evident over this time (Plate XXXVII, Figures 144-147). After 30 minutes, little or no brown color is visible in the loricas.

Plate XXXVI. A Comparison of Structure, Surface Morphology and SEM-EDS Spectra of Colorless and Dark Loricas.

Figure 141. Uncoated, unstained TEM whole mounts of isolated colorless loricas shows loose meshwork of lorica matrix and marginal projections. X 12,400.

Figure 142. Uncoated, unstained TEM whole mount of isolated dark loricas. Note the coarser matrix (left) which results in a much more dense structure. Surface projections are evident along the margins of both loricas. X 10,575.

Figure 143. Comparative spectra of colorless (top) and dark (bottom) loricas. The white pointer on the bottom spectrum is at 5.89 KeV, the $K\alpha$ for manganese. There is no manganese recorded in the top (colorless lorica) spectrum. Both spectra show a peak for calcium (white arrows).

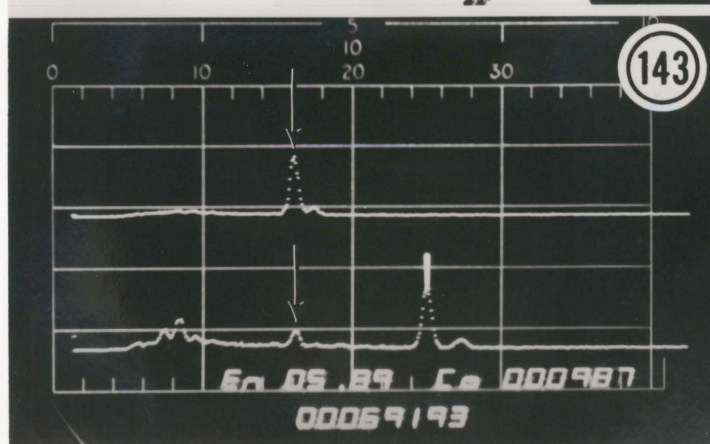
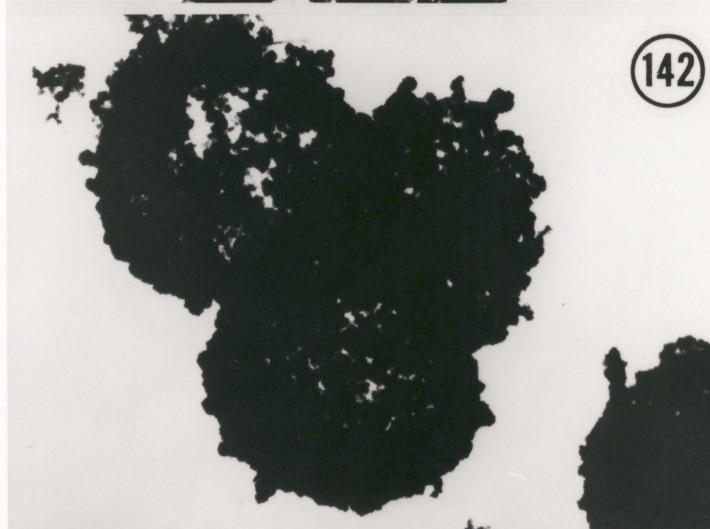
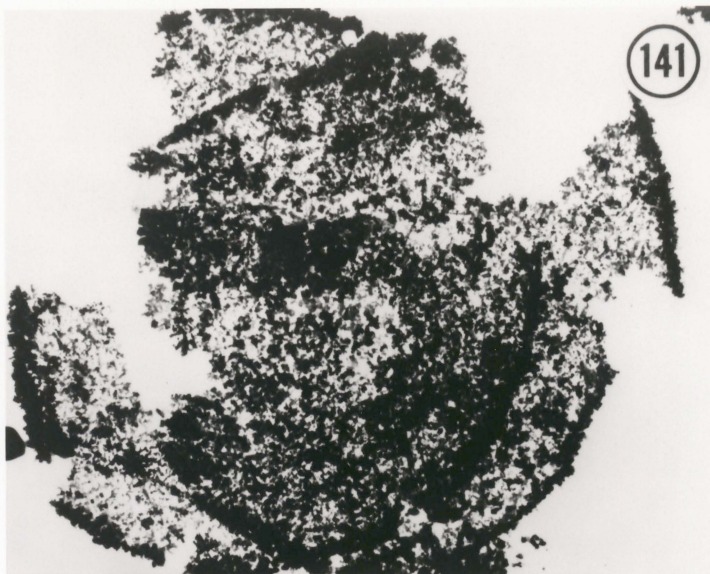


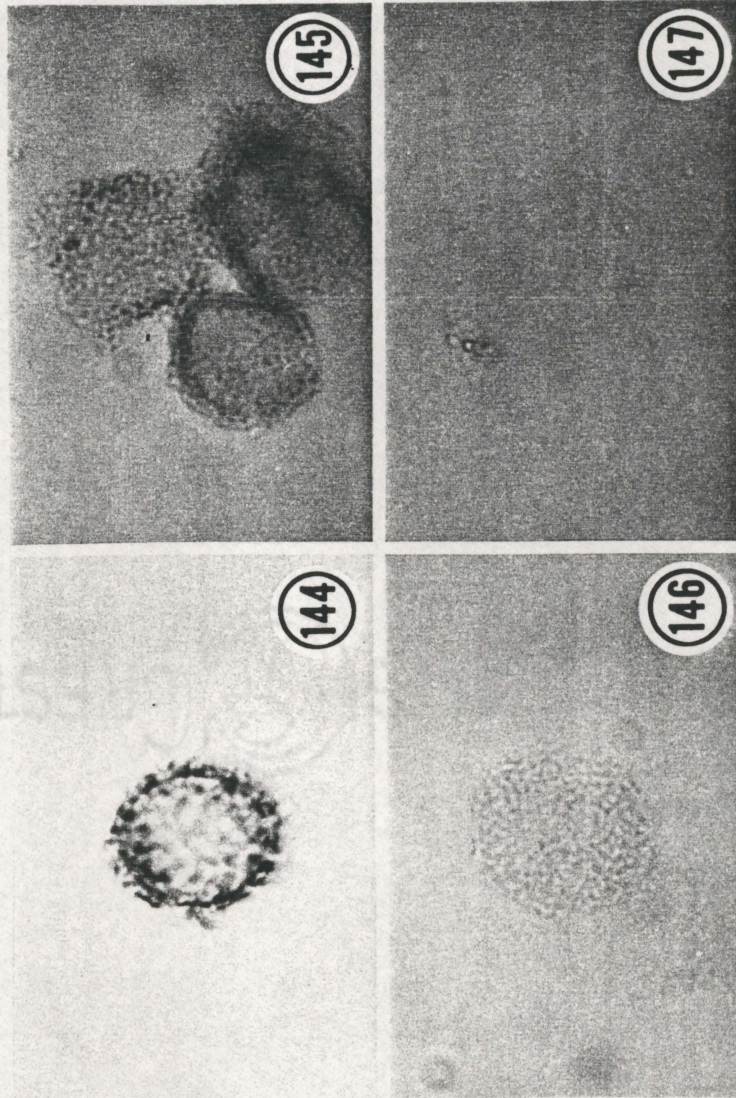
Plate XXXVII. Light Microscopy of EDTA-Treated Loricas.

Figure 144. Dark (brown) lorica at 0 min., 5 mM EDTA. LM. Bright-field illumination. X 2,100.

Figure 145. Dark lorica after 5 min., 5 mM EDTA. Some loss of color is visible. LM. Bright-field illumination. X 2,100.

Figure 146. Dark lorica after 15 min., 5 mM EDTA. Very little color remains. LM. Bright-field illumination. X 2,100.

Figure 147. Dark lorica after 30 min., 5 mM EDTA. No color is visible and the outline of the lorica is difficult to discern. LM. Bright-field illumination. X 2,100.



EDTA-treated loricas and dark controls have very different SEM-EDS spectra (Figures 148 and 149). Control brown loricas have prominent manganese K_{α} and K_{β} peaks. Calcium and aluminum are the next most prominent elements, with sodium, arsenic, silicon, phosphorus, sulfur, potassium and barium (or titanium) present in lesser amounts (Figure 148).

In loricas treated for 30 minutes with 5 mM EDTA, no manganese peak is present and the calcium and aluminum peaks are much smaller (Figure 149). Sodium is the most prominent element in this spectrum; it is a result of treatment with the disodium salt of EDTA and should be considered an artefact. Other minor elements (e.g., phosphorus, silicon, sulfur, chlorine and potassium) are present in variable trace amounts.

EGTA treatment. Isolated dark loricas from 2-month-old soil-water cultures were treated with 1 mM EGTA for 0 to 30 minutes (Figures 150 and 151). The loss of brown color from these loricas, as seen with light microscopy, is similar to that observed with EDTA treatment except that with EGTA, the loss of color was more rapid.

SEM-EDS analysis of a brown control lorica (Figure 150) reveals that manganese is the principal element with calcium and aluminum found in lesser amounts. Several trace elements are also recorded.

A spectrum derived from a lorica treated for 10 minutes in 1 mM EGTA is somewhat unusual (Figure 151). Manganese is present in negligible amounts; however, calcium, silicon and phosphorus are

Figure 148. Representative spectrum from a dark, untreated lorica, with a prominent manganese peak.

Figure 149. Spectrum from dark lorica treated with 10 mM EDTA for 30 minutes. Manganese is absent; relative amounts of other elements are also reduced. The sodium peak is a result of the treatment.

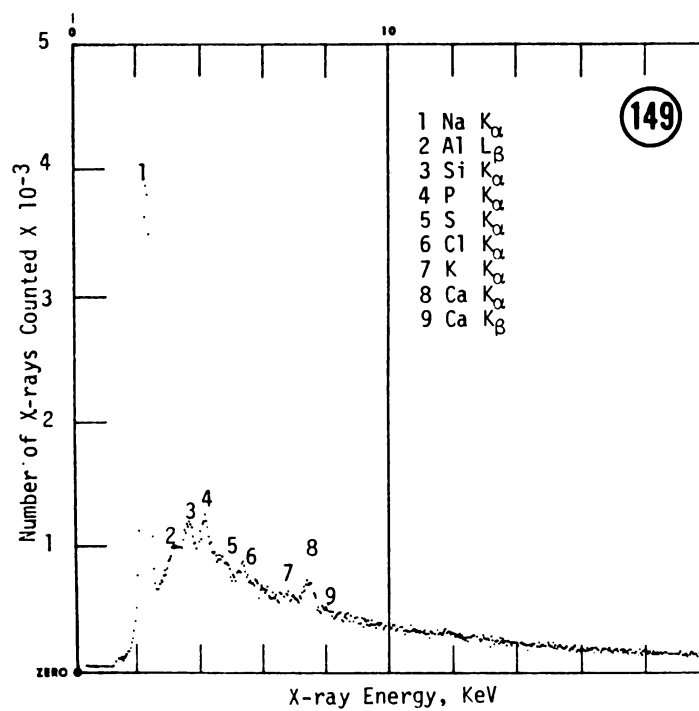
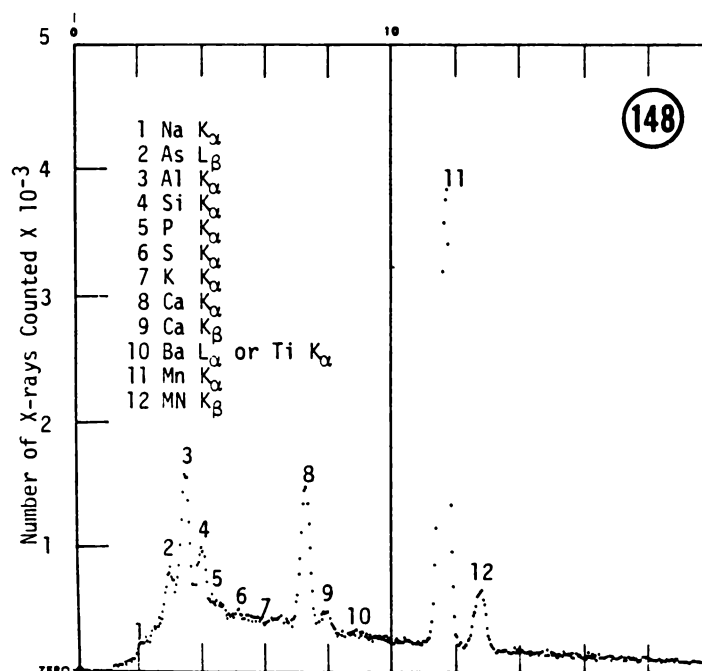
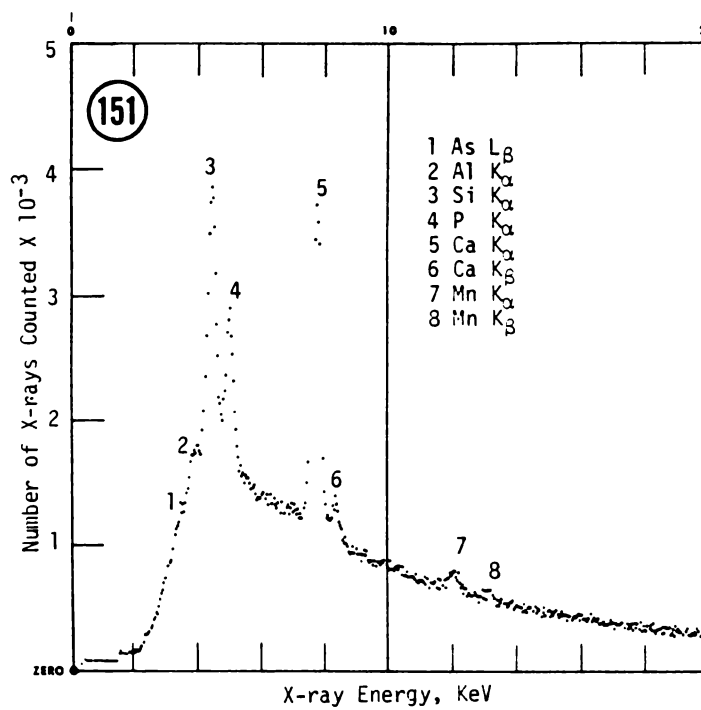
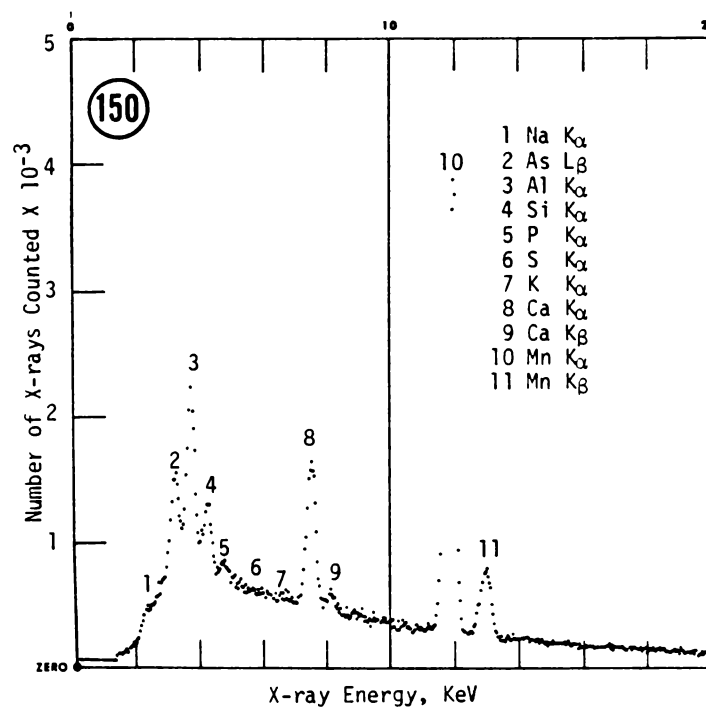


Figure 150. Representative spectrum from a dark, untreated lorica. Note manganese peak.

Figure 151. Spectrum of a dark lorica treated with 1 mM EGTA for 10 minutes. The remaining manganese is negligible, whereas some elements (i.e., Ca, Si and P) are present in somewhat higher amounts than in the untreated lorica.



found in equal or greater amounts than in the untreated loricas. Arsenic and aluminum appear to be in lesser amounts, whereas several other trace elements (sodium, sulfur and potassium) are absent.

Characterization of the Lorica Matrix

Cytochemical tests. Loricas from MVM-SWE cultures were harvested from the bottoms of cultures, washed five times with distilled water and then stained with one of several cytochemical stains. This particular culture medium was chosen since most loricas of cells grown in MVM-SWE are colorless, and the staining reaction is, therefore, readily seen.

Positive reactions are obtained with the following stains, selective for carbohydrates: Alcian Blue (AB), pH 0.5; Alcian Yellow (AY), pH 2.5; PAS; Methylene Blue (MB) and Toluidine Blue 0 (TBO), pH 5.6, 4.2 and 1.0. A positive reaction to AB at pH 0.5 is indicative of the presence of sulfate groups in the carbohydrate moiety of the lorica, while only carboxyl groups bind to AY at pH 2.5 (Plate XXXVIII, Figures 152 and 159). PAS is a general indicator of polysaccharides, while MB suggests the presence of pectic substances in the lorica (Plate XXXVIII, Figures 154 and 155). Metachromatic staining of the lorica with TBO at pH 5.6 reveals the presence of an acid polymer (Plate XXXVIII, Figure 156). Most of the pink stain is abolished at pH 4.2, a result of carboxyl groups in the polymer (Plate XXXVIII, Figure 157). Loricas stained at pH 1.0 have a small amount of metachromatic stain, indicative of either sulfate or polyphosphate groups (Plate XXXVIII, Figure 158).

A positive reaction to bromphenol blue in the loricas suggests a protein component in the matrix (Plate XXXVIII, Figure 153). Tests

Plate XXXVIII. Cytochemical Stains.

Figure 152. Lorica stained with Alcian Blue at pH 0.5, indicating the presence of sulfated polysaccharides. LM. Bright-field illumination. X 2100.

Figure 153. Positive reaction to Mercuric Bromphenol Blue, suggesting a protein component in the lorica. LM. Bright-field illumination. X 2100.

Figure 154. Positive reaction to PAS, a general stain for carbohydrates. LM. Bright-field illumination. X 800.

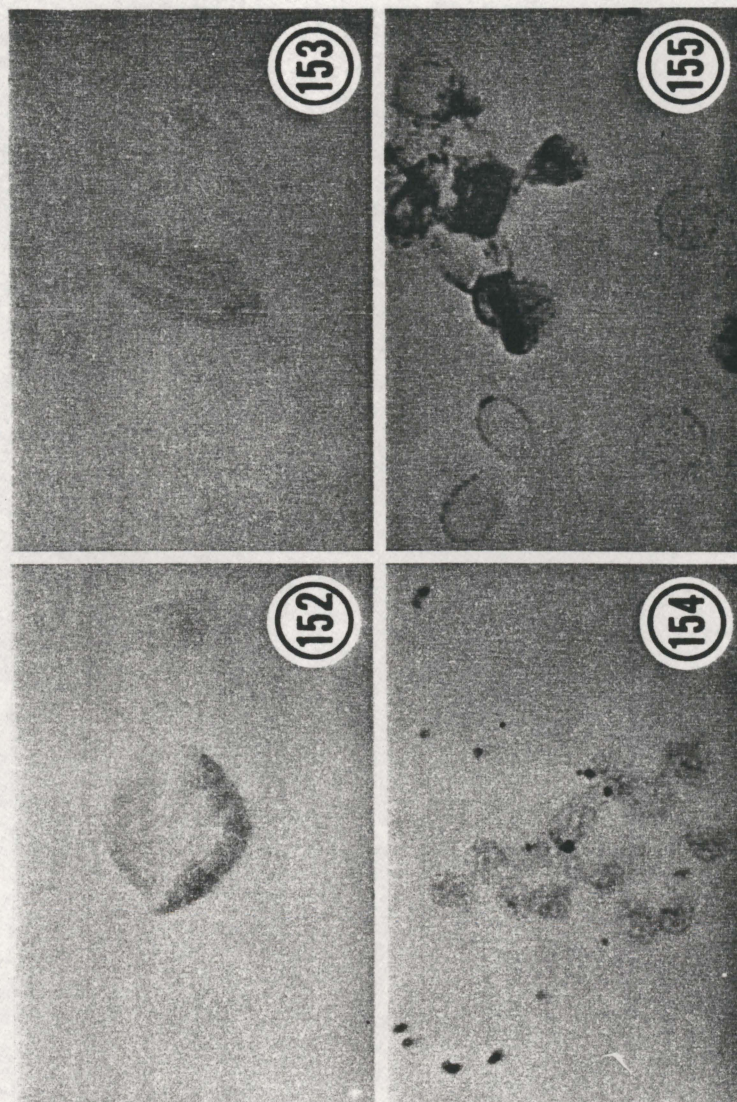
Figure 155. Loricas stained with Methylene Blue, indicating a pectin-like compound in the lorica matrix. LM. Bright-field illumination. X 800.

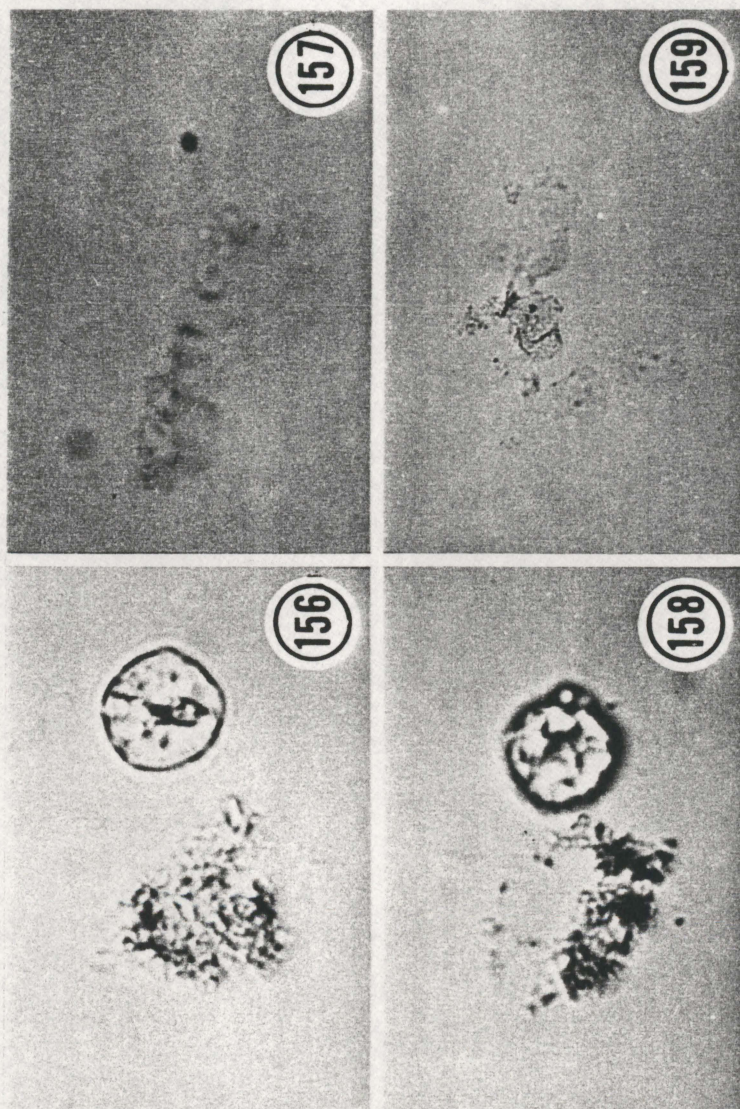
Figure 156. Lorica stained with Toluidine Blue O at pH 5.6. Metachromatic color indicates the presence of acidic polymers in the lorica matrix. LM. Bright-field illumination. X 2100.

Figure 157. Lorica stained with TB0 at pH 4.2. The disappearance of pink color (c.f. Figure 156) is due to the presence of carboxylated non-sulfated polysaccharides in the lorica. LM. Bright-field illumination. X 2100.

Figure 158. Lorica stained with TB0 at pH 1.0. Metachromasia suggests the presence of sulfate and/or polyphosphate groups in the lorica. LM. Bright-field illumination. X 2100.

Figure 159. Faint positive reaction of the lorica matrix with Alcian Yellow, pH 2.5, suggesting the presence of carboxylated polysaccharides. LM. Bright-field illumination. X 800.





for the presence of iron with potassium ferrocyanide are negative.

Enzyme digestion. When loricas are subjected to enzyme digestion, structural alteration is evident. Trypsin treatment results in a loss of lorica matrix components. Depending upon the thickness and condition of the lorica, removal of the material is partial to complete (Plate XXXIX, Figures 160-165). Mild trypsin digestion results in a perforated lorica, with a fibrous meshwork of dense cores separated by large gaps (Plate XXXIX, Figure 164). Further digestion leaves only large clumps of densely stained fibrils and sections of the granulo-fibrillar basal layer (Plate XXXIX, Figure 163). In both cases, trypsin removes much of the lorica matrix material before it acts on the dense core material.

Protease has a more pronounced effect on lorica structure and causes a greater disruption of lorica integrity. The lorica usually separates into fibrous mats or becomes completely amorphous after protease digestion (Plate XXXIX, Figure 165).

With both trypsin and protease, the loss of one or more lorica constituents results in a loss of the integrity of the lorica as a whole and dissociation of the remaining components.

Colorimetric analysis of the lorica. Loricas used for all chemical analyses were isolated from either MVM-SWE cultures or soil-water cultures. Loricas were separated from cellular debris by differential centrifugation, washed with distilled water and subsequently analyzed. The composition of the isolated loricas, by weight, is presented in Table X, which shows that the lorica has a

Plate XXXIX. Enzyme Treatments.

Figure 160. Transverse section of a protease-digested lorica. A basal layer remains close to the surface of the cell, while most of the lorica matrix has separated. Most of the remaining matrix is a dense mat of fine fibrillar material. A few dense cores are visible (arrows). AB/TEM thin section. X 21,500.

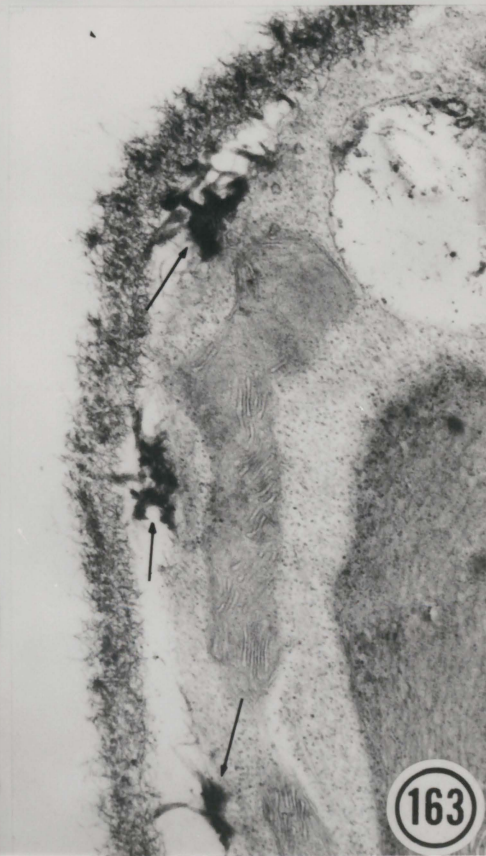
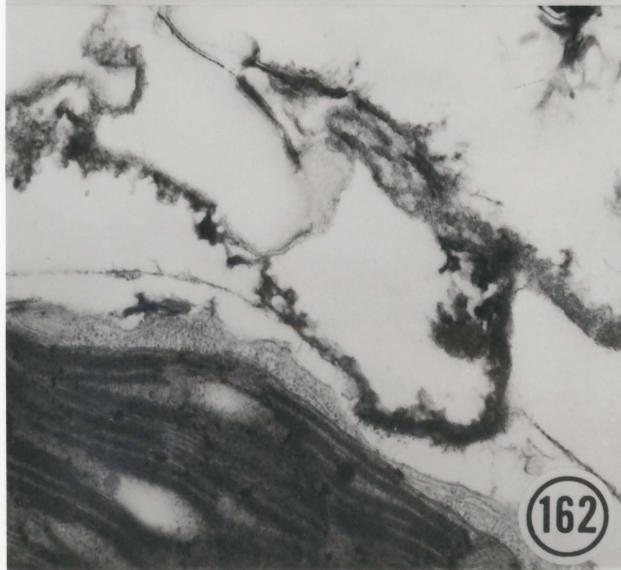
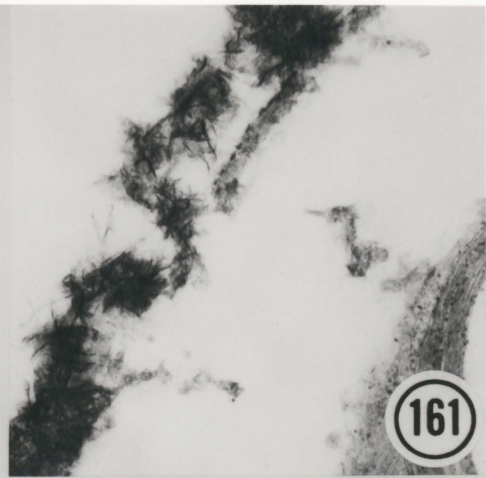
Figure 161. Lorica which has undergone extensive protease digestion. Structural alteration is severe; only an amorphous fibrous mat remains. AB/TEM thin section. X 23,700.

Figure 162. Cross section of a trypsin-digested lorica. Treatment has removed portions of the lorica matrix, leaving fibrous mats attached to a granulo-fibrillar basal layer. AB/TEM thin section. X 33,200.

Figure 163. Trypsin-digested lorica. Only a loose fibrillar mat remains. Some dark core material is visible (arrows). AB/TEM thin section. X 34,400.

Figure 164. Cross section of trypsin-treated cell and lorica shows perforated lorica matrix, revealing dense cores (c.f. Figure 78). AB/TEM thin section. X 59,000.

Figure 165. Protease-treated lorica shows disruption of structural integrity. AB/TEM thin section. X 20,900.



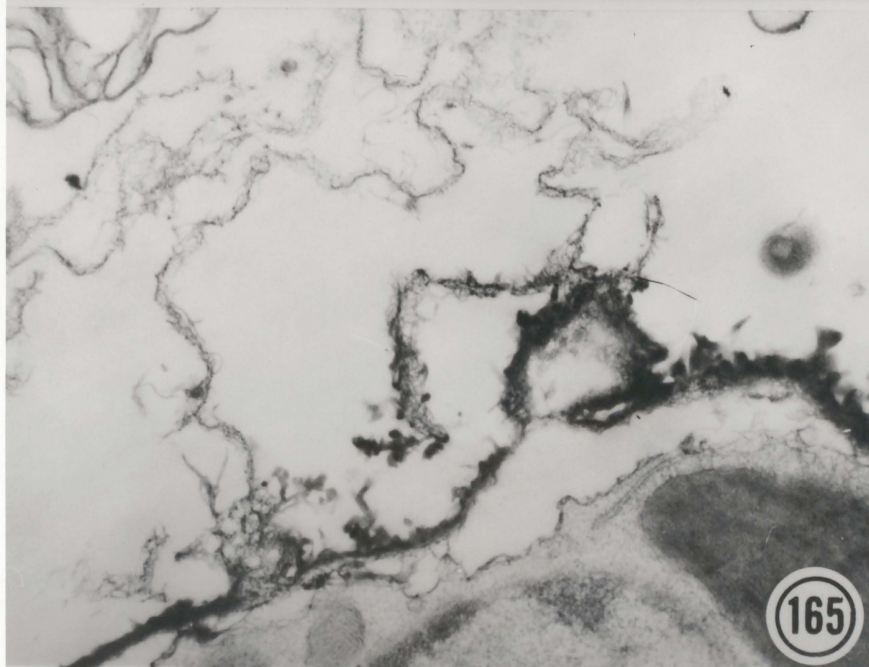
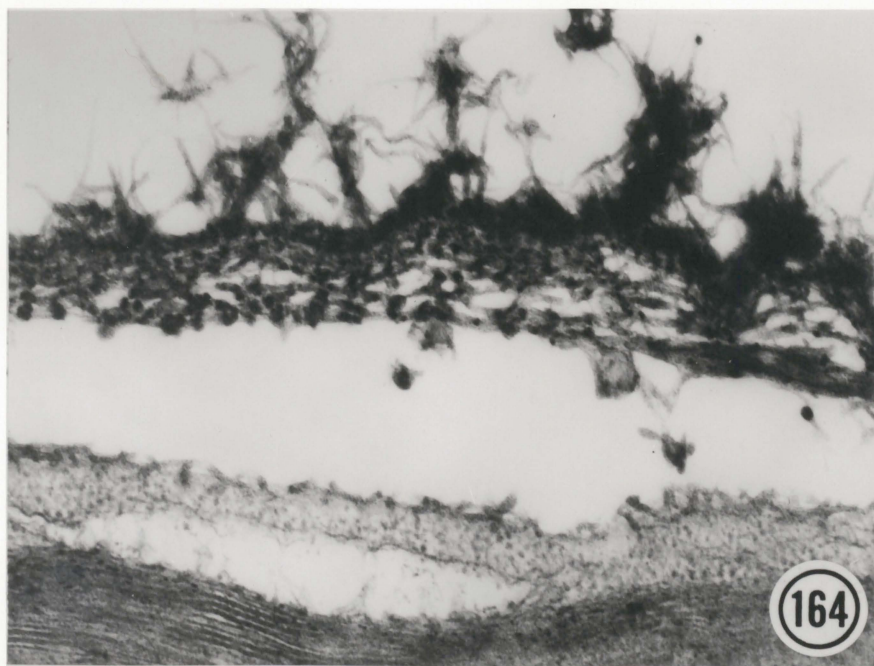


TABLE X

CHEMICAL COMPOSITION OF THE LORICA MATRIX OF
PHACOTUS LENTICULARIS

Constituent	Percentage of Dry Weight
Neutral Sugars	36
Protein	26
Sulfate	4
Total	66

lower carbohydrate and sulfate content than the ECM, but a higher amount of protein (cf. page 162). These three components account for only 66% of the total weight of the loricas; it is probable that the metals in the lorica account for the balance of the dry weight.

Carbohydrate composition of the lorica: Qualitative thin-layer chromatography. The carbohydrate composition of the lorica was investigated by thin-layer chromatography of TFA hydrolysates on cellulose supports using Solvent System I.

Thin-layer chromatography reveals only two components, as visualized with anisidine-HCl (Figure 166 and Table XI). By comparison with standards, the lower spot is identified as glucose; however, the spot with greater mobility remains unidentified.

Carbohydrate composition of the lorica: Qualitative gas-liquid chromatography. To gain corroborative information about the sugar(s) identified with TLC, hydrolyzed loricas were also analyzed with gas-liquid chromatography.

Only two peaks are consistently found in lorica hydrolysates (Figure 167). The larger peak, with a retention time of 10.4 minutes, corresponds to glucose. The smaller peak, at 7.7 minutes, remains unidentified and may correspond to the unknown spot visualized with TLC.

Amino acid composition of the lorica. Because of the limited quantities of isolated loricas available, only preliminary information regarding the amino acid composition of the loricas was obtained. Two-dimensional thin-layer chromatography produced very light spots

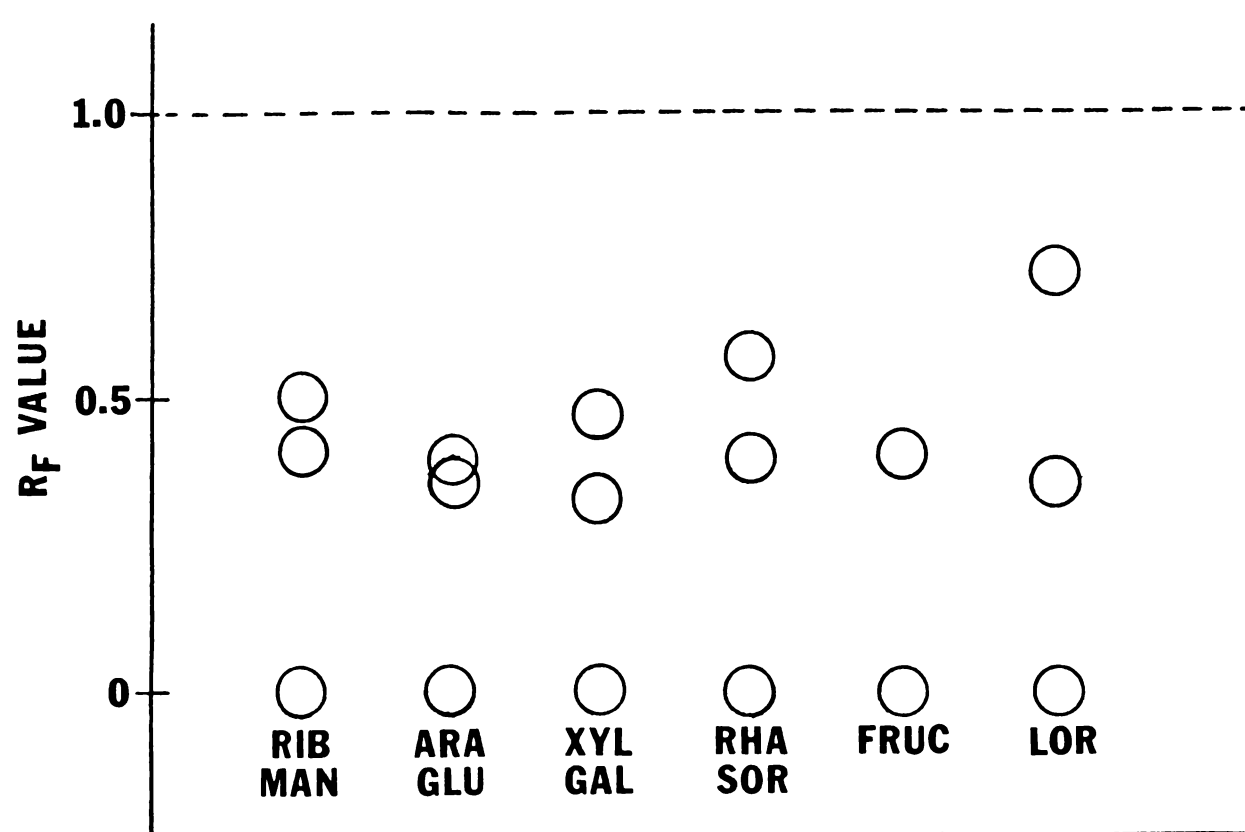


Figure 166. TLC profile of the neutral sugars in a typical lorica hydrolysate. Standards used for comparison are also shown (lanes 1-5). See Table XI for R_f values.

TABLE XI

A COMPARISON OF CARBOHYDRATE STANDARDS AND CARBOHYDRATE
COMPONENTS OF ISOLATED LORICAS ANALYZED BY
THIN-LAYER CHROMATOGRAPHY

Lane	Substance	R _f	Color, Anisidine-HCl
1	Ribose	0.51	Pink
	Mannose	0.41	Yellow-Brown
2	Arabinose	0.41	Pink
	Glucose	0.37	Brown
3	Xylose	0.48	Pink
	Galactose	0.34	Brown
4	Rhamnose	0.59	Gold
	Sorbose	0.41	Gold
5	Fructose	0.41	Yellow
6	Lorica #1	0.37	Brown
	Lorica #2	0.73	Gray-Brown

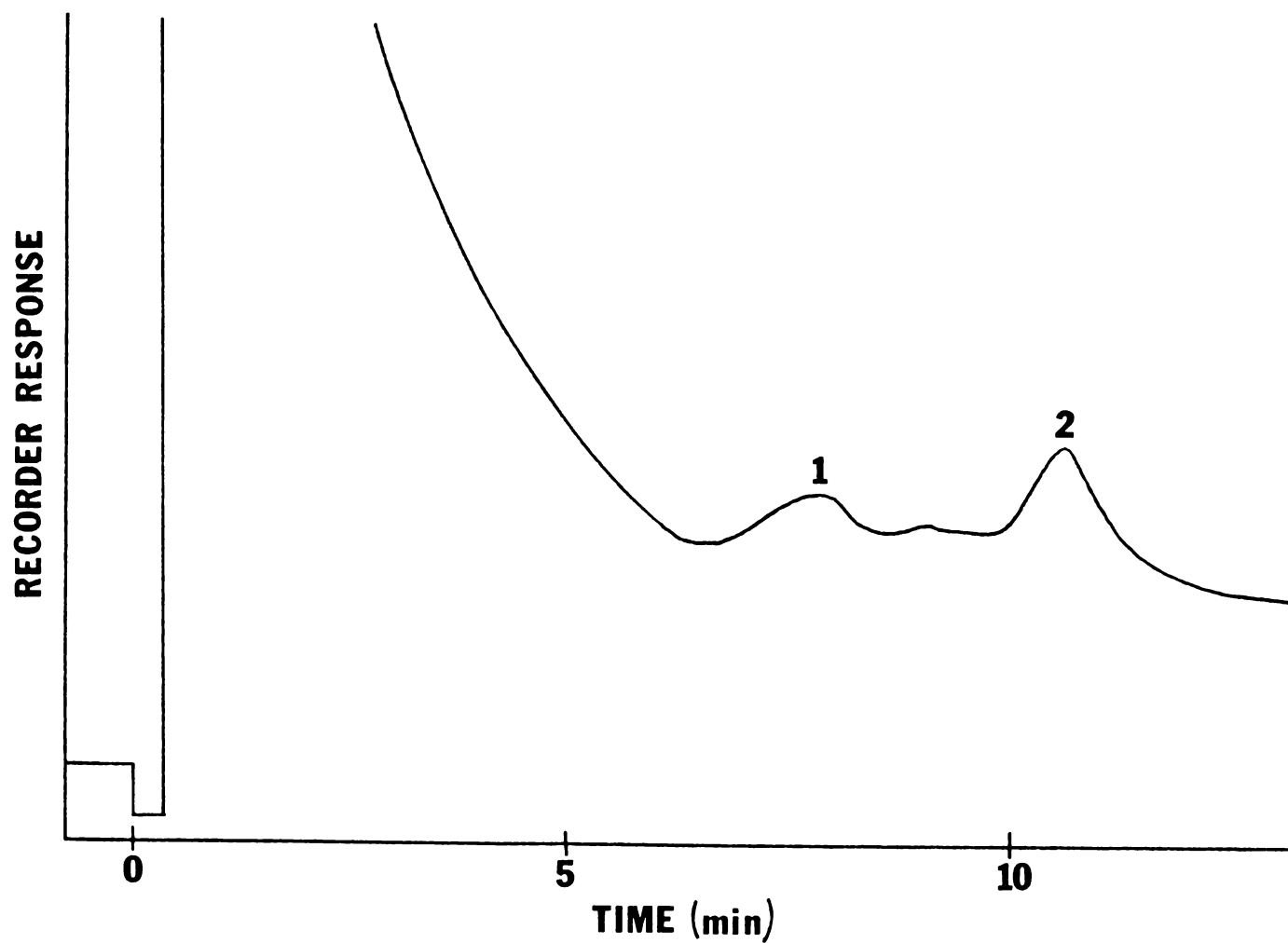


Figure 167. GLC tracing of the neutral sugars in the lorica hydrolysate. Two peaks are resolved. See text for retention times and identities of peaks.

when visualized with ninhydrin. Greater amounts of the lorica hydrolysate are needed for positive identification of most amino acids; however, a yellow spot corresponding to either proline or hydroxyproline was identified on the chromatogram. The Leech colorimetric assay for hydroxyproline produced positive results indicating that up to 1% of the protein (by weight) in the lorica is hydroxyproline.

Characterization of Extracellular Mucilage

Mucilage secretion. Cells used for this study were subcultured from late log/stationary axenic cultures, which typically exhibit a sigmoidal growth curve (Figure 168). A lag phase of approximately four days is followed by exponential log phase growth. During exponential growth, a doubling time of 80 hours is typical. Stationary phase begins at a culture age of 11-12 days. Cells remain motile for approximately 21 days; after this, many cells become palmelloid and highly vacuolate.

The amount of mucilage secreted is not proportional to cell numbers (Figure 168). After inoculation of cells and during lag phase, the mucilage content of the culture decreases to its lowest concentration (61 mg/L) which coincides with the onset of log phase. Mucilage content peaks at late log phase (98 mg/L) and then remains at a somewhat lower level throughout stationary phase. The greatest amount of mucilage (dry weight) isolated from the medium was 0.1214 g/L culture supernatant. Aged cultures become highly viscous from the accumulation of this water-soluble mucilage, a result indicating that the mucilage is probably a high molecular weight compound.

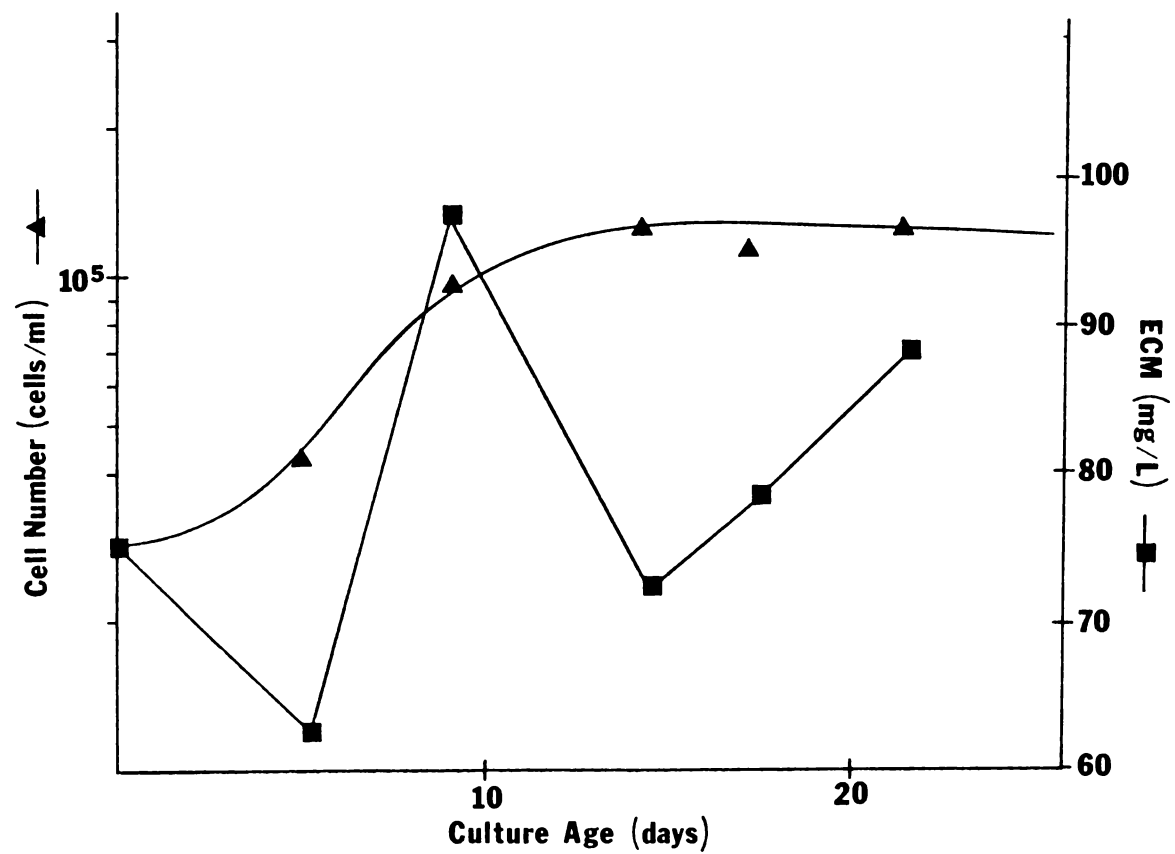


Figure 168. The amount of mucilage excreted into the culture medium during the culturing of Phacotus lenticularis. See text for details.

Determination of procedures for the isolation of extracellular mucilage. Extracellular mucilage (ECM) of Phacotus lenticularis was isolated from the culture medium by two basic methods. The first involved precipitation of the ECM as a calcium salt with the compound cetylpyridinium chloride (CPC). This method made use of the high affinity of CPC for molecules with a high anionic charge density. Although this method yielded large quantities of mucilage (up to 150 mg/L culture supernatant, depending upon culture age), difficulties were encountered. The CPC-precipitated mucilage did not chromatograph well; there was an excessive amount of tailing in the spots, as well as incomplete separation. This is due to an incomplete dissociation of the ECM from the CPC salt.

The method of choice in isolating mucilage from the culture medium was an ethanol precipitation. Although slightly lower amounts of mucilage were extracted with ethanol (up to 120 mg/L, depending upon culture age), no problems with salt interference in chromatographic procedures were encountered. For this reason, ethanol-precipitated mucilage was used throughout all subsequent analytical studies.

Colorimetric analyses of mucilage. The extracellular mucilage precipitates from the culture supernatant as a stringy white material which dries to a white or off-white fibrous solid. The dried ECM will solubilize in hot water to yield a cloudy, viscous liquid at concentrations of 1 mg/ml or greater.

Isolated ECM is composed of carbohydrate, protein and sulfate as determined by colorimetric assay. The major portion is carbohydrate,

accounting for 50% of the dry weight of the ECM. The weight percents of each of the ECM components are shown in Table XII. Sulfate and uronic acid moieties contribute a major portion of the acidic groups in the mucilage. The components investigated account for only 83% of the total dry weight of the ECM. This may be due to decomposition of carbohydrate or protein constituents during analysis or may be accounted for in components such as lipid or ash which were not measured.

The amount of uronic acid present in the ECM of P. lenticularis does not fluctuate much over the course of culturing (Figure 169). Although the uronic acid content drops slightly during log phase growth, it increases again during stationary phase, a result indicating that, in terms of dry weight, uronic acid is a persistent component, lending a negative charge to the ECM at all phases of cell culture.

Carbohydrate composition of the mucilage: Qualitative thin-layer chromatography. The technique of thin-layer chromatography was employed as a means of separation and characterization of the carbohydrate components of the ECM. Neutralized TFA hydrolysates of the isolated ECM were chromatographed on cellulose TLC plates. A pyridine-butanol-water (6-4-3) solvent system effected the best separation of neutral sugars. Figure 170 illustrates a typical chromatographic profile of the hydrolyzed mucilage. The identities of the sugar components were confirmed by a comparison of their R_f values and spot colors with those of known sugar standards (Table XIII). Neutral sugars identified from the ECM include arabinose, mannose (and/or sorbose), xylose, fucose, ribose and rhamnose. A fast-migrating spot ($R_f = 0.92$) which is brown

TABLE XII
CHEMICAL COMPOSITION OF THE EXTRACELLULAR
MUCILAGE OF PHACOTUS LENTICULARIS

Constituent	Percentage of Dry Weight
Neutral Sugars	50
Uronic Acid (calculated as galacturonic acid)	5
Protein	12
Sulfate	16
Total	83

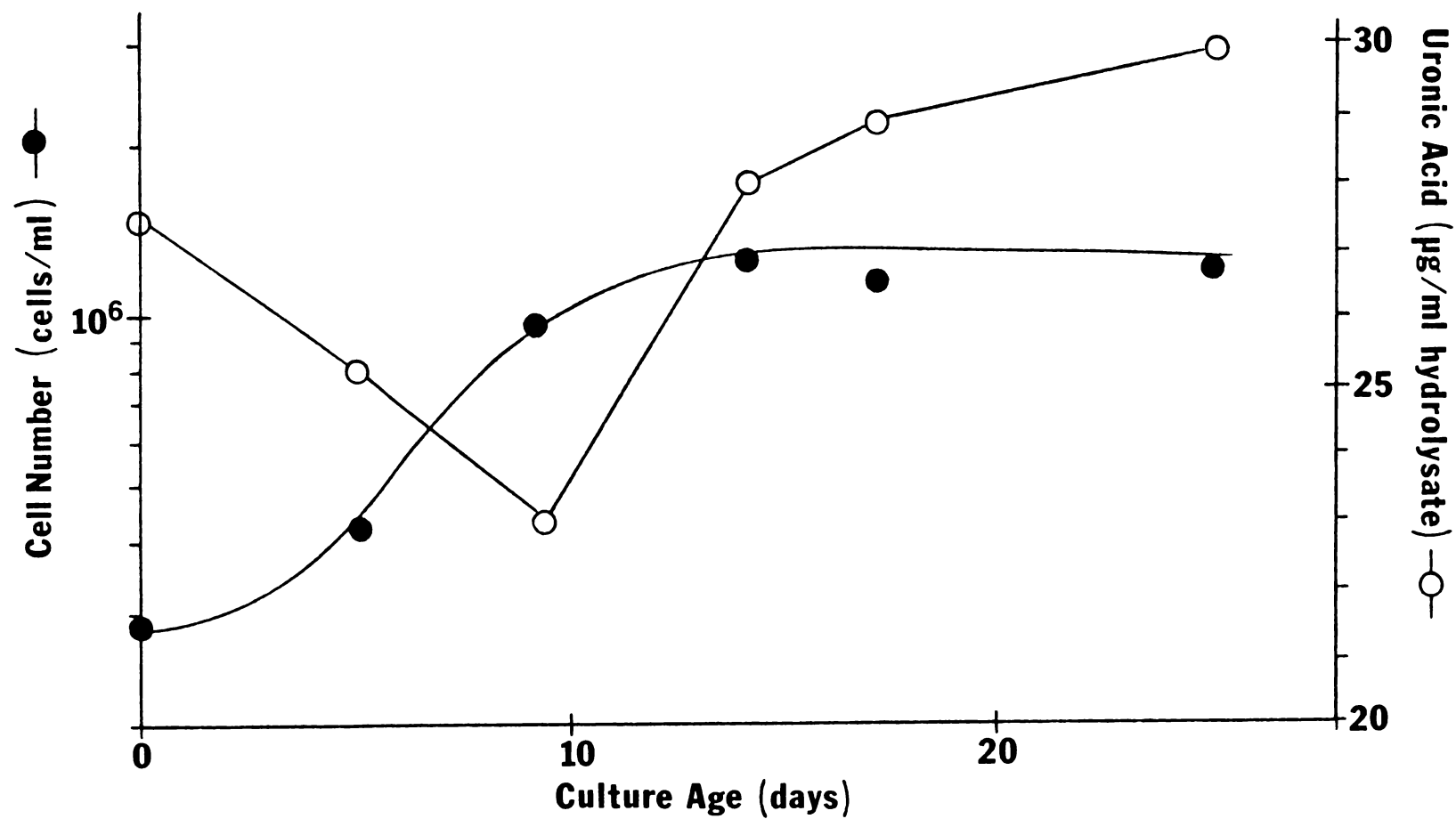


Figure 169. A comparison of uronic acid present in the ECM and the growth of cells in MVM medium. Uronic acid is calculated as μg equivalents of galacturonic acid/mg ECM.

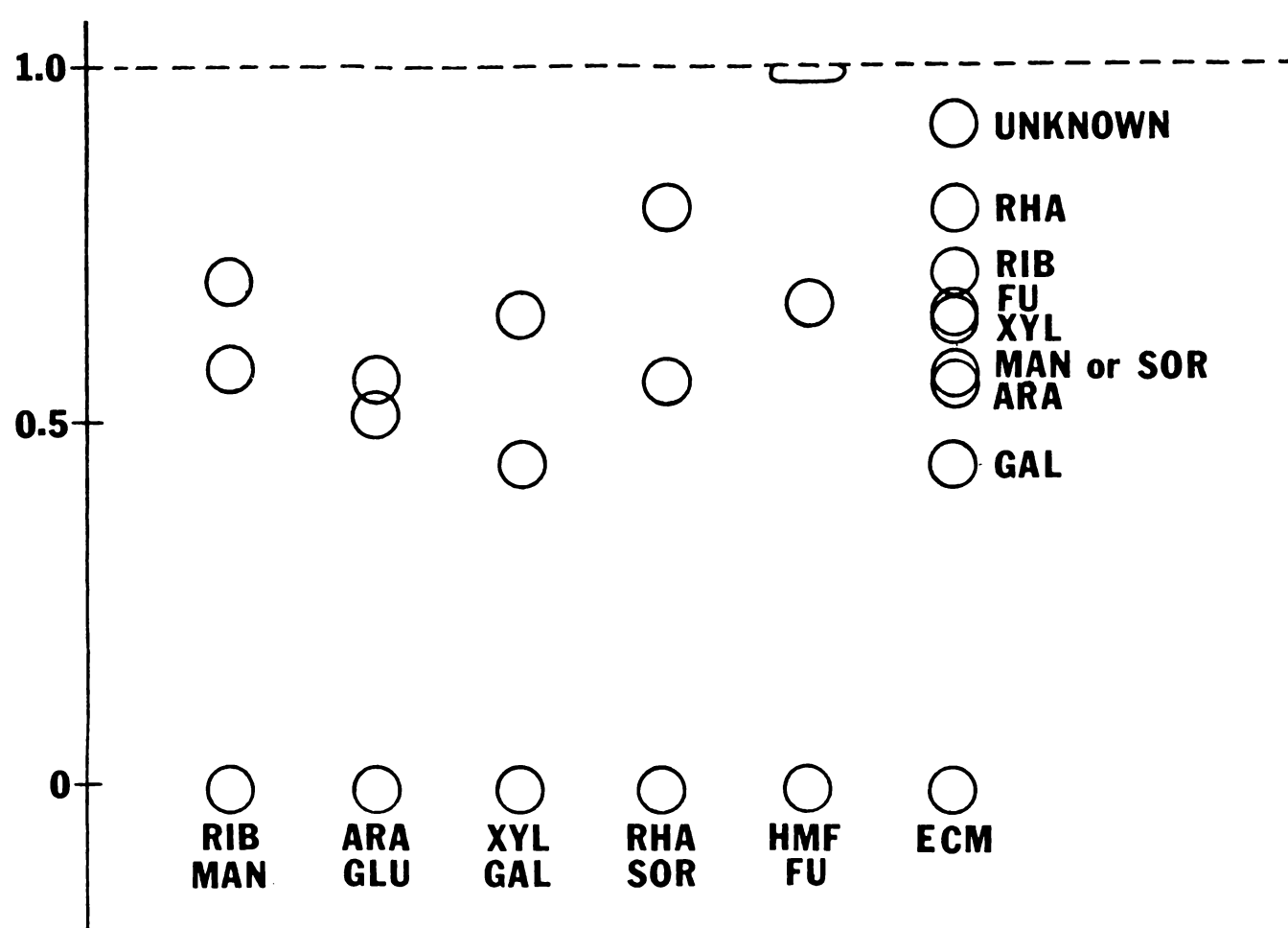


Figure 170. TLC profile of the neutral sugars in a typical ECM hydrolysate. Standards used for comparison are also shown (lanes 1-5). R_f values are presented in more detail in Table XIII.

TABLE XIII
CARBOHYDRATE COMPONENTS OF ISOLATED EXTRACELLULAR MUCILAGE
IDENTIFIED BY THIN-LAYER CHROMATOGRAPHY

Component	Color, Anisidine-HCl Spray	R _f	Identity
1	Brown	0.45	Galactose
2	Rose	0.57	Arabinose
3	Yellow-Brown	0.58	Mannose (or Sorbose)
4	Rose	0.65	Xylose
5	Yellow	0.67	Fucose
6	Rose	0.71	Ribose
7	Gold	0.80	Rhamnose
8	Gray	0.92	Unknown

or brown-gray in color is consistently present in the hydrolysates. None of the sugar standards co-chromatographed with this compound, although the gray-brown color suggests that it may be a 2-deoxyaldose sugar.

Uronic acid constituents, indicated by colorimetric assay, were also investigated by thin-layer chromatography on cellulose supports. Best separation was achieved with a solvent system of ethyl acetate-pyridine-water-acetic acid (5-5-3-1). This solvent system separates the uronic acids, if present, from any neutral sugars in the hydrolysate. Identities of uronic acids were confirmed by comparison of R_f values and color of spots after visualization with those of known standard uronic acids. The results of the chromatography of hydrolyzed ECM for uronic acids are summarized in Figure 171 and Table XIV. Galacturonic acid is confirmed as a constituent of the ECM by its R_f value of 0.50 and rose color. The violet/brown spot with an R_f of 0.65 contains the unresolved neutral sugar components of the ECM.

Carbohydrate composition of mucilage: Qualitative gas-liquid chromatography. To confirm the identities of the neutral sugar components resolved with TLC, neutralized TFA hydrolysates were also analyzed by gas-liquid chromatography (GLC). Peaks produced from the hydrolysate were identified by a comparison of their retention times with those of authentic sugar standards. A representative chromatogram of the hydrolyzed ECM is shown in Figure 172. Twelve peaks are routinely seen; the identities of these peaks and their respective retention times are shown in Table XV. The carbohydrates identified

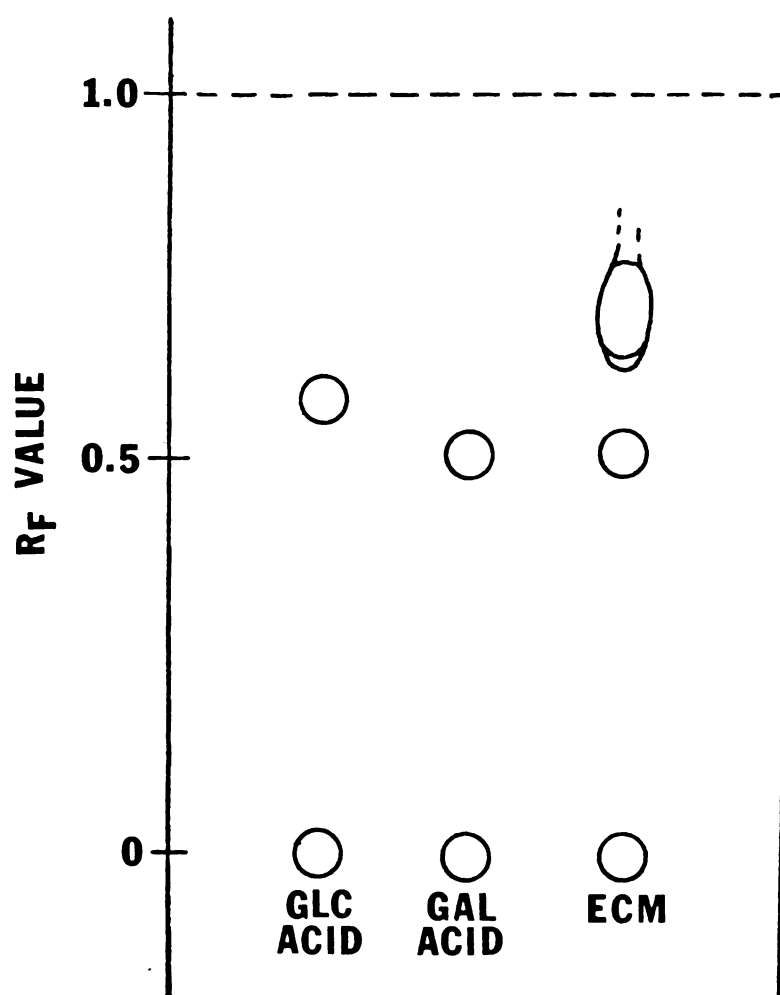


Figure 171. TLC uronic acid profile of the hydrolyzed ECM. The ECM contains only galacturonic acid. The other component seen in the ECM represents unresolved neutral sugars.

TABLE XIV
URONIC ACID COMPONENTS OF ISOLATED EXTRACELLULAR
MUCILAGE IDENTIFIED BY THIN-LAYER
CHROMATOGRAPHY

Component	R _f	Color, Aniline-TCA Spray
Glucuronic Acid	0.56	Red-Brown
Galacturonic Acid	0.50	Rose
ECM Spot 1	0.50	Rose
ECM Spot 2	0.65	Violet/Brown

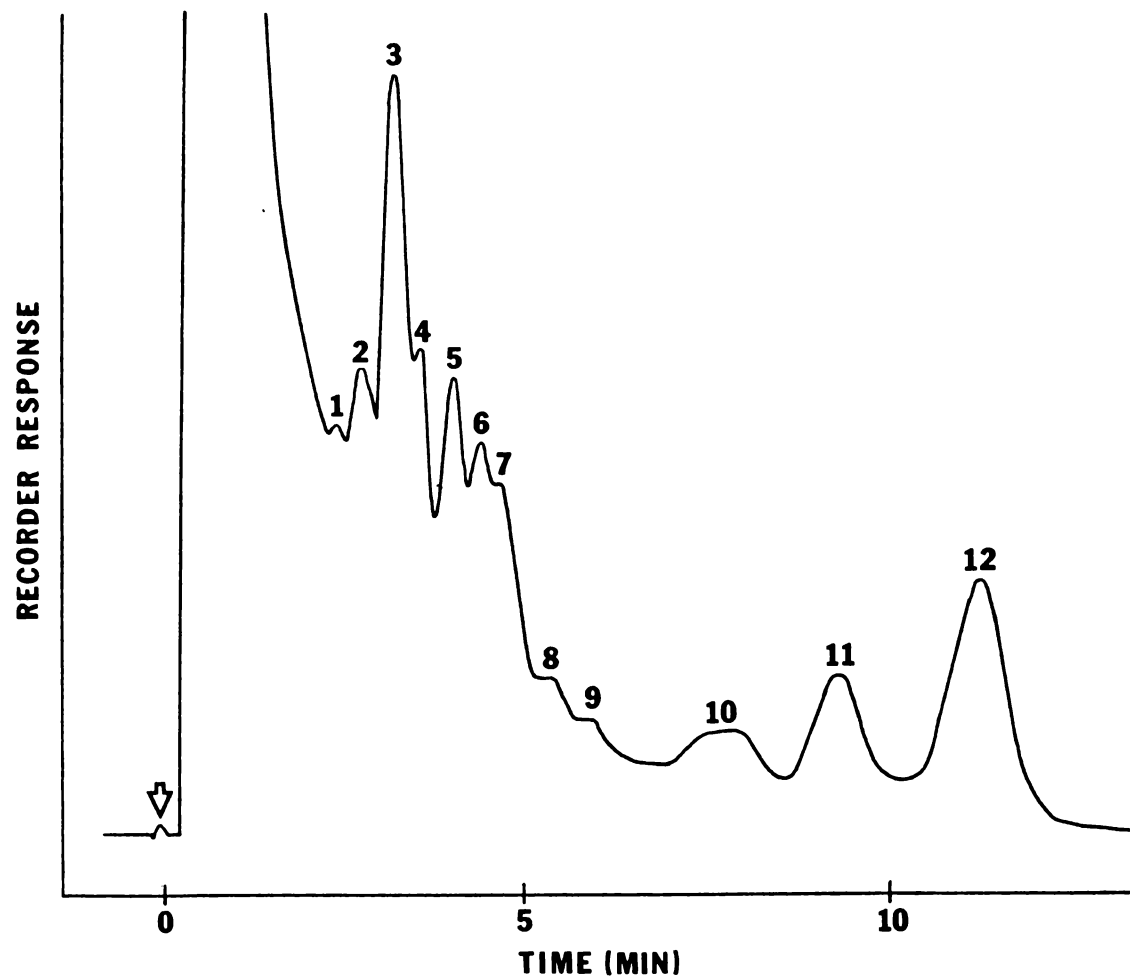


Figure 172. Typical GLC tracing of the neutral sugar components present in the isolated mucilage of Phacotus lenticularis. The identities of components 1-12 are shown in Table XV.

TABLE XV
CONSTITUENTS SEPARATED BY GAS-LIQUID CHROMATOGRAPHY FROM THE
HYDROLYZED MUCILAGE OF PHACOTUS LENTICULARIS

Peak	TMS Retention Time, Min.	Components
1	2.6	Unknown
2	2.9	Rhamnose anomer (?)
3	3.3-3.5	Arabinose
4	3.7	Ribose/Rhamnose
5	4.1-4.2	Fucose
6	4.5	Unknown
7	4.7	Xylose
8	4.9	Unknown
9	6.0	β -Xylose
10	7.3-7.4	Mannose
11	9.2	Galactose
12	10.9-11.0	β -Galactose (?)

with GLC agree well with those determined by TLC; however, the identities of three peaks (6, 8, and 12) are uncertain. Based upon comparisons with known galactose isomers, peak 12, with a retention time of 10.9-11.0 minutes, could be, in part, β -galactose. No other isomers or anomers of any identified sugars positively correspond to peaks 6 or 8.

Thick-layer chromatography/Gas-liquid chromatography-- In conjunction with the above analyses, a combined thick-layer chromatography (THC) and gas-liquid chromatography procedure was devised both to match GLC peaks with specific TLC spots and to attempt to gain information about the unknown components. The cellulose support used for the thick-layer analysis is 500-1000 μm thick; this does not give as clear a resolution of sugar components as the thinner (100 μm) support used in TLC. As a result, fewer spots are visualized and minor components are masked by major ones. The constituents visualized with thick-layer plates are shown in Figure 173 and Table XVI. Fractions corresponding to each spot were eluted from the plates with methanol and analyzed with GLC. The GLC components of each fraction, shown in Figures 174-180, confirm the presence of galactose, arabinose, mannose, fucose, xylose, ribose and rhamnose. The presence of sorbose, suggested by thin-layer chromatography, was ruled out. Other data collected clarify the identities of some previously unidentified GLC peaks and TLC spots.

THC fractions 5 and 6 contain GLC peaks which do not compare with any sugar isomer or anomer used in this study. Fraction 5, which is known to contain rhamnose, has a GLC component with a retention time of

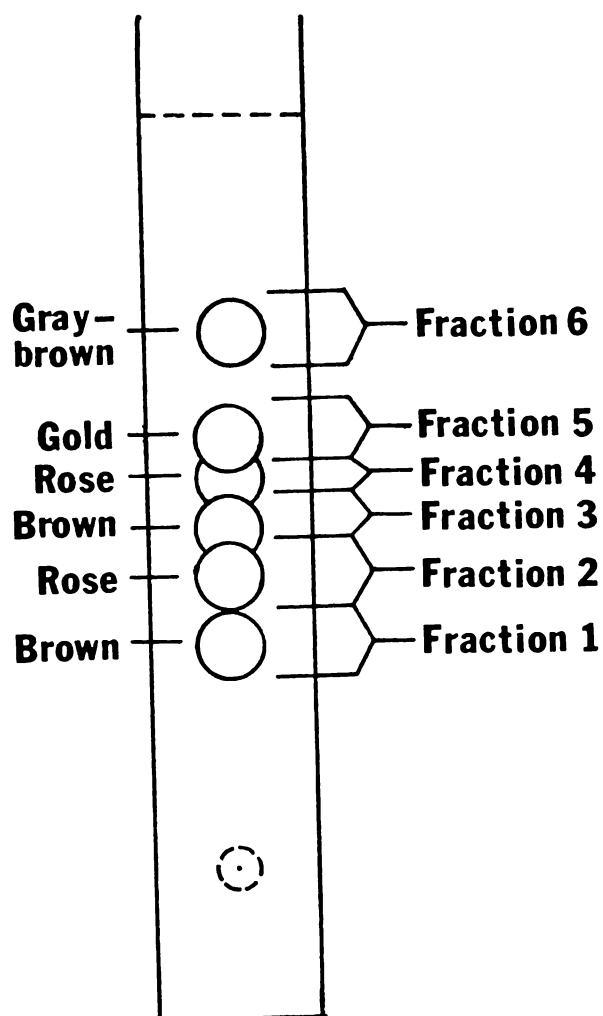


Figure 173. Representative diagram of a thick-layer cellulose plate used for thick-layer/GLC analysis. Each fraction corresponds to a particular neutral sugar component. For identities of sugars and components of each fraction, see Table XVI.

TABLE XVI
 CONSTITUENTS SEPARATED AND ANALYZED BY COMBINED THICK-LAYER CHROMATOGRAPHY AND
 GAS-LIQUID CHROMATOGRAPHY FROM THE HYDROLYZED MUCILAGE
 OF PHACOTUS LENTICULARIS

Fraction	R _f	Color, Anisidine HCl Spray	TMS Retention Time, Min.	Component(s)
1	0.29	Brown	9.2 11.0	Galactose β-Galactose (?)
2	0.38	Rose	3.4	Arabinose
3	0.46	Yellow-Brown	7.4	Mannose
4	0.51	Dark Rose	3.7 4.2 4.7 6.0	Ribose Fucose Xylose β-Xylose
5	0.57	Gold	1.1 2.8 3.5 4.5	Unknown Rhamnose Anomer Rhamnose Unknown
6	0.70	Gray-Brown	2.9	Unknown

Figure 174. GLC tracing of fraction 1, confirming the presence of galactose (peaks 11 and, possibly, 12).

Figure 175. GLC tracing of fraction 2, confirming the presence of mannose (peak 10).

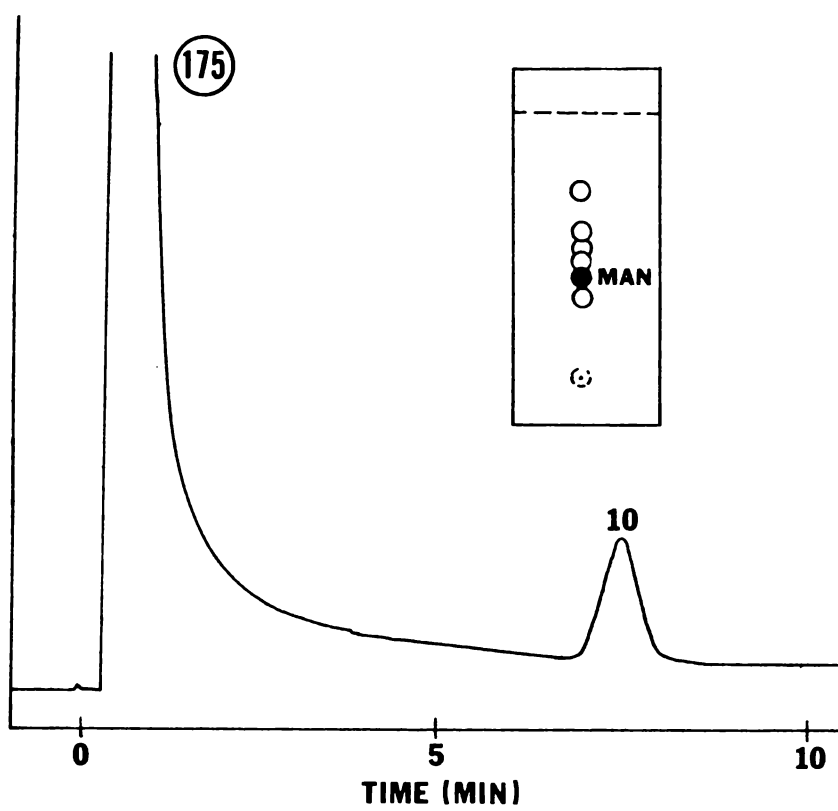
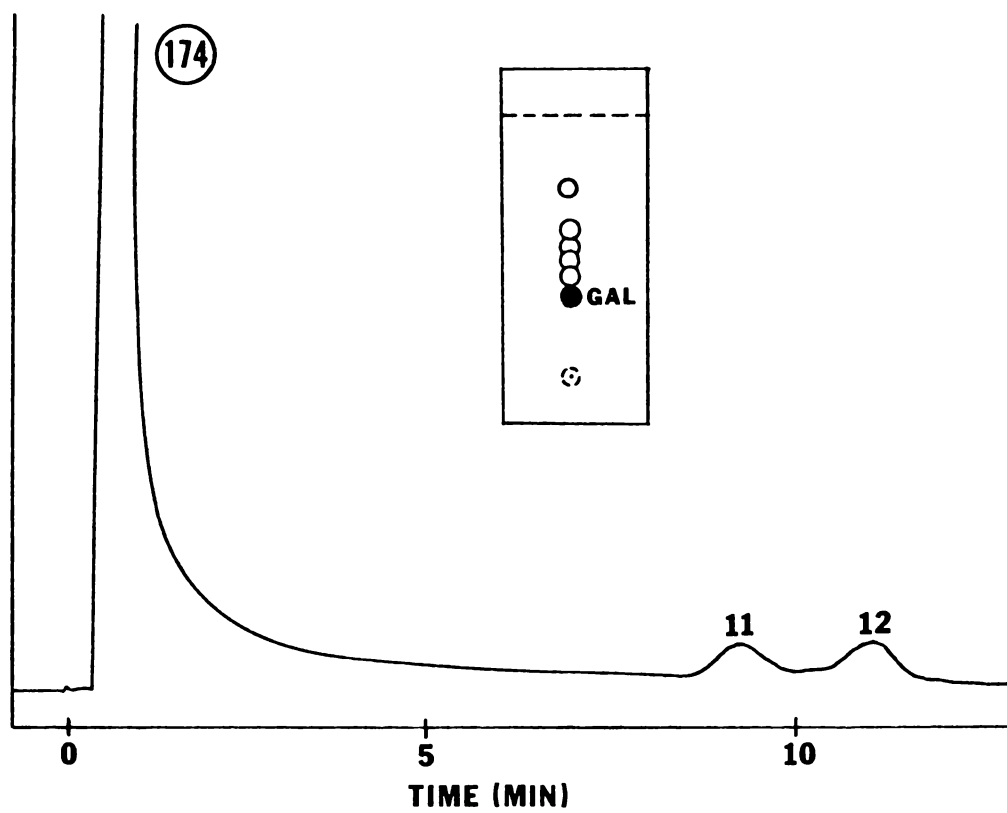


Figure 176. GLC tracing of fraction 3, confirming the presence of arabinose (peak 3).

Figure 177. GLC tracing of fraction 4, confirming the presence of ribose (peak 4).

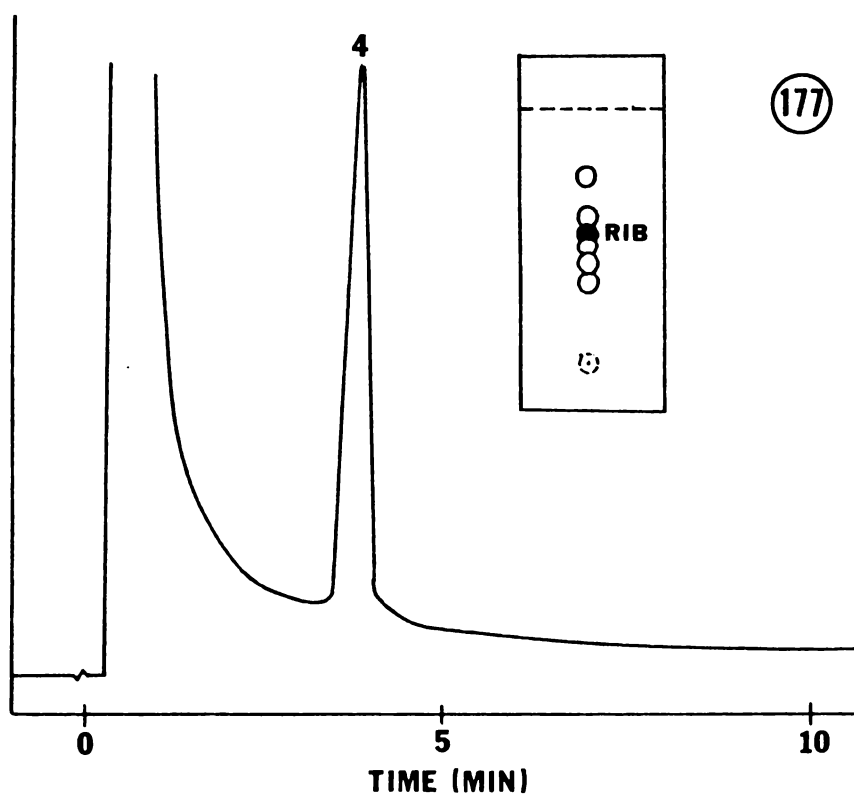
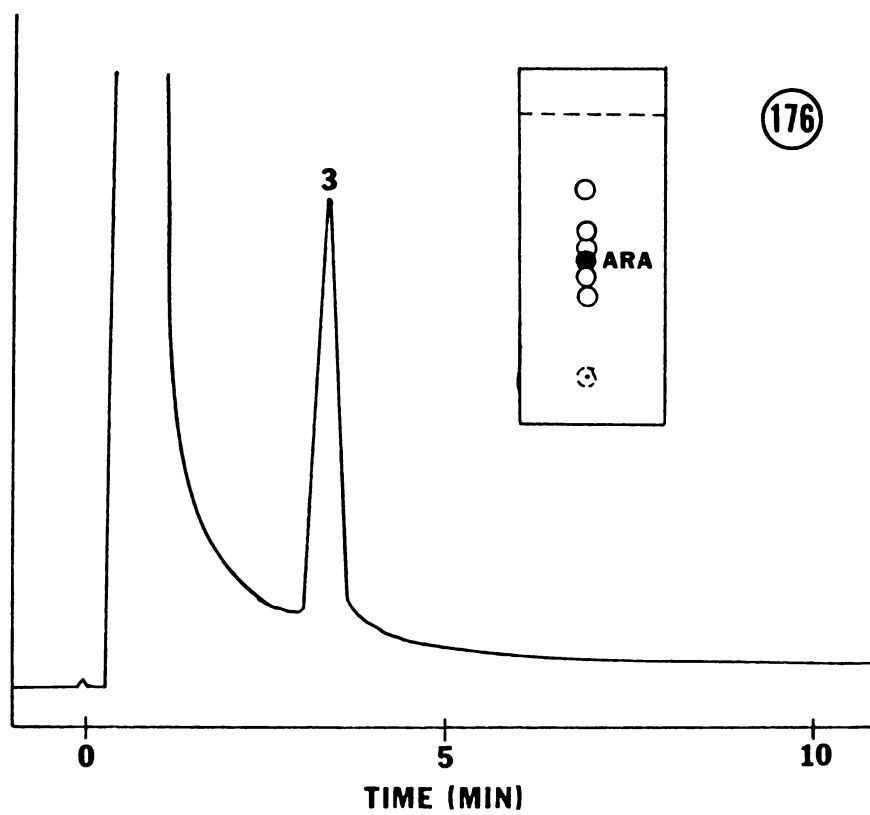
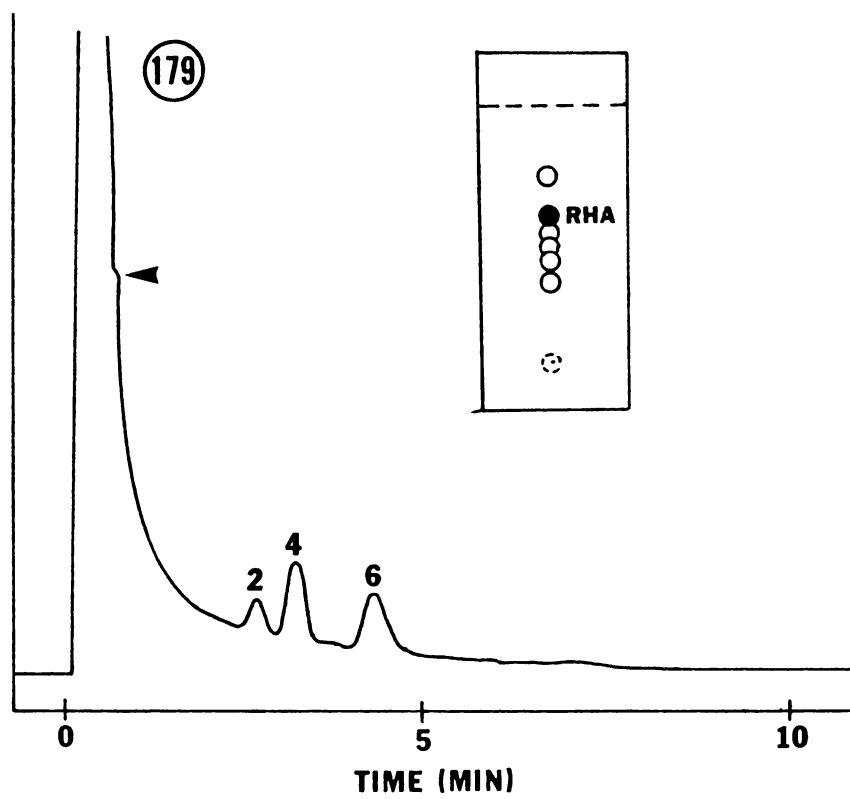
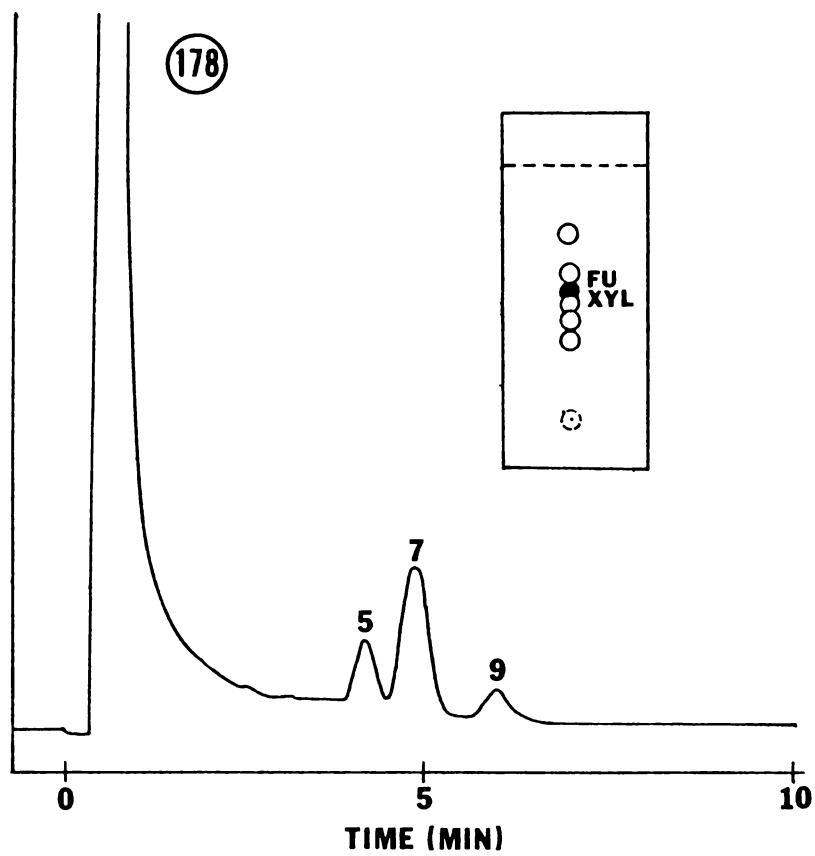


Figure 178. Additional components of fraction 4 include fucose (peak 5), and xylose (peaks 7 and 9).

Figure 179. GLC tracing of fraction 5, confirming the presence of rhamnose (peak 4). Two other components, a possible rhamnose anomer (peak 2) and an unknown (peak 6), are also resolved. A previously undetected shoulder peak (arrowhead) is also seen.



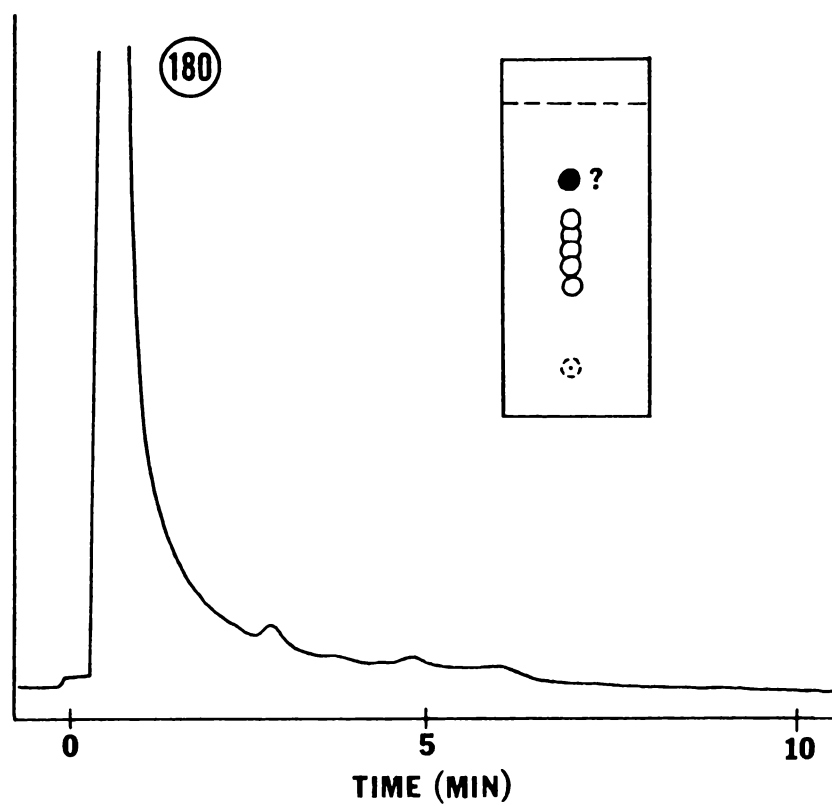


Figure 180. Fraction 6 chromatogram produces no identifiable components.

2.8-2.9 minutes, identical to that of a minor rhamnose anomer. However, this same retention time is also seen in Fraction 6, whose identity was never determined with TLC. It is probable that these two peaks represent different carbohydrates that have extremely close retention times. A small peak with a short retention time of 1.1 minutes, seen in the Fraction 5 chromatogram (Figure 179), is only resolved when the solvent peak is very small. Its identity is uncertain.

Time course study-- To study changes in the carbohydrate components of the extracellular mucilage during culturing, a time course study was carried out. The use of TLC data in such a study is of limited value since components with very close mobilities are often difficult to distinguish. For this reason, the various components were separated by gas-liquid chromatography. Although no absolute values are assigned to amounts of each compound detected, relative amounts may be compared and the presence or absence of compounds may be noted. The times selected are as follows: Day 1, day of culture inoculation; Day 5, onset of log phase; Day 9, end of log phase; Days 14 and 17, stationary phase and Day 25, onset of culture decline. ECM from cultures at each of the above times was analyzed with GLC and the resulting components compared with the twelve GLC components commonly detected in the mucilage of P. lenticularis (c.f. Table XV, page 170).

Twelve characteristic GLC peaks are present at all times except Day 9 (Figures 181-186). The relative amounts of those carbohydrates resolved by GLC agree well with the absolute amounts calculated in colorimetric assays. Between Days 1 and 5, a decrease in peak height,

Figure 181. Time course study, Day 1. Chromatogram shows all components present in low amounts.

Figure 182. Time course study, Day 5. Peak heights are further reduced (c.f. Figure 181), but all components are still present.

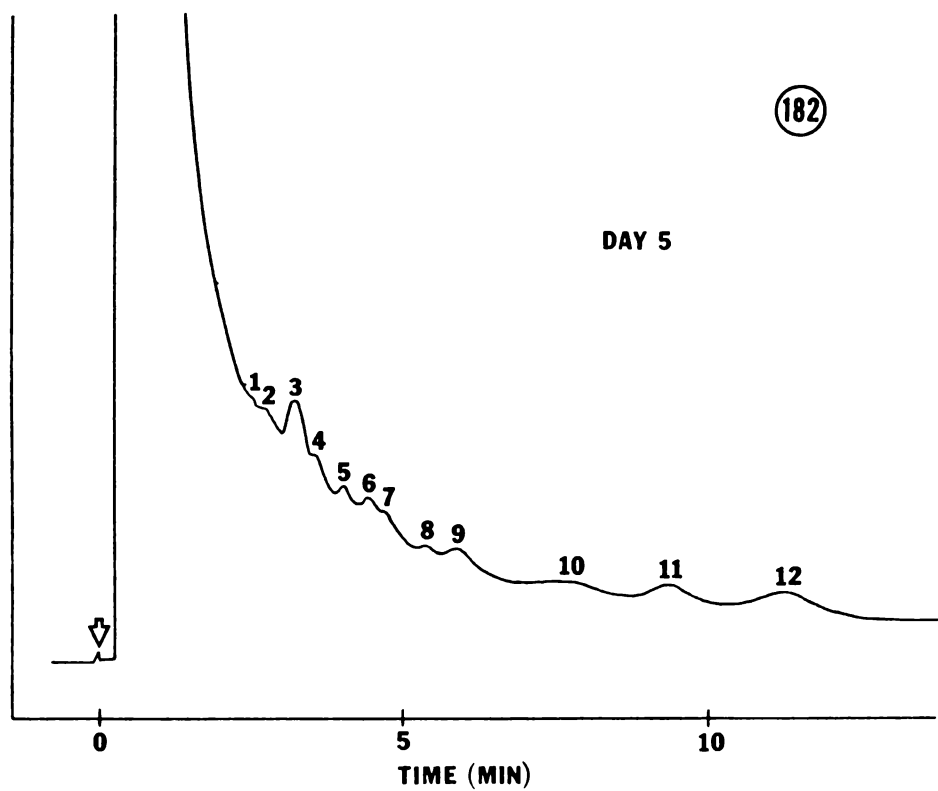
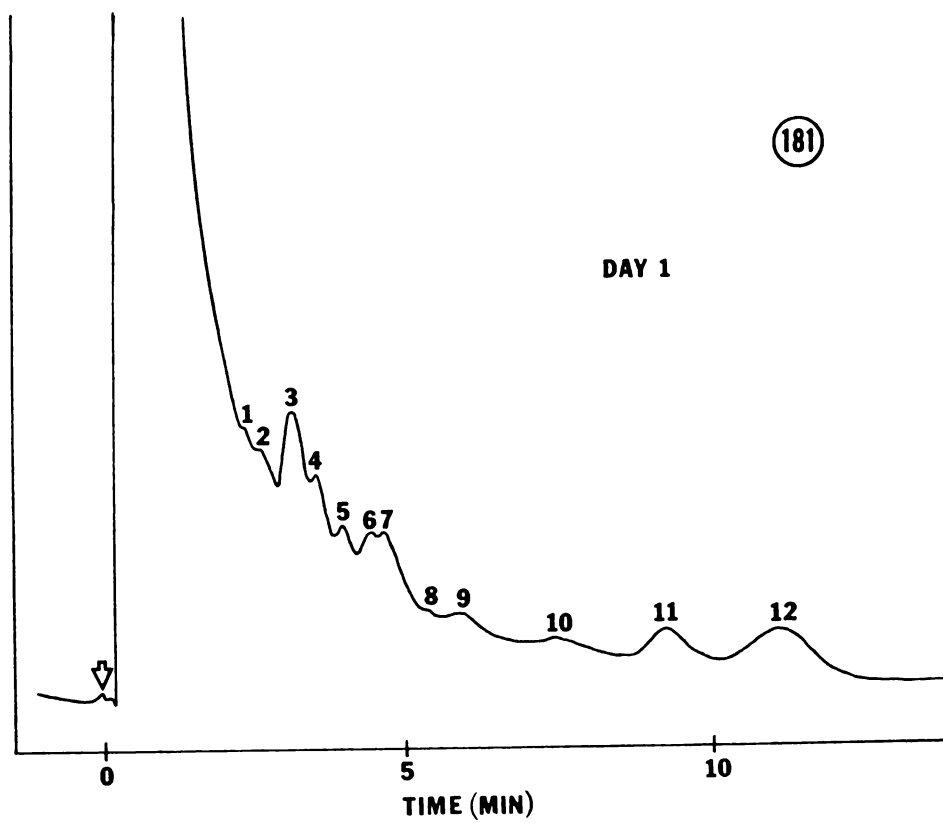


Figure 183. Time course study, Day 9. A slight increase in most sugar components is obvious, with the exception of peak 1, which is not present in detectable amounts.

Figure 184. Time course study, Day 14. An increase in the peak height of galactose (11) and arabinose (3) is obvious. Other components also increase slightly.

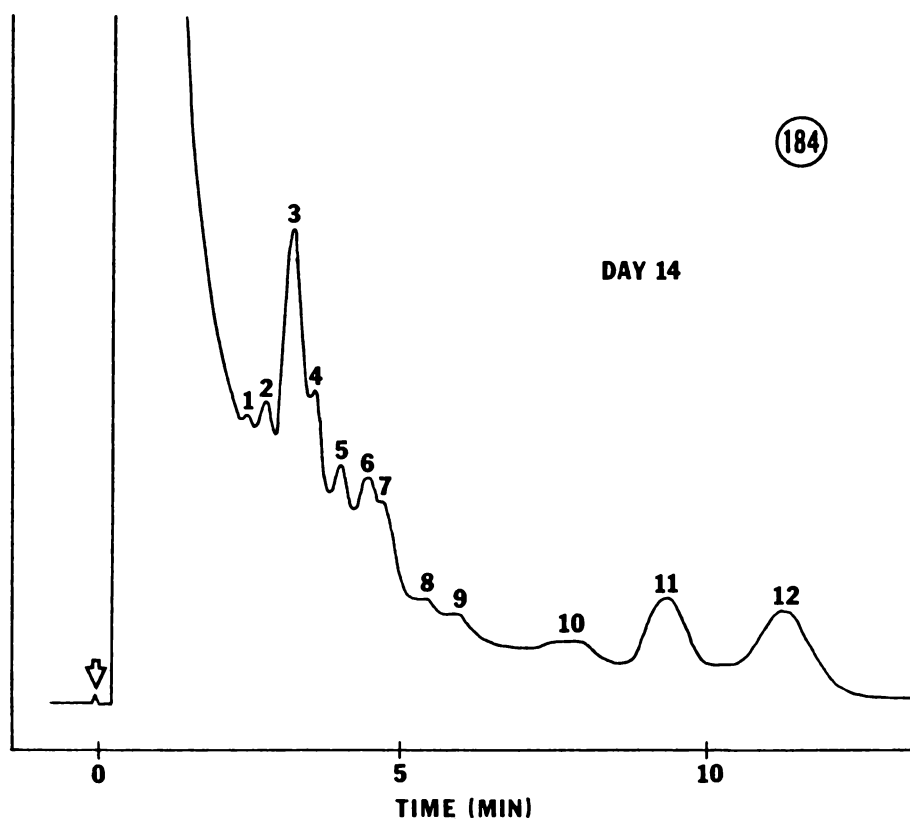
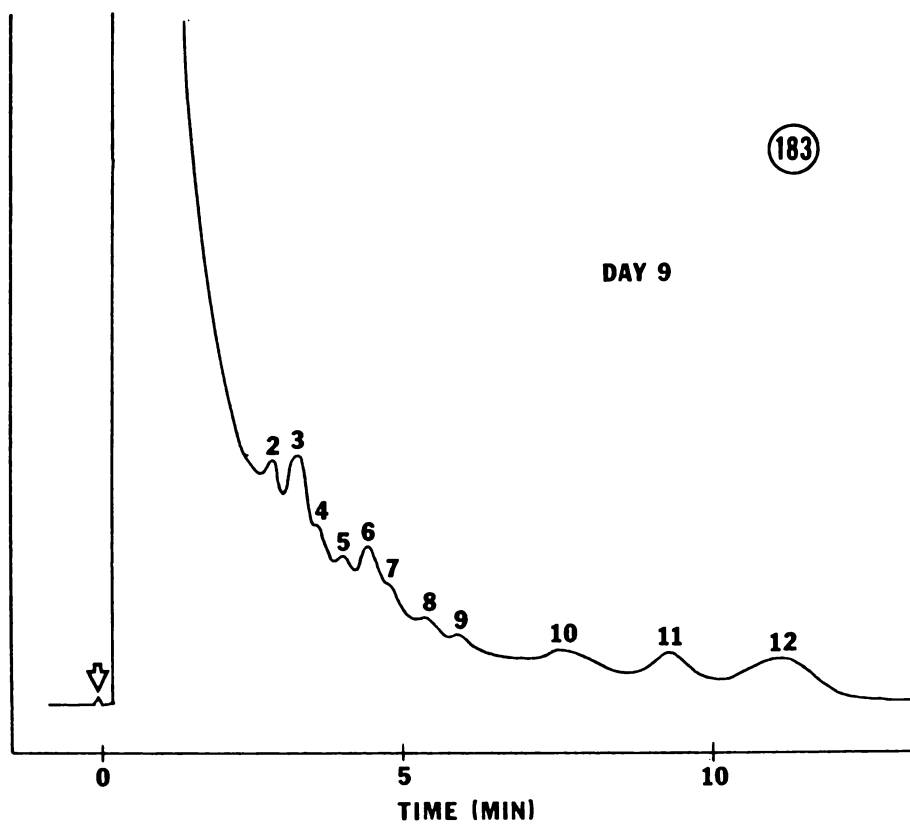
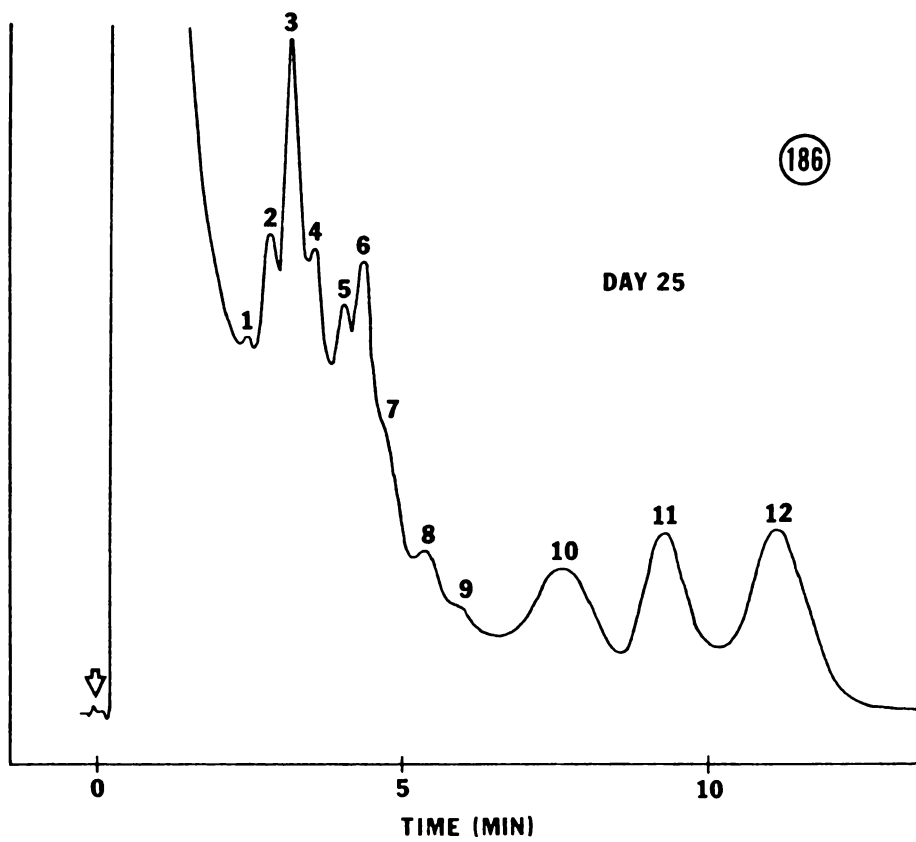
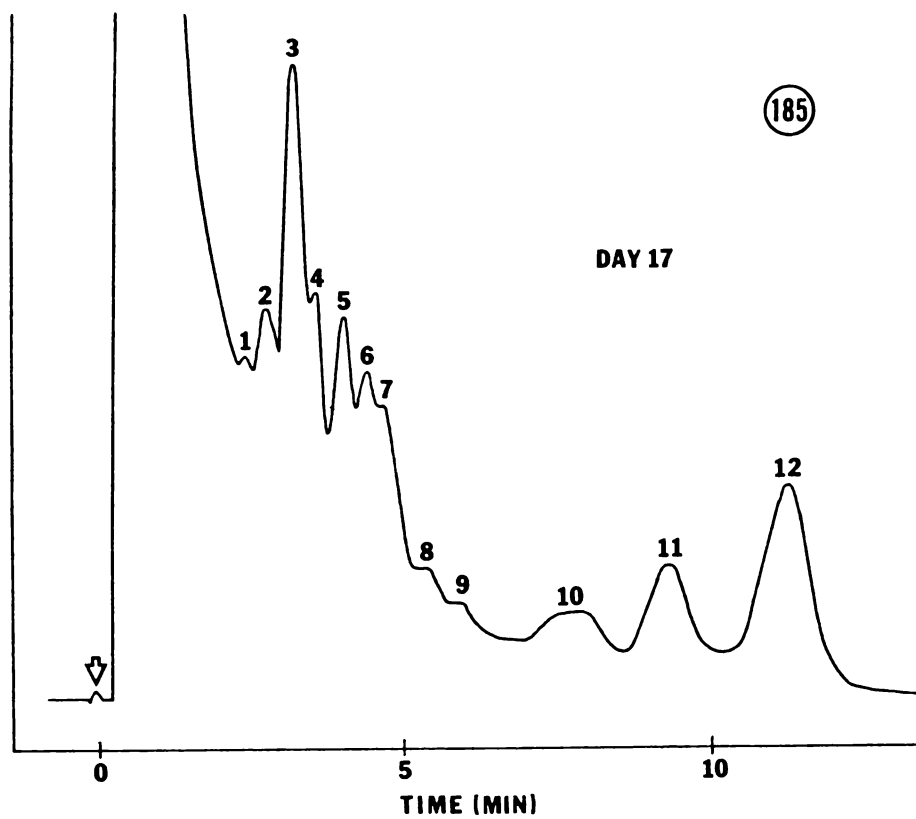


Figure 185. Time course study, Day 17. All sugar components continue to increase in amount.

Figure 186. Time course study, Day 25. At this time, some reduction is seen in the amount of mannose (10) and an unknown compound (6).



especially with regard to arabinose (3), ribose (4), xylose (7) and galactose (11) is obvious, indicating a decrease in the amount of these sugars in the mucilage. An increase in the amount of sugars in the ECM is indicated at Day 9, with the exception of peak 1 (an unknown compound) which disappears completely. At Day 14, an increase in arabinose (3) and galactose (11) is obvious. Peak 1 also reappears. Arabinose, galactose and mannose (10) continue to increase in amount throughout the remainder of the time course study. Little or no change is seen in peaks 1 and 2, rhamnose and ribose (4) and xylose (7 and 9). Fucose (5) reaches a maximum at Day 17, as does Peak 12, which is tentatively identified as a galactose isomer.

Amino acid composition of the mucilage. A protein component was indicated as part of the ECM through colorimetric assays with the Coomassie Blue dye-binding assay. In an attempt to characterize the protein moiety more completely, several additional techniques were employed: thin-layer chromatography for amino acids, automated amino acid analysis and the Leech colorimetric assay for hydroxyproline.

Thin-layer chromatography -- Neutralized HCl-acid hydrolysates of isolated ECM were chromatographed on cellulose TLC plates in a two-dimensional solvent system. A representative chromatogram is shown in Figure 187. Twenty-six spots were visualized, of which 12 were identified by comparison with authentic standard amino acids and characteristic color reactions after ninhydrin visualization. The most intensely colored spots were assumed to represent those amino acids present in the greatest amounts. These include aspartic acid, glutamic

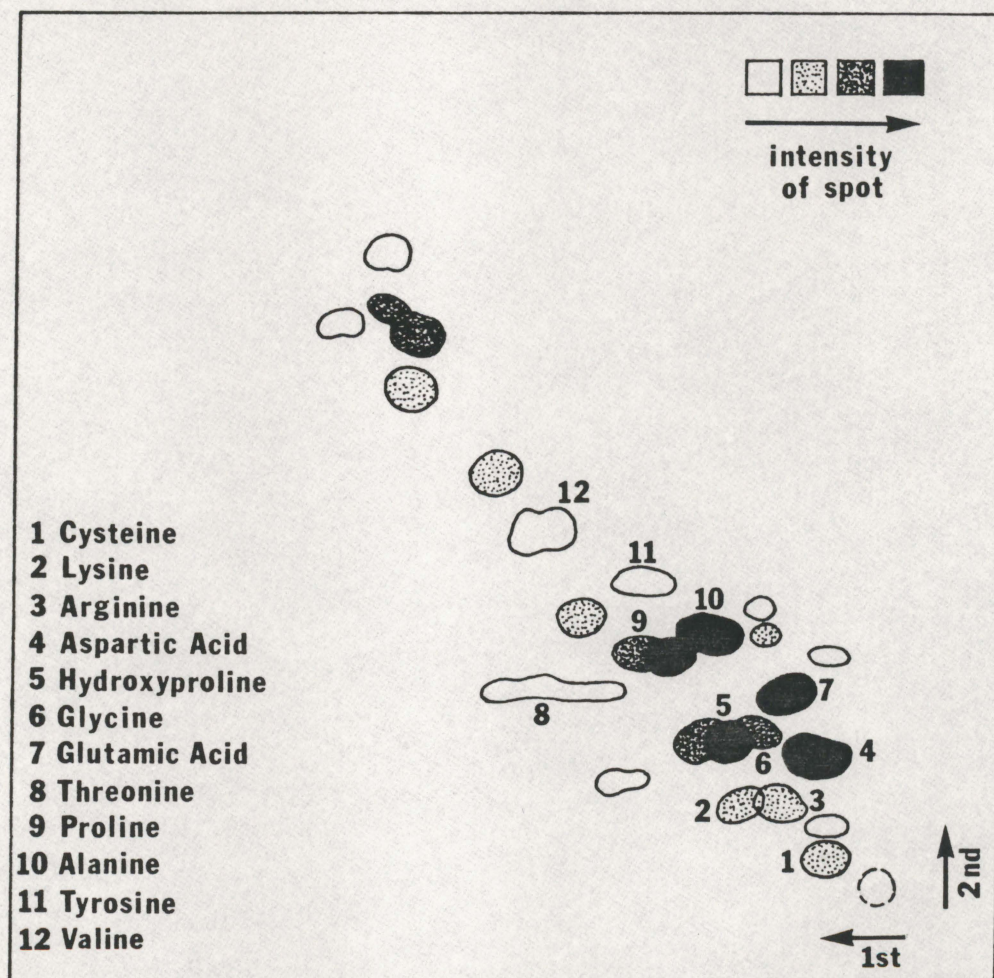


Figure 187. TLC amino acid profile of hydrolyzed ECM. Twelve amino acids were identified by comparison with standards.

acid and alanine. Hydroxyproline and proline are readily identified by a characteristic yellow or yellow-orange color, in contrast to the usual blue-purple produced by ninhydrin. This color difference, however, makes comparisons of intensity somewhat difficult. For this reason, these two amino acids may be underestimated as to relative amounts present on the chromatogram.

Automated amino acid analysis-- Automated amino acid analysis of HCl-hydrolyzed ECM produced limited information. Due to the small amount of protein (ca. 12%) in the ECM, quantitative estimates were difficult. The amount of ECM available for analysis was not sufficient for nanomole estimates of the amino acid content; therefore, only qualitative estimates were made.

With automated analysis, seven amino acids were detected: alanine, arginine, glycine, histidine, hydroxyproline, lysine and proline, of which hydroxyproline and proline were present in the greatest amounts.

Colorimetric assay for hydroxyproline-- Since hydroxyproline appears consistently in amino acid hydrolysates of extracellular mucilage, an attempt was made to quantify the amount present by a spectrophotometric assay, the Leech assay. Time points identical to those employed for the GLC time course study were used for this assay. Isolated ECM was hydrolyzed with either TFA or HCl; it was found that HCl hydrolysates give a 2-2.5 X greater hydroxyproline yield than the TFA hydrolysates. The results of the assay are shown in Figure 188. A decrease in the amount of hydroxyproline to undetectable levels occurs

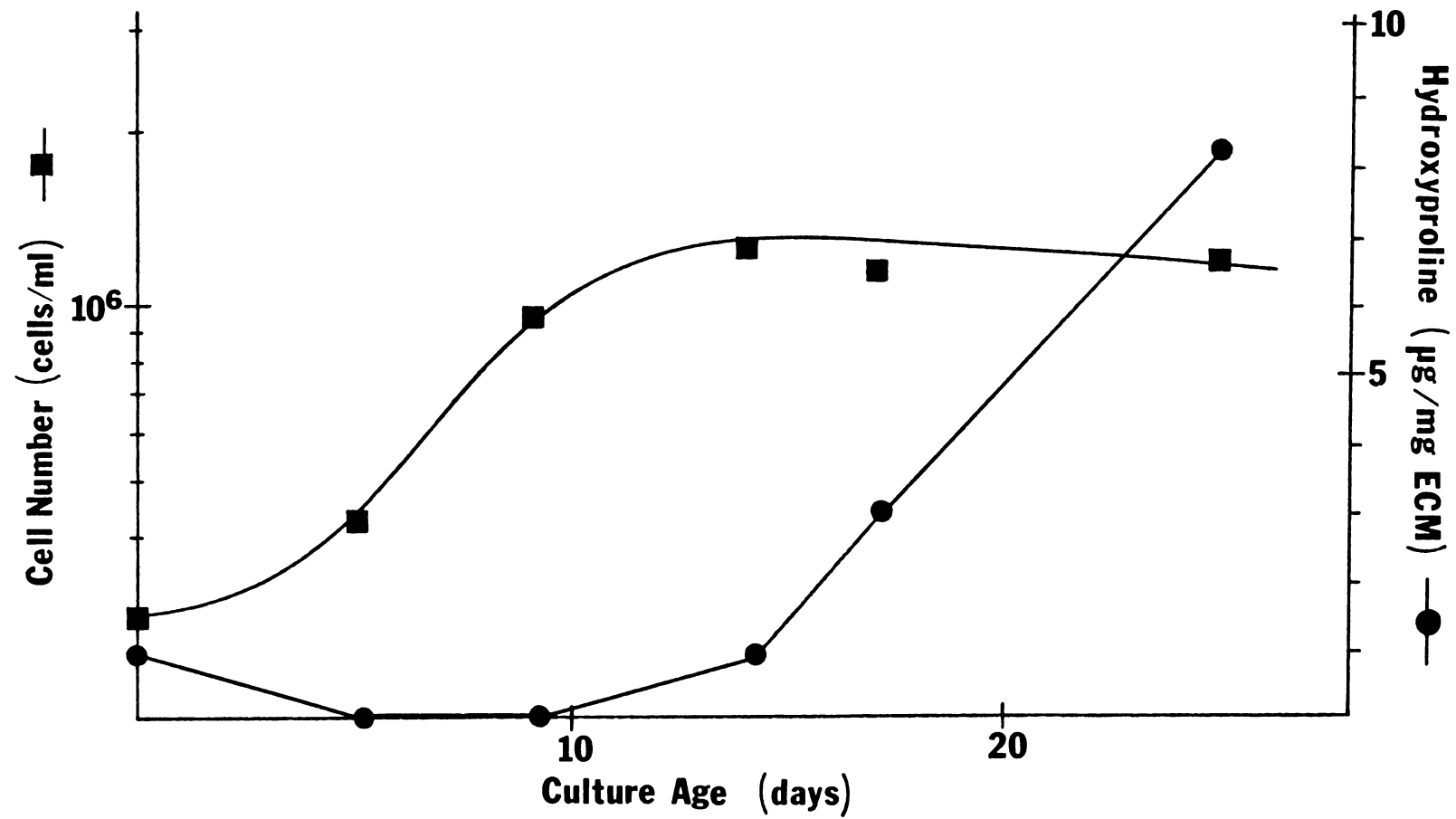


Figure 188. A comparison of the hydroxyproline content of the ECM and the growth of cells in MVM medium. Hydroxyproline is calculated as $\mu\text{g/mg ECM}$.

during early log phase of culture growth but rises to a maximum during late stationary growth. A comparison of all techniques used to detect amino acids in the ECM is shown in Table XVII.

Electrophoresis of mucilage. Colorimetric and chromatographic results indicate the presence of both protein and carbohydrate in the ECM. The technique of gel electrophoresis was selected to investigate the possibility that these entities exist together as a glycoprotein.

In preliminary experiments, ECM was solubilized and applied to polyacrylamide gels without the addition of SDS. Under these conditions, the mucilage never migrated from the top of the gel, a result suggesting that the molecule was extremely large. In subsequent trials, SDS was added to the sample preparation and gels to solubilize the ECM and to cleave the component molecules into smaller subunits. The results of a typical electrophoretic run are shown in Figure 189. This densitometer scan indicates that several bands in the gel stain with both the PAS stain for carbohydrates and Coomassie Blue for protein. These data suggest that the carbohydrates and proteins are parts of the same glycoprotein molecule. The mucilage has a slow mobility, further indicating a high molecular weight. One large protein peak with a relative mobility of 0.64 has no corresponding carbohydrate peak, suggesting that some of the protein in the ECM does exist independently of the carbohydrate moiety.

Ultrastructural Localization of Carbohydrates

Data derived from colorimetric and chromatographic analyses indicate the presence of carbohydrate in both the lorica matrix and

TABLE XVII
AMINO ACID COMPOSITION OF ISOLATED EXTRACELLULAR MUCILAGE
OF PHACOTUS LENTICULARIS

Amino Acid	Methods of Analysis		Colorimetric
	Automated Analysis	TLC	
Alanine	+	++++	
Arginine	+	++	
Asparagine			
Aspartic Acid		++++	
Cysteine		++	
Glutamic Acid		++++	
Glutamine			
Glycine	+	+++	
Histidine	++		
Hydroxyproline	++++	++++	++++ ¹
Isoleucine			
Leucine			
Lysine	+	++	
Methionine			
Phenylalanine			
Proline	++++	++++	
Serine			
Threonine		+	
Tryptophan			
Tyrosine		+	
Valine		+	

¹Colorimetric tests were done only for hydroxyproline.

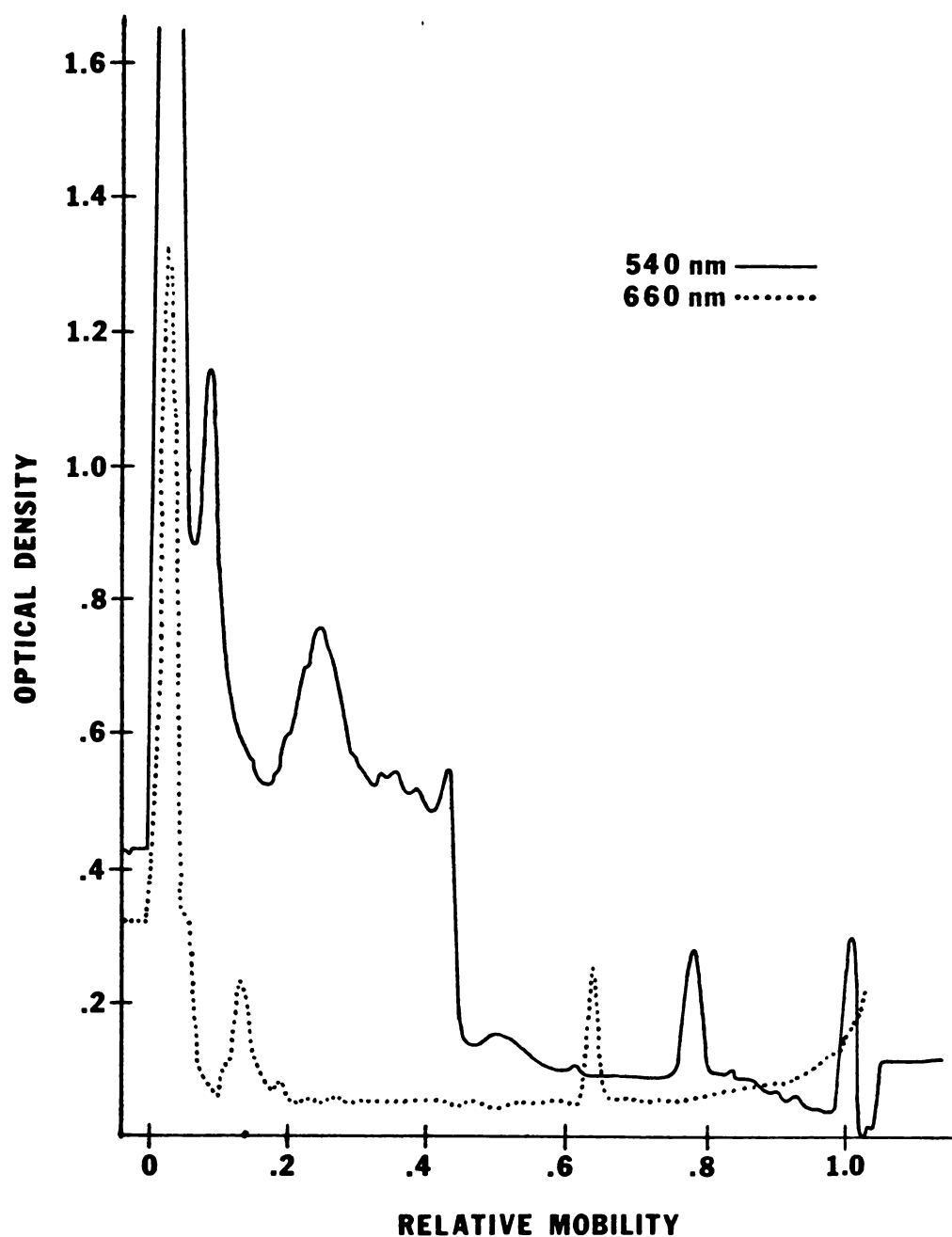


Figure 189. Densitometer tracing of bands produced from SDS-polyacrylamide gel electrophoresis of hydrolyzed ECM. Gels were scanned for protein at 660 nm and for carbohydrates at 540 nm. Each peak corresponds to a protein and/or carbohydrate component of the ECM.

extracellular mucilage. Cytochemical stains such as PASH and PA-TCH-SP can be used not only to localize these moieties in the lorica and ECM, but also for intracellular localization of carbohydrates which ultimately might be incorporated into the lorica and/or ECM.

Periodic Acid-Silver Hexamine (PASH). Silver grains are heavily deposited in the lorica matrix (Plate XL, Figure 190) and the extracellular mucilage (Plate XL, Figure 191). A limitation of the particular method is excessive background staining. When staining times are shortened sufficiently to remove the background silver, staining is significantly reduced in other areas. For this reason, the choice was made to tolerate low levels of background silver in order to enhance details of deposition elsewhere.

With this technique, the lorica matrix appears fibrillar. Newly formed loricas are composed of a thin, wispy fibrillar matrix (Plate XL, Figure 192), whereas stain deposition in older loricas indicates a much thicker, denser structure (Plate XL, Figure 193). A homogeneous dense layer subtends a fine fibrillar coat which extends along the entire lorica surface (Plate XL, Figure 194). The extracellular mucilage is seen as large, evenly stained areas either in the surrounding medium or contiguous with the outer surface of the cell (Plate XL, Figure 191). The even distribution and pattern of silver grains in the mucilage suggests a homogeneous, fibrillar substructure.

Periodic Acid-Thiocarbohydrazide-Silver Proteinate (PA-TCH-SP). Using the PA-TCH-SP stain, carbohydrates are readily visualized inside of the cell. A heavy label is most often seen in dictyosome cisternae

Plate XL. Carbohydrate Deposition: PASH Reaction.

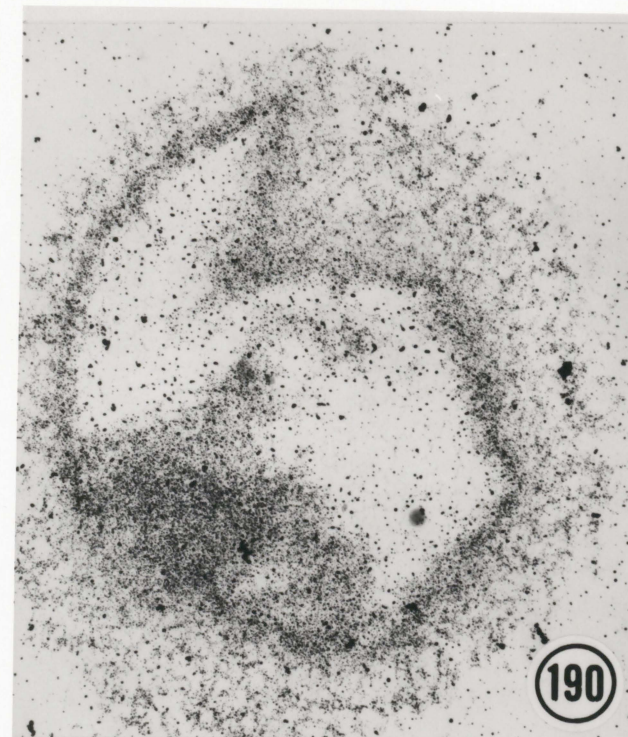
Figure 190. Glancing section through empty lorica. The heavily staining areas are surrounded by a less-dense region, suggesting that the lorica may be differentiated with respect to carbohydrate content. TEM thin section. X 20,900.

Figure 191. Transverse section through lorica and associated mucilage showing abundance of carbohydrate in both structures. TEM thin section. X 20,600.

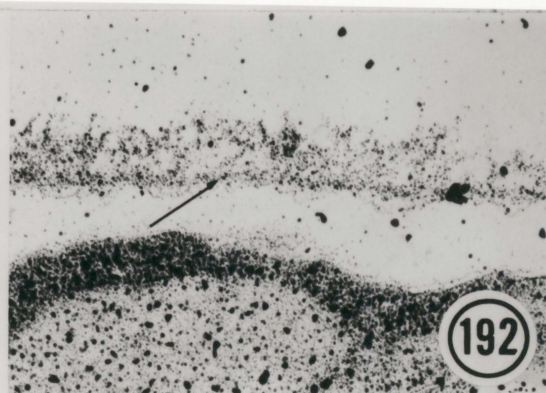
Figure 192. Transverse section through young lorica. Silver deposition is not uniform throughout the lorica; a thin layer of carbohydrate (arrow) is seen where the label is more intense. TEM thin section. X 34,400.

Figure 193. Transverse section of a mature lorica. Silver is deposited in a wide band with wispy fibrillar projections on the outer margin. TEM thin section. X 25,800.

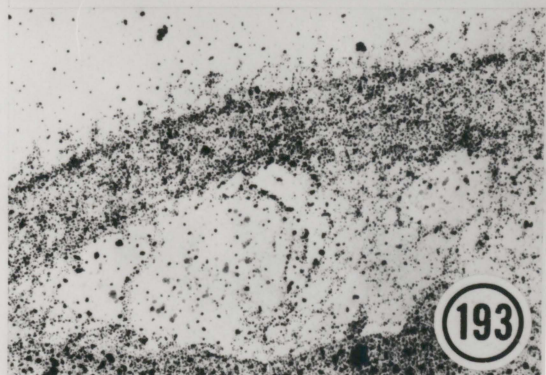
Figure 194. Slightly oblique section through mature lorica. Silver label is present throughout the lorica. Fibrillar projections, similar to those seen in Figure 79, page 93, are also apparent here. TEM thin section. X 21,500.



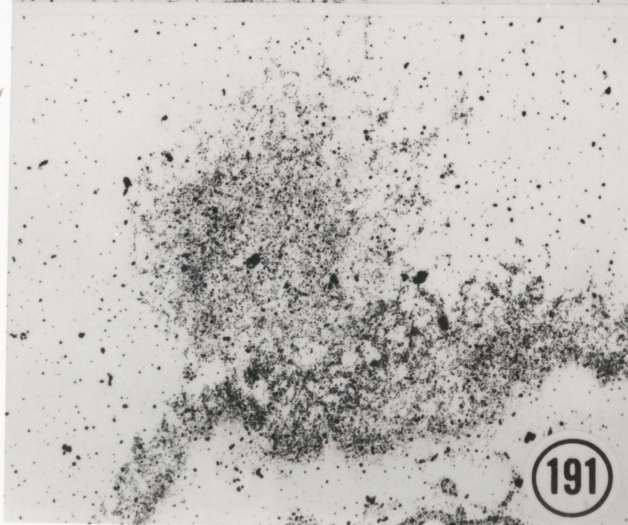
190



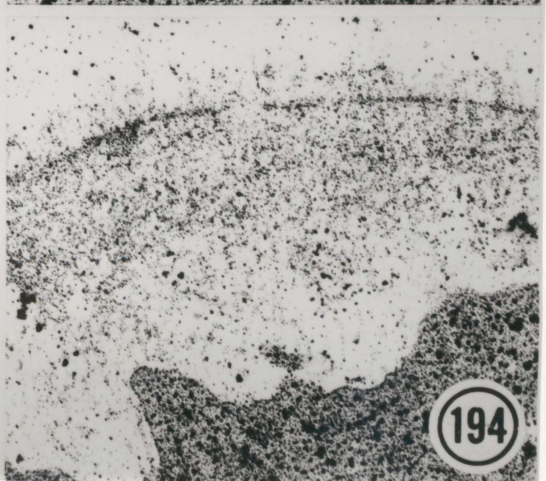
192



193



191



194

and closely associated vesicles (Plate XLI, Figures 195-201). In a few instances, a light silver deposit is also noted in the transitional endoplasmic reticulum (Plate XLI, Figures 200 and 201). Intracisternal staining is not always uniform; the maturing face often manifests a heavier silver deposit than the forming face (Plate XLI, Figures 198 and 200). Moreover, not all dictyosomes within the same cell react to the same degree. In some cases, even adjacent dictyosomes do not display the same intensity of stain; one may stain intensely and the other not at all (Plate XLI, Figure 201). Lorica matrix material also stains heavily (Plate XLI, Figure 202), a result indicating the presence of carbohydrate in the lorica.

Plate XLI. Carbohydrate Deposition: PA-TCH-SP Reaction.

Figure 195. General view of cellular components. Silver precipitate is localized in several dictyosomes and associated cytoplasmic vesicles, a result indicating the presence of carbohydrate in these organelles. TEM thin section. X 31,500.

Figure 196. PA-TCH-SP control. There is no localization of the stain, only a slight background precipitate. TEM thin section. X 22,100.

Figure 197. The presence of carbohydrate is indicated in this dictyosome and in some associated vesicles (arrows). TEM thin section. X 55,100.

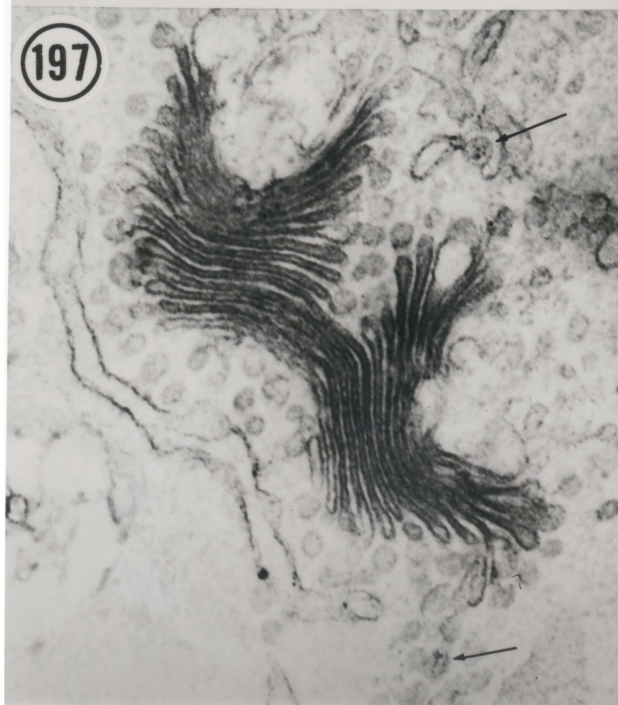
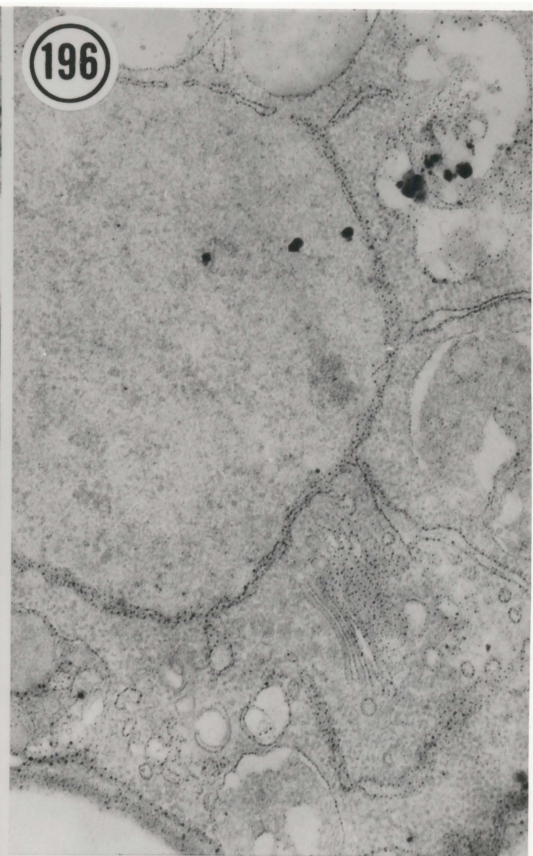
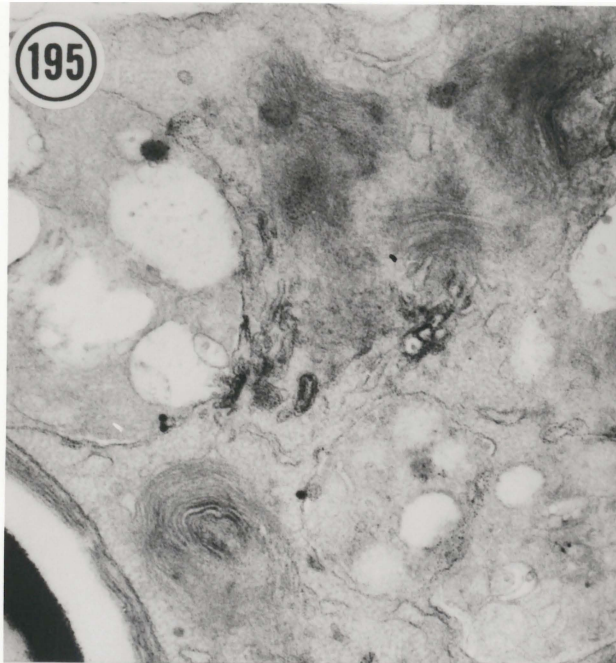
Figure 198. Uneven silver deposition in a labelled dictyosome. An intense precipitate occurs at the maturing face of the dictyosome, in contrast to almost none at the forming face. Note that no stain is apparent in the associated endoplasmic reticulum. TEM thin section. X 61,200.

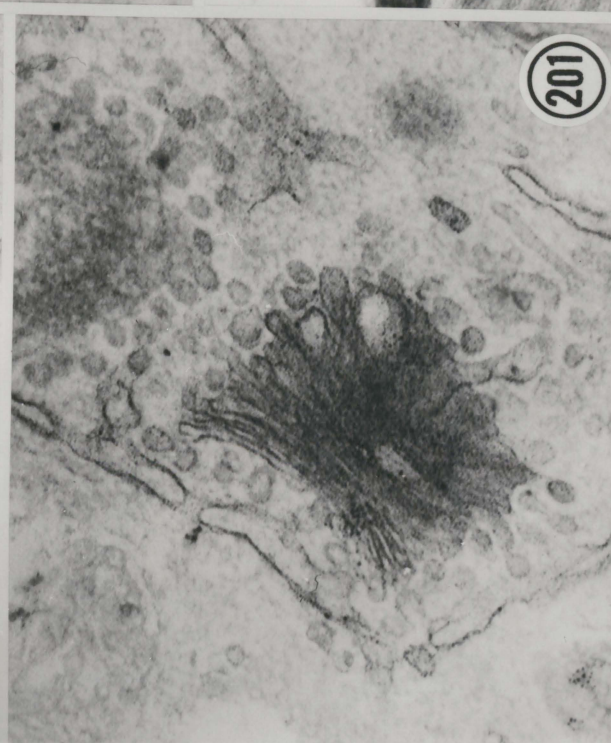
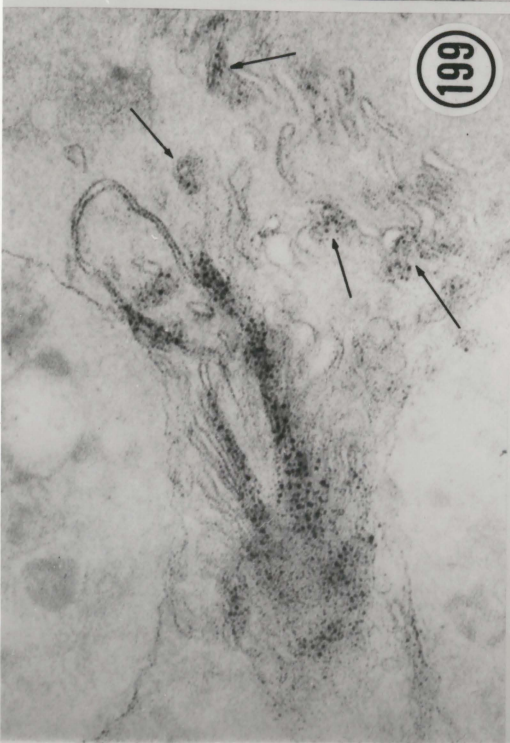
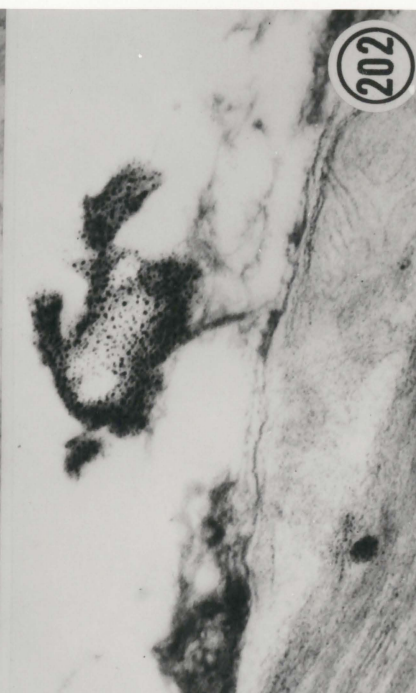
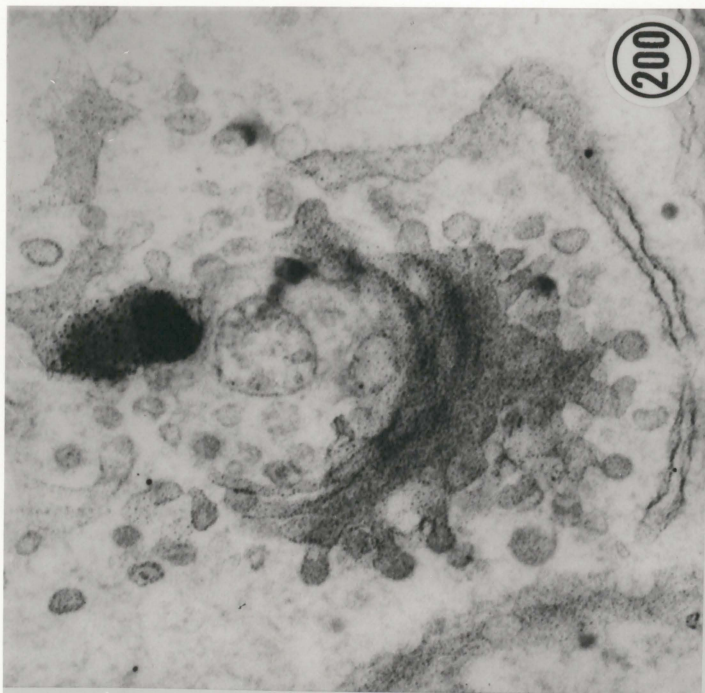
Figure 199. In this glancing section through the maturing region of a dictyosome, a heavy deposit of silver is seen in all cisternae. Many associated vesicles are also labelled (arrows). TEM thin section. X 65,400.

Figure 200. Oblique section through a labelled dictyosome. Heavier silver precipitate is seen in the maturing face and associated vesicles, while the nearby endoplasmic reticulum is faintly labelled. TEM thin section. X 68,900.

Figure 201. Two adjacent dictyosome profiles. The lower dictyosome is heavily labelled with silver, whereas the other has no precipitate. The associated ER is lightly stained. TEM thin section. X 68,900.

Figure 202. Silver precipitate on the outside of the cell, a result indicating the presence of polysaccharide in the lorica. TEM thin section. X 62,600.





CHAPTER IV

DISCUSSION

1. NUTRITIONAL STUDIES

Data derived from nutritional studies of Phacotus lenticularis indicate that the presence of nitrogen in the medium influences the structure and mineral composition of the lorica. P. lenticularis is able to utilize several different forms of nitrogen, although nitrate-N promotes the most luxuriant growth. Ammonium-N is the preferred nitrogen source for most algae; however, nitrate is preferentially utilized by some unicellular green algae (Proctor, 1957; Stross, 1963; Cain, 1965).

The addition of nitrogen to cells in soil-water medium causes subsequent generations of Phacotus to develop extremely thin loricas which are colorless (i.e., they contain no manganese). Conversely, nitrogen-starved cells or those grown in SW produce ornate loricas which are manganese rich. Lorica morphology in other members of the Phacotaceae is also media dependent. For example, the lorica of Pteromonas varians varies in both shape and surface texture depending upon the age of the culture and type of medium used (Evans, 1942; Jane, 1944). Gerard (1980) also reported differences in surface texture and elemental content of the lorica of Pteromonas protracta, depending on whether defined medium or soil-water medium was used.

It has been reported for many different algae that nitrogen deficiency causes a decrease in cellular nitrogen content with a

concomitant change in the major product of photosynthesis, from protein to carbohydrate (Syrett, 1962). Cells in a N-starved condition have an excess of carbohydrate which may be stored as starch or excreted. In other studies, Marker (1965) also found that nitrogen-starved Isochrysis produced more extracellular carbohydrate than normal cells, presumably because of the increased amount of available intracellular carbohydrate. Similarly, cells of Phacotus which are grown in nitrogen-poor conditions (such as soil-water medium) have excess carbohydrate, as is evident from the large amounts of starch stored in the cell. Formation of a large elaborate lorica should present no problem for these cells since there is sufficient excess carbohydrate for incorporation into an extracellular structure. On the other hand, cells which have an ample nitrogen source have lesser amounts of carbohydrates available for extracellular release and may not be able to supply enough carbohydrate to construct a large lorica.

The relationship, if any, between nitrogen availability and manganese mineralization in the lorica is unclear. It may be that in a thin lorica, produced when nitrogen is abundant, fewer metal binding sites are available. Calcium, which appears to be the primary mineralizing element in thin loricas, may bind most available sites and thereby competitively exclude manganese (for additional discussion on mechanisms of mineralization, see section 4, p. 220).

2. ULTRASTRUCTURE OF THE VEGETATIVE CELL

The ultrastructure of the vegetative cell of Phacotus lenticularis (excluding the lorica) is similar to numerous other volvoclean organisms.

The cellular organization differs, however, in several aspects from those phacotacean algae that have been examined with transmission electron microscopy (Porcella, 1976; Gerard, 1980). Noteworthy differences appear in pyrenoid structure and in chloroplast and stigma morphology.

Pyrenoid

The pyrenoid of P. lenticularis has two starch plates surrounding a matrix which is commonly traversed by chloroplast lamellae. No other phacotacean alga examined to date has such a pyrenoid, although several other types have been described. Dysmorphococcus globosus has a pyrenoid with one circular starch plate (Porcella, 1976), while Pteromonas protracta (Gerard, 1980), Pteromonas angulosa (Belcher and Swale, 1967) and Wislouchiella planktonica (unpublished observations) all possess pyrenoids with up to six surrounding starch plates. Dysmorphococcus globosus and Dysmorphococcus africana (Dawson and Harris, 1979), like Phacotus lenticularis, normally have 3-8 pyrenoids, while Pteromonas protracta and Wislouchiella planktonica never have more than one pyrenoid except when the cell is dividing.

The morphology and number of pyrenoids in the Phacotaceae may be helpful in establishing taxonomic relationships among members of the group. Pringsheim (1953) used pyrenoid morphology as a taxonomic feature in the euglenoid genus, Trachelomonas. Together with other cellular attributes, this feature may be helpful in the taxonomic re-evaluation of the Phacotaceae.

Chloroplast Lamellae Lattice

In Phacotus lenticularis, a highly organized lamellar lattice may occur in the chloroplast. The lattice is formed by a regular infolding of chloroplast thylakoids and may account for a large portion of the chloroplast. A similar lattice structure was reported by McLean and Pessoney (1970) in the chloroplasts of the filamentous green alga Zygnema and by Palisano and Walne (1972) in the chloroplasts of Euglena. In contrast to Zygnema, however, in which the lattice occurs only during stationary phase, lattices in P. lenticularis are found in cells of all culture ages and may even be evident in newly divided cells.

In plant cells, the most commonly encountered three-dimensional lattice structure is the prolamellar body, found in chloroplasts of dark grown higher plants (Gunning, 1965). Prolamellar bodies are not well documented among the algae, however. Only in Euglena and Chlamydomonas do lattices occur which are similar in development and structure to the prolamellar bodies of higher plants (Dodge, 1973). The lattice in P. lenticularis is unlike a typical prolamellar body in that it occurs within fully formed chloroplasts and does not require darkness for its initiation.

Although internal lattice structures have been reported in structures other than chloroplasts in a variety of organisms, their function is not clear. The prolamellar body is thought to be a type of membrane storage in the chloroplast, while in certain plant nectaries and fish glands, lattice structures seem to function in secretion and excretion (Gunning, 1965; VanLennep and Lanzig, 1967). The trigger for formation of lattice structures may be related to environmental

conditions. The lattice of Zygnema forms during stationary phase, suggesting a nutrient depletion phenomenon, while in the trypanosome Leptomonas collosoma, lattice formation varies with the osmolarity of the medium (Linder and Staehelin, 1980).

The induction and function of lattice formation in Phacotus lenticularis remain uncertain. Closer attention to environmental parameters during lattice formation might help to clarify the role of this structure.

Stigma

The stigma (eyespot) of Phacotus lenticularis is more complex than that of any other phacotacean alga thus far examined. The stigma is large, consisting of up to four layers of osmophilic granules (60 obvious in some oblique sections), in contrast to only one layer reported in Dysmorphococcus globosus (Porcella, 1976) and from one to three layers in Pteromonas protracta (Gerard, 1980). The complexity of the stigma is somewhat unusual, since many unicellular algae have only one or two layers of stigma granules (Dodge, 1973). In the Chlorophyceae, many multilayered stigmata are found in the colonial members of the Volvocales (Lang, 1963).

Of particular interest in P. lenticularis is the close relationship of the stigma and the flagellar microtubular system. Fortuitous sections reveal a band of four microtubules in close association with the stigma, suggesting a possible functional relationship with the flagella. Few precedents for this type of relationship exist in the green algae (for review, see Moestrup, 1978). In the Volvocales, "eyespot roots"

(sensu Moestrup, 1978) have been noted in Volvox (Pickett-Heaps, 1975) and Eudorina (Hobbs, 1971). Recently, microtubules were also noted in close proximity to the stigma in the phacotacean alga, Pteromonas protracta (Gerard, 1980). Whether this relationship is only structural, mechanical or physiological too, is still a matter of conjecture. The eyespot root in Phacotus lenticularis is a broad root (i.e., more than two strands), as is also true for most other green algae and euglenoid algae thus far examined (Moestrup, 1978).

3. CHARACTERIZATION OF EXTRACELLULAR MUCILAGE

Organic Constituents

The chemical composition of the extracellular mucilage of Phacotus lenticularis is similar in many aspects to the mucilages of other algae. The carbohydrate portion of the mucilage is a heteropolysaccharide, whose main constituents are galactose, arabinose and galacturonic acid. Galactose and arabinose are common constituents of the polysaccharide of both red and green algae (Percival, 1979) and many higher plants (Lamport and Miller, 1971). In the Volvocales, these two monosaccharides have been identified in the extracellular mucilage and/or matrix of Chlamydomonas (Lewin, 1956), Platydorina (Crayton, 1980), Eudorina (Tautvydas, 1978) and Volvox (Kochert, 1975). The presence of galacturonic acid in Phacotus lenticularis is unusual since it is more commonly found in marine seaweeds than in freshwater forms. Uronic acids are particularly good metal ion scavengers, an example of which is the guluronic acid-rich alginic acid of some brown algae (Percival, 1979). Such uronic acids have a high affinity for calcium and sodium

ions, which in turn promotes a highly organized molecular structure; this type of molecule is responsible for the gel strength of the polysaccharide (Rees, 1972).

The mucilage also contains a protein moiety which comigrates with a carbohydrate component on electrophoretic gels, a result suggesting that at least a portion of the mucilage is glycoprotein in nature. Although many marine algae have mucilages which contain carbohydrates exclusively (Percival, 1979), glycoproteins have been located in a large number of freshwater mucilages and cell walls (Roberts, 1974). Many of the glycoprotein matrices, including the mucilage of P. lenticularis, contain the amino acid, hydroxyproline. This amino acid is present in all higher plants and green algae (except Nitella) thus far examined, and in most cases is concentrated in the cell wall (Gotelli and Cleland, 1968). Its regular occurrence is unusual since there is no codon for hydroxyproline; it is a result of a post-translational modification of peptidyl-proline (Chrispeels and Sadawa, 1971; Varner and Burton, 1980).

In the Volvocales, hydroxyproline-containing glycoproteins have been most extensively studied in Chlamydomonas (Catt, Hills and Roberts, 1976; 1978; Roberts, 1979; Roberts, Gay and Hills, 1980; O'Neill and Roberts, 1981), but have also been located in other members of this group. Eudorina californica has a high molecular weight hydroxyproline glycoprotein component in the mucilage surrounding the colony (Tautvydas, 1978). Cytochemical data indicate that the sheath of Volvox also contains some type of glycoprotein substance(s) (McCracken and Barcellona, 1976).

The studies of extruded slimes and cell walls have, in many instances, shown that the extracellular mucilages are solubilized glyco-protein components (Keegstra et al., 1973; Takeuchi and Komamine, 1980). This is not true for Phacotus, since the chemical composition of the lorica and mucilage is not similar, either in weight percent or in composition.

The question as to which membranous cell component is responsible for transport of hydroxyproline-rich glycoproteins to the cell surface remains unresolved. In animal cells, the Golgi apparatus is responsible for both transport and terminal glycosylation of mucopolysaccharides and extracellular glycoproteins (Neutra and LeBlond, 1966a and b). More recently, Rothman (1981) proposed that the refinement of proteins destined for export is carried out in the Golgi apparatus by way of a two-compartment system, the cis and trans Golgi. The removal of "escaped" ER membrane proteins is accomplished in the cis compartment, while the sorting of "refined" proteins for their export to various parts of the cell occurs in the trans compartment.

The biosynthesis and secretion of hemicelluloses and various mucilages of higher plants is also regulated in the Golgi apparatus (Chrispeels, 1976). Studies with secretory mutants of Volvox indicate that synthesis and initial glycosylation of proteins destined for export occur at the rough ER, with the synthesis of carbohydrate and subsequent remodelling of the glycoprotein molecule at the level of the dictyosome (Dauwlder, Whaley and Starr, 1980). The actual site of hydroxyproline-glycoprotein transfer is disputed, however. In Acer cells, Dashek (1970) found that the bulk of hydroxyproline transport was by a smooth

membranous component, most probably the endoplasmic reticulum. However, in carrot root phloem parenchyma cells, most of the labelled glycoprotein was located in the Golgi apparatus (Gardiner and Chrispeels, 1975).

In cells of Phacotus lenticularis which are actively secreting mucilage, silver deposition from the PA-TCH-SP staining reaction is found over many dictyosomes, nearby vesicles and ER profiles. This stain is a good indicator of glycoprotein or mucopolysaccharide molecules since it detects 1,2-glycol and α -amino-alcohol groups, both abundant in glycoproteins (Thiery, 1969). Results from Phacotus indicate that assembly of the mucilage begins at the level of the endoplasmic reticulum or at the transitional region between the ER and Golgi apparatus. Stain intensity increases from the forming to maturing face (cis to trans Golgi, sensu Rothman, 1981) of the dictyosome, a result indicating either an accumulation of product or a modification of the product so that it stains more heavily. Sugars in glycoprotein molecules are often added sequentially to the protein portion, both in the ER and Golgi cisternae (Whaley, Dauwalder and Kephart, 1972). If this is also true of glycoprotein assembly in Phacotus, the increase in stain intensity from the forming to maturing dictyosome cisternae may indicate the presence of a growing glycoprotein molecule. Autoradiographic studies with specific sugars known to occur in the glycoprotein molecule of Phacotus mucilage would be useful in transport studies.

Inorganic Constituents

It is somewhat unusual that the mucilage of Phacotus is strongly sulfated. Although many marine algae have ester sulfate linkages in their extracellular matrices (Percival, 1979), this type of linkage is uncommon in freshwater algae and land plants. Sulfated polysaccharides have been located in the cell wall and/or extracellular matrix of several volvoclean algae. Crayton (1980) confirmed the presence of a sugar-ester sulfate linkage in the matrix of Platydorina caudata using cytochemical staining and infrared spectroscopy. Fulton (1978) located sulfate groups in the sheath and capsule of Pandorina, Astrephomene and Volvulina using diaminobenzidine and Alcian Blue staining. The most complete study, to date, has been with Chlamydomonas cell glycoproteins and excreted glycoproteins. Roberts et al. (1980) found that between 1 and 4% sugar-O-sulfate esters were located asymmetrically in the glycoprotein molecules.

The high sulfate content of the mucilage of Phacotus suggests that it may serve in an important functional role for the organism. In animal connective tissues, for example, sulfated mucopolysaccharides and glycoproteins are important in structural organization, cell differentiation, recognition and adhesion (Lindahl and Hook, 1978; Dietrich et al., 1980). In plants, sulfate groups may promote interchain binding between oligosaccharides or may participate in cell wall assembly (Roberts et al., 1980). Sulfated polysaccharides have been shown to be responsible for cell wall assembly in Fucus (Hogsett and Quatrano, 1978) and for the assembly of scales in Pleurochrysis (Brown and Romanovicz, 1976). Sulfated mucilages may also provide the organism with a mechanism to

withstand drought, since these substances are hygroscopic and can bind large amounts of water.

Sulfated glycoproteins or polysaccharides in algae are synthesized in the Golgi apparatus (Ramus and Groves, 1972; Callow et al., 1978). This is a likely site for the sulfation of the glycoprotein in Phacotus also, although tracer studies are needed to establish this.

Dynamics of Extracellular Release

Most of the extracellular mucilage of Phacotus lenticularis is produced during late log and stationary phase, but secretion does occur at all stages of the cell cycle. The amount of extracellular mucilage in the medium actually decreases slightly during log-phase growth. This is not a unique phenomenon; a similar profile of secretion occurs with the capsular polysaccharide of the red algae, Porphyridium (Ramus, 1972) and Rhodella (Evans et al., 1974), in which there is a decrease in mucilage synthesis during log phase, when cells are actively dividing. Some algae are also capable of re-utilizing excreted extracellular products, in turn, causing a decrease in the amount that can be detected in the medium (Nalewajko, 1977). This may be true of Phacotus but is unlikely, since large-molecular-weight mucilages are rarely re-absorbed (Fogg, 1952).

Previous investigators have questioned the physiological condition of cells which excrete large amounts of mucilage. Marker (1965) observed that extracellular carbohydrate production in the haptophyte Isochrysis was greatest during later growth stages and concluded that

most of the mucilage originated from dead or dying cells. This is not true for Phacotus, since mucilage is produced at all culture phases and can be visualized inside of actively growing cells with TEM.

Ecological Implications

Fogg (1971) described two types of extracellular products in aquatic systems, based on their molecular weights and metabolic origins. Type I extracellular products are low-molecular-weight metabolic intermediates, while Type II products are usually high-molecular-weight compounds which are end products of metabolism. Both types may be liberated by passive diffusion, but Type II compounds are also commonly transported by vesicles which ultimately fuse with the plasma membrane (Fogg, 1971; Nalewajko, 1977). The mucilage produced by Phacotus is clearly a Type II product. Its very high molecular weight is demonstrated by its behavior on electrophoretic gels and by its viscosity in solution. Its transport by vesicles rather than liberation by diffusion is evident in thin sections where fibrous mucilage strands may be visualized inside of intracellular vesicles, at the plasma membrane and subsequently outside the cell.

Type II extracellular products of phytoplankton are important components of aquatic systems. Algal mucilages are potential carbon sources for heterotrophs which may be spatially associated with the algae (Nalewajko, 1977). Bacterial populations often increase in number with an increase in algal photosynthetic activity and excretion, suggesting that the bacteria can utilize the algal products as a carbon source (Vela and Guerra, 1966; Maksimova and Pimenova, 1969; Nalewajko, 1977).

Observations of bacterially contaminated cultures of Phacotus support this type of relationship. In mixed cultures, bacteria preferentially accumulate within large masses of algal mucilage, suggesting a commensal relationship between the two organisms. This relationship may be mutually beneficial, however, since a pronounced increase in the growth of Phacotus occurs when the bacteria are present in the culture. Further research on the nature of the relationship between Phacotus and the bacteria is needed, specifically the role of the mucilage in bacterial nutrition. This could be accomplished by monitoring the excretion of ^{14}C -labelled carbohydrates by the alga and subsequent uptake by the bacteria (e.g., Nalewajko et al., 1980).

Function and Significance

Between 5 and 35% of the photosynthate of phytoplankton is released as some type of extracellular product (Fogg, 1966). Although mucilages are widely accepted as a source of carbon for heterotrophic organisms, the benefit of the release of these organic compounds to the organism itself is less clear. Mucilages of many algae have long been implicated in purely physical phenomena such as the reduction of turbulent friction in flow (Hoyt and Soli, 1965) and prevention from dessication (Percival, 1979). Recently, the elucidation of the chemical structure of many mucilages has also implicated them as physiological and biochemical regulators for the organisms which produce them.

Many mucilages are anionic polymers, due to the presence of carboxylated polysaccharides, uronic acid residues or ester sulfate groups in the molecule. Colorimetric and cytochemical data indicate

that the mucilage of Phacotus contains, at least in part, all of these entities, resulting in an overall negative charge. These free anionic groups have been implicated in the binding and redistribution of biologically important cations in the mucilages of both algae and higher plants (Fogg and Westlake, 1955; Lange, 1976; Leppard, Massalski and Lean, 1977; Ramamoorthy and Leppard, 1977). At the surface of carrot roots, mucilage fibrils rich in polygalacturonic acid can remove cations from the soil for subsequent nutrient ion uptake by the root (Ramamoorthy and Leppard, 1977). The extracellular material released by the cyanobacterium, Anabaena cylindrica, complexes with micronutrient cations and holds them in a form available to the cell (Fogg and Westlake, 1955).

A negatively charged mucilage is also capable of binding to and holding toxic cations in a form less toxic to the cell. Algal extracellular material reduced the toxicity of copper when added to cultures of Anabaena cylindrica by reducing the concentration of free copper ions to a tolerable level (Fogg and Westlake, 1955; Whitton, 1970). The mucilage of Phacotus may also act as a nutrient cation exchanger since it resembles, in structure and composition, the fibrillar pectin of growing root surfaces.

4. CHARACTERIZATION OF LORICA

Organic Constituents

Colorimetric assays of the lorica of Phacotus lenticularis indicate that it has an organic matrix composed primarily of a glucose polymer which is associated with a hydroxyproline-rich protein.

The matrix also contains ester-linked sulfate groups at undetermined positions, giving the lorica an overall negative charge. An acidic polysaccharide component is indicated by positive staining reactions with Ruthenium Red and Alcian Blue, although none was detected by chromatographic or colorimetric methods.

Glucose polymers, such as cellulose or chitin, have been established as major components of the loricas of other unicellular algae. For example, the chrysophyte, Dinobryon has a fibrillar lorica, composed of a weakly crystalline cellulose I associated with a protein (Herth and Zugenmeyer, 1979). Poterioochromonas also has a microfibrillar lorica, but has chitin as its principal component instead of cellulose (Schnepf et al., 1975; Herth et al., 1977). The type of glucose polymer present in the lorica of Phacotus has not been established; however, the type of microfibrillar constituents seen in Dinobryon and Poterioochromonas are not obvious in Phacotus. If the glucose is present as cellulose or chitin, it does not appear to be organized into microfibrillar units.

Compositional similarities to the organic matrix of other phacotacean loricas are unclear. Cytochemical data indicate a positive staining reaction in the loricas of Dysmorphococcus (Porcella, 1976) and Pteromonas (Gerard, 1980) to Ruthenium Red and Alcian Blue, respectively. There are, however, no chromatographic or colorimetric data available to establish whether glucose polymers are part of the matrices of these loricas.

Many algal cell walls have been found to have a crystalline glycoprotein structure, composed primarily of hydroxyproline arabinosides

(Roberts, 1974). The presence of hydroxyproline in Phacotus loricas has been established with colorimetric and chromatographic assays, but the question of a crystalline component remains unresolved. It is possible that the major organic structural units of the lorica, composed of a glucose polymer, are embedded in a hydroxyproline glycoprotein matrix, which may have within it the acidic properties responsible for the cytochemical staining reactions observed.

Dense stellate cores and wispy fibrillar material similar to that found in the lorica matrix have been visualized in Golgi cisternae and Golgi-derived vesicles in Phacotus. These two lorica components are visible in thin sections, both in the same vesicle and in separate vesicles. Both components have the same staining properties and general appearance as elements of the complete lorica.

The data indicate that the lorica components are synthesized in the Golgi apparatus and perhaps the adjacent ER, and are subsequently transported to the outside of the cell where they are correctly assembled into the lorica. This is similar to the pattern established for lorica assembly in Pteromonas protracta (Gerard, 1980). The transport of cell wall or cell covering material by way of Golgi-derived vesicles that ultimately fuse with the plasma membrane is fairly well established. In higher plants, pectic and hemicellulosic wall components are synthesized in the dictyosomes and are subsequently transported to outside the cell where they are incorporated into the cell wall (Chrispeels, 1976). In the algae, there are numerous examples of a similar type of transport for both cellulosic and non-cellulosic wall or extracellular surface components (Manton and Parke, 1965;

Manton, 1966; Moestrup and Thomsen, 1974; Brown and Romanovicz, 1976; Moestrup and Walne, 1978).

Inorganic Constituents

In addition to an organic matrix, the lorica of Phacotus lenticularis also contains a number of inorganic constituents. In colorless loricas, calcium is the predominant element, while in dark loricas, manganese is present in the greatest amounts. In either type, several other elements--P, K, Na, Al, Si and S--are found, although they are usually minor-to-trace constituents.

Loricas from cells grown in nitrogen-supplemented medium or those found on very young cells are never dark in color and have calcium as their major element. In Pteromonas, calcium was a major element in loricas from both soil-water and defined-medium cultures; these loricas also never darkened (Gerard, 1980). These results suggest that calcium may be the primary mineralizing agent in the lorica, upon which other minerals are added. The presence of calcium is not surprising since it is a common mineral in the tests of many protozoans (Zajic, 1969; McEnery and Lee, 1970; Isenberg and Lavine, 1973; Angell, 1979) and is also found in the coccoliths of some haptophycean algae (Isenberg, Lavine and Weissfellner, 1963; Isenberg and Lavine, 1973).

The form of calcium in the lorica of Phacotus is uncertain. Based on chemical tests with HCl, Gerard (1980) suggested that the calcium in loricas of Pteromonas was in the form of calcite. This may also be true in Phacotus, since preliminary results with HCl treatment are similar to those of Pteromonas. Other polymorphs of calcium are

found in the mineralized parts of microorganisms, however. Electron diffraction of a probable coccolithophorid, Polycrater galapagensis, indicates that the calcium carbonate of the scales is present as aragonite and not calcite (Manton and Oates, 1980). All three types of CaCO_3 --calcite, aragonite and vaterite--have been identified in the coccolithophorid, Coccolithus huxleyi (Wilbur and Watabe, 1963). X-ray diffraction of both colorless and dark loricas of Phacotus would confirm the identity of the calcium compound in the lorica. It is possible that different calcium polymorphs are present in light and dark loricas and that this might have a bearing on subsequent mineralizing events.

Manganese appears to be responsible for the darkening of loricas on soil-water cells, although the addition of excess manganese to cultures with colorless loricas does not effect their darkening. Dark loricas treated with EDTA or EGTA retain little or no manganese, seen visually as a loss of brown color and with SEM-EDS as a reduction in the K_{α} peak of manganese. Manganese has also been implicated in the darkening of the loricated euglenoid Trachelomonas (Donnelly, 1979; West, Walne and Bentley, 1980).

Data derived from this study refute early taxonomic descriptions which state that the lorica of Phacotus darkens as a result of iron oxide deposits (Pascher, 1927; Huber-Pestalozzi, 1961). No iron was ever detected in colorless or dark loricas of Phacotus with either SEM-EDS or a potassium ferrocyanide cytochemical test. These observations support those of Kamptner (1950), who reported no iron in the loricas of Phacotus lendneri. The lack of iron in the lorica of Phacotus lenticularis is unusual since this element is recorded in

colorless loricas of Pteromonas protracta (Gerard, 1980) and of Trachelomonas lefevrei (Donnelly, 1979) and in dark loricas of Dysmorphococcus globosus (Porcella, 1976).

The presence of sulfur in the lorica is indicated by its frequent occurrence in SEM-EDS spectra. The sulfur is, for the most part, in the form of inorganic sulfate, as indicated by the barium chloranilate assay and low pH staining with Alcian Blue and Toluidine Blue O. The presence of sulfur in the lorica, therefore, does not necessarily indicate the presence of protein as has been suggested by some investigators (Lang and Chrispeels, 1976; West, 1977; Donnelly, 1979).

Mechanisms of Mineralization

Mineralization of the lorica of Phacotus lenticularis, although affected by nutrient availability, occurs in all cells. Calcium is found in all loricas, regardless of culture conditions and is probably the basic mineralizing element.

Studies with bacterial cell walls have shed some light on the mechanisms of mineral incorporation into wall structures. Bacterial cell walls are composed in large part of an organized network of anionic hetero- and homopolymers, most of which are polysaccharide in nature, much like the organic lorica matrix of Phacotus (Beveridge and Murray, 1976; 1980; Marquis, Mayzel and Carstensen, 1976). Cations in the surrounding medium are free to interact with and bind to the polyanions of the wall matrix. In Bacillus, a two-step metal deposition process is proposed for the cell wall. The first is a stoichiometric interaction of a discrete metal species with the reactive (anionic) groups in the

wall. This is followed by an inorganic deposition of increased amounts of the metal, forming crystalloids which are thermodynamically more favorable for increased deposition (Beveridge and Murray, 1976). This may be the type of mechanisms involved for the minerals found in trace amounts in the lorica of Phacotus or for those which are not persistent in their occurrence.

In his "General Theory of Mineralization," Moss (1964) proposed several factors which he believed were integral to all mineralizing systems. They are especially appropriate to the mineralization of the lorica of Phacotus, since experimental data indicate that the formation of the lorica closely corresponds to the following ideas set forth in Moss' theory:

Mineralization is a cellular process. Cellular energy must be expended to produce at least the organic matrix of the mineralized structure and, possibly, the inorganic matrix. Ultrastructural data show the presumptive lorica matrix precursor material in Phacotus is transported to the surface of the cell by Golgi-derived vesicles, but it is not yet clear whether the inorganic components are deposited in this fashion.

Mineralization is biphasic. The organic matrix of the mineralized tissue is deposited first, according to this idea, after which the actual mineralization occurs. Phacotus deposits a thin polysaccharide or polysaccharide-protein layer soon after cell division, which is later mineralized with calcium and/or manganese.

The organic matrix has two components. The first, an "organized fibrillar-like meshwork" is seen in Phacotus as stellate-shaped

fibrillar cores. The stellate cores are organized into a regular, scalloped pattern along the margin of the lorica. The second, an "unorganized amorphous ground substance-like phase" is obvious especially at the inception of lorica formation but is later obscured by the stellate cores. Both phases are carbohydrate in composition and one or both have a protein moiety associated with them. Moss proposed in his theory that both phases are a protein-carbohydrate complex, with the fibrils embedded in the amorphous layer. In Phacotus, the amorphous matrix is probably an acidic mucopolysaccharide in which is embedded fibrils which are largely composed of a glucose polymer (i.e., chitin or cellulose).

The form (i.e., size and shape) of the mineralized tissue is determined by the organic phase. This seems to be true in Phacotus, since not only is the organic phase deposited first, but it is also deposited in an orderly array, providing a template for further mineralizing events.

Mineralization is not a simple chemical precipitation. The major mineralizing elements in Phacotus, calcium and manganese, are not precipitated onto the lorica surface by an excess of the elements in the medium. Although there is no direct proof for cellular secretion of calcium and/or manganese in Phacotus at the present time, this is a plausible explanation based on studies with other algal systems. The deposition of silicon in diatoms was long believed to be a result of chemical reactions only. The "autopolycondensation" of Si(OH)_4 was postulated to occur without any cellular metabolic intervention. It has been recently shown by Sullivan (1980), however, that silicification in

Navicula pelliculosa is dependent on cellular energy since it was interrupted by inhibitors of oxidative phosphorylation.

The biological deposition of calcium has been investigated for many microorganisms, including several haptophycean algae (Manton and Leedale, 1969; Outka and Williams, 1971; Isenberg and Lavine, 1973). These algae are surrounded by calcium-containing scales, which are liberated to the cell exterior by fusion of Golgi-derived vesicles with the plasma membrane. The calcium-containing cores of the lorica of Pteromonas protracta also are produced inside the cell, rather than precipitated from the medium (Gerard, 1980). It is likely that the calcium in the lorica of Phacotus, and probably the manganese, is deposited under careful biological control, possibly through processing in the Golgi apparatus. Additional studies with labelled manganese and calcium salts may give some insights as to the precise mode of the deposition of these minerals on the surface of the cell.

Lorica Development

Development of a new lorica in Phacotus lenticularis begins before the release of daughter cells from the old "mother" lorica. Lorica formation involves the deposition of a granular layer which is closely appressed to the newly formed cells. This thin, flexible layer is probably analogous to the "immature envelope" or "skin" described by Leedale (1975) for the euglenoid Trachelomonas and by Porcella and Walne (1980) for Dysmorphococcus globosus. Subsequent addition of fibrillar units in a regular array external to the "skin" results in a dense, rigid structure in the mature lorica. This pattern of lorica

development closely resembles that of Pteromonas protracta (Gerard, 1980) and several species of Trachelomonas (Donnelly, 1979). It is very different, however, from that observed in Trachelomonas oblonga var. punctata and Dysmorphococcus globosus, where deposition of the lorica occurs internal to the "skin" or "immature lorica" (Leedale, 1975; Porcella and Walne, 1980).

The regular periodicity of fibrillar deposition in Phacotus may be the result of a template originating in the granular immature lorica. This layer is closely appressed to the plasma membrane in the early stages of lorica development, as is found in Dysmorphococcus (Porcella, 1976; Porcella and Walne, 1980). The plasma membrane may provide a template for the organization of the immature lorica, which in turn promotes further ordered deposition of fibrillar elements. A template mechanism has been implicated in the wall deposition of several other green algae (Kiermayer, 1970; Pickett-Heaps, 1972; Millington and Gawlik, 1975; Porcella and Walne, 1980). Gerard (1980) suggested that the same type of process might be responsible for the hexagonal surface pattern of the lorica of Pteromonas.

The deposition of the lorica of the chrysophyte, Poterioochromonas, is also regulated at the level of the plasmalemma (Schnepf et al., 1975). The lorica is composed of chitin microfibrils which are synthesized at the surface of the plasma membrane and positioned by microfibril synthetases in the plasma membrane. The synthetases are positioned by cytoskeletal microtubules which are located immediately beneath the plasma membrane. It is possible that cytoplasmic microtubules are involved in the regulation of lorica deposition in Phacotus, since

microtubules are frequently seen in thin section immediately below the plasma membrane. However, unlike Poterioochromonas, lorica precursors are visible in Golgi-derived vesicles in Phacotus, indicating that although they may be assembled in final form at the plasma membrane, the lorica precursors are synthesized inside of the cell, and not on the plasma membrane.

Lorica Autolysis

The release of daughter cells from the mother lorica involves a unique pattern of the dissolution of the old lorica into its component fibrillar and granular constituents. The ultrastructure of the disintegrating loricas is similar to that seen after enzyme digestion of the lorica. It is possible that the lorica itself contains autolytic enzymes which are activated at the onset of cell division. These enzymes would then promote dissolution of the lorica to facilitate the exit of the newly formed daughter cells.

It is not improbable that lytic enzymes are present in the lorica, since the presence of protein in the lorica has been demonstrated. It is well established that cell wall hydrolases are present in the cell walls of bacteria, fungi and higher plants, and that they contribute to the autolytic degradation of the wall (Lee, Kivilaan and Bandurski, 1967; Huber and Nevins, 1979). Cell wall hydrolases are involved in the loosening of cell wall structural components to allow for the growth of young plant cell walls (Lee, Kivilaan and Bandurski, 1967). It is significant to note that in bacteria the autolytic process is closely linked to cell division and cytokinesis (Shockman, Kolb and Toennies, 1958) as it appears to be in Phacotus.

Function and Significance

Lorica function in Phacotus lenticularis, as well as other loricate organisms, is somewhat problematic. This investigation has shown that cells of Phacotus which have much smaller loricas as a result of manipulations of media grow in culture equally as well or better than those which produce elaborate loricas. Although the cell expends a large amount of energy in the production of large ornate loricas, their benefit to the cell is unclear.

The lorica probably does not function in the support of the cell, as do many plant cell walls. The cell is physically separated from the lorica, except for a short time at the inception of lorica formation. In addition, thin sections of the cell reveal a rather extensive cytoskeletal system of microtubules, indicating little need for a further support system.

A more likely function for the lorica is that of a biochemical regulator. For example, the composition of the lorica is especially well suited for this. The prevalence of acidic groups in the lorica matrix of Phacotus make it ideal for the trapping and retention of cations. Gram-positive bacterial cell walls with a similar anionic charge have been shown to possess ion-exchange ability which is comparable to that of Dowex A1 resin (Marquis, Mayzel and Carstensen, 1976). The wall (or lorica) may sequester or release ions depending upon the needs of the cell. Some metals may be concentrated to satisfy particular trace element requirements, such as enzyme cofactors. The wall may also provide a short-term source of ions for cells in nutrient-poor medium.

On the other hand, detrimental cations could also be bound to this type of wall or lorica, resulting in a form which no longer poses a threat to the cell. Heavy metal tolerance in plants often involves an ion sink, such as the wall or specialized vacuoles, where metal ions are trapped to prevent cytoplasmic damage (Silverberg, 1975). The green alga Spirogyra submargaritata grows well in areas with high concentrations of heavy metals due to the exclusion of these ions by binding them within the cell wall (Chan, Wong and Wong, 1981). An examination of Stigeoclonium tenue with X-ray microanalysis reveals the accumulation of lead in the walls of cells exposed to lead nitrate (Silverberg, 1975). It has been suggested, in the case of lead, that the anionic groups in the cell wall polyuronides bind the toxic cations (Brown and Bates, 1972). This type of selective exclusion could also be at work in Phacotus, since the data indicate that the lorica does have an abundance of anionic groups.

5. PHYLOGENY AND TAXONOMY

The phylogenetic affinities of the members of the Phacotaceae are problematic due to a lack of a "true" cell wall, such as in Chlamydomonas. Organisms in this family may or may not be a natural group, since their only common feature is a lorica, which itself varies greatly in structure and composition among genera. The homology of the lorica with cell walls in other members of the Volvocales is uncertain, since the chemical composition and substructure of loricas in the group were unknown until recently. Some investigators believe that the Phacotaceae are more closely allied to the naked Volvocales, the Polyblepharidaceae, than to

walled forms since the cells are essentially naked, with no closely appressed cell wall (Higginbotham, 1942). In most schemes, the taxonomic status of the Phacotaceae is at a place close to the Chlamydomonadaceae, with the Phacotaceae as the climax of its particular evolutionary line (Smith, 1950).

Current thought regarding the phylogeny of the green algae centers around cytological rather than gross morphological characters. An examination of highly conserved features such as the flagellar and mitotic apparatus and, recently, the cell wall architecture has led investigators to reconsider the classical taxonomic relationships among the green algae. The algal class Chlorophyceae has been reorganized to include those algae which have a collapsing telophase spindle and a phycoplast during mitosis and whose motile cells have a cruciate flagellar root system (Pickett-Heaps, 1975; Stewart and Mattox, 1975; 1978; Mattox and Stewart, 1977). The ancestor of the Chlorophycean algae is most likely a scaly green flagellate, similar to the members of the class Prasinophyceae (Mattox and Stewart, 1977).

Data derived from thin sections support the inclusion of Phacotus lenticularis in the Chlorophyceae. The nuclear membrane persists during mitosis until at least metaphase and the daughter nuclei remain very close at the time of cytokinesis. A phycoplast is present at telophase and cytokinesis is effected by means of a cell furrow. The flagellar root system is cruciate, with each root normally composed of 2 or 4 microtubules, although roots with 3 microtubules are not uncommon.

The relationship of P. lenticularis to other chlorophycean algae becomes especially interesting when the structure of the lorica is compared with the cell walls of some other members of the group. The lorica of P. lenticularis is composed of stellate cores which are organized outside of the cell into a continuous cell covering; the individual units are no longer discernible after they aggregate. The stellate units bear a striking resemblance to the scales produced by the prasinophycean algae. The origin of the stellate units and their mode of transport to the outside of the cell also strongly suggest that they are homologous to the scales of such algae as Heteromastix and Pyramimonas (Manton and Parke, 1965; Mattox and Stewart, 1977). The consolidation of the scale-like fibrillar units into a lorica strongly resembles the situation found in the prasinophyte, Platymonas (Manton and Parke, 1965). Platymonas is surrounded by a theca formed from the coalescence of small stellate particles liberated to the outside of the cell from Golgi-derived vesicles.

A phylogenetic scheme based on cell wall structure and composition was proposed by Domozych, Stewart and Mattox (1980), who suggest that extracellular scales fused to form a theca and from this, "typical" chlorophycean walls evolved. If this scheme is correct, Phacotus is most likely derived from this evolutionary line at a point before true wall development. The lorica has no structural similarity to the 7-layered wall of Chlamydomonas but, instead, shares the wall architecture of Platymonas. The theca of ancestral phacotacean organisms was probably modified to the lorica of present-day forms by the addition of more polysaccharides and/or glycoproteins to the cell

coat and with a secondary acquisition of inorganic constituents (Figure 203).

Chemical and structural analyses are now needed from other phacotacean algae in order to determine if similar patterns of structure and composition exist. Ultrastructural studies of Pteromonas protracta indicated that the lorica is also produced in scale-like units (Gerard, 1980), whereas no such units were reported for the lorica of Dysmorphococcus globosus (Porcella, 1976; Porcella and Walne, 1980).

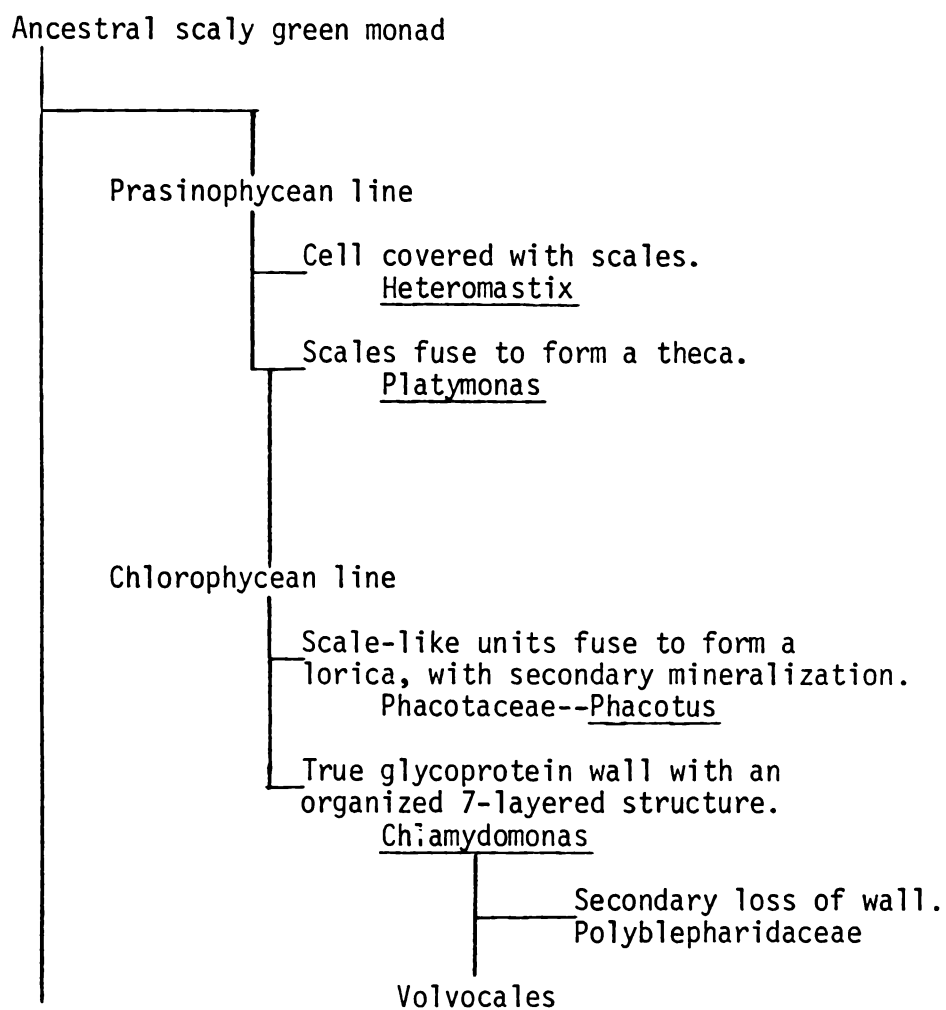


Figure 203. Theoretical phylogenetic relationship of the Phacotaceae to other Chlorophycean algae, based on wall architecture. Adapted from Domozych, Stewart and Mattox, 1980.

CHAPTER V

SUMMARY

Cells of Phacotus lenticularis are distinguished by the presence of a non-living, bivalved, laterally compressed wall-like lorica which is separated from the protoplast. The shape, size and color of the lorica are highly variable and are dependent upon culture conditions. Cells cultured in biphasic soil-water medium have brittle loricas which are ornamented with blunt surface projections and are brown in color. Cells cultured in a defined medium such as Chu #10 or MVM do not develop ornate loricas but instead have thin and colorless loricas which are not readily observed at the light-microscopic level. The addition of nitrate, ammonium or organic nitrogen to soil-water cultures alters the appearance of the loricas so that they resemble those grown in defined media. The presence of nitrogen in the medium not only suppresses the production of an ornate lorica but also promotes more luxuriant growth than in soil-water cultures.

Elemental analysis of isolated loricas with SEM-EDS reveals a striking difference between colorless and dark loricas. Dark (brown) loricas have Mn as the principal element, whereas in colorless loricas, Ca is the predominant element; Na, As, Al, Si, P, S, Cl and K are found in all loricas as trace constituents, while Rh and Ba(Ti) are present sporadically. These results, together with nutritional data, suggest that the presence of nitrogen in the medium affects the elemental composition of the lorica, specifically its ability to bind manganese.

The organic matrix of isolated cell-free loricas was examined with colorimetric assays, TLC and GLC. The matrix is 36% carbohydrate, 26% protein and 4% ester-linked sulfate. The amino acid, hydroxyproline, is estimated to account for ca. 1% of the protein in the lorica. Analyses of the carbohydrate of the lorica by TLC and GLC indicate that only one sugar, glucose, is present in large enough amounts to be resolved with these methods. Cytochemical tests of isolated loricas indicate the presence of both carbohydrate and protein constituents, with carboxyl, sulfate and acidic moieties present in the carbohydrate portion. These data suggest that the lorica contains a structural polymer of glucose with a supporting acid mucopolysaccharide matrix.

The substructure of the lorica was investigated with the aid of the cationic dyes, Alcian Blue and Ruthenium Red, since without the stains, the lorica was barely visible in thin-section. The lorica is composed of two structural elements: 1) a granulo-fibrillar component which is deposited first during lorica development and 2) stellate cores, which are deposited in a regular array on the granular layer. Lorica development begins immediately after cytokinesis, with the formation of a thin, granulo-fibrillar layer which is closely appressed to each daughter cell. Dense stellate cores are deposited outside of this initial layer and subsequently are interconnected to form a continuous structure. Production of the lorica precursor materials occurs in the Golgi apparatus, where both types of lorica components are visualized in thin section.

Loricas lose their structural integrity by the separation of core and fibrillar components and subsequent disintegration, thus

accommodating the release of daughter cells during asexual reproduction. It is suggested that this is a type of autolysis which is triggered by cell division, thereby allowing the release of newly formed cells.

Extracellular mucilage is produced by the cells in copious amounts at all stages of culture, but reaches a maximum at late log-early stationary phases. The mucilage is composed of threadlike strands which aggregate in large mats on the surface of the cell or are released into the surrounding culture medium. The chemical composition of the extracellular mucilage was investigated by a variety of techniques. Colorimetric assays indicate that the mucilage is 50% carbohydrate, 12% protein, 16% ester-linked sulfate and 5% uronic acid by weight. Thin-layer and gas-liquid chromatography suggest the persistent presence of 7 sugars-galactose, arabinose, mannose, xylose, fucose, rhamnose and ribose. Twelve amino acids are present in the protein portion of the mucilage, with hydroxyproline in the greatest amount. Electrophoresis of the mucilage confirms both its large molecular weight and the presence of a glycoprotein component in the mucilage molecule.

At the ultrastructural level, the cellular organization of Phacotus lenticularis is generally similar to vocalean and phacotacean algae. It differs from previously described phacotacean algae, Pteromonas protracta and Dysmorphococcus globosus, in the structure of the pyrenoid and stigma. The stigma of Phacotus is complex and consists of up to 4 layers of osmophilic, polygonal granules. Pyrenoids are surrounded by two starch plates and may number as many as eight per cell. An unusual structure, the chloroplast lamellar lattice, is described for Phacotus lenticularis and is the first such report for any volvocalean

alga. The lattice is formed by an infolding of chloroplast thylakoids into a three-dimensional "quasi-crystalline" structure. The function and cause of the lamellar lattice are not known.

The phylogenetic relationship of Phacotus to other green algae is re-evaluated in terms of its mode of cell division and type of cell covering. Cell division is effected by a phycoplast, warranting the inclusion of Phacotus in the Chlorophyceae (sensu Stewart and Mattox, 1975). The structure of the lorica of Phacotus is similar to the theca of the prasinophyte, Platymonas, rather than to the highly organized wall of Chlamydomonas. This suggests that Phacotus may have diverged from the chlamydomonad line before the evolution of a true cell wall.

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APPENDIXES

APPENDIX A

CULTURE MEDIA

Biphasic Soil-Water Medium (Pringsheim, 1946; Starr, 1978)

Add the following to a half-pint milk bottle:

- a. CaCO_3
- b. Soil 20 g
- c. Double-distilled water 180 ml

Autoclave for one hour on 3 consecutive days. Let stand for 5 days to one week until the liquid clears.

Chu #10 (Chu, 1942)

Prepare the following stocks and store refrigerated:

Stock Solution A	0.5g/L
K_2HPO_4	
Stock Solution B	0.8g/L
FeCl_3	

To make one liter of medium, add one ml of each stock to one liter of glass-distilled water. To this solution, add the following:

$\text{Ca}(\text{NO}_3)_2$	0.04g/L
$\text{Mg}(\text{SO}_4)$	0.025g/L
Na_2SiO_3	0.025g/L

Dispense into culture vessels and autoclave.

Volvox Medium (Starr, 1969)

Prepare stock solutions of macronutrients separately. The micronutrient solution is prepared as a separate stock.

A. Macronutrients

<u>Chemical</u>	<u>Stock</u>
1. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	11.8 g/100 ml H_2O
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4.0 g/100 ml H_2O
3. Na_2 glycerophosphate $\cdot 5\text{H}_2\text{O}$	5.0 g/100 ml H_2O
4. KCl	5.0 g/100 ml H_2O
5. Glycylglycine	5.0 g/100 ml H_2O
6. Biotin	25.0 μg /100 ml H_2O
7. Cyanocobalamine	15.0 μg /100 ml H_2O

B. Micronutrients

Dissolve 0.750 g Na_2EDTA in 500 ml glass distilled H_2O . Add the following:

- 97 mg $\text{FeCl}_3 \cdot \text{H}_2\text{O}$
- 41 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
- 5 mg ZnCl_2
- 2 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
- 4 mg Na_2MoO_4

Add one ml of macronutrients 1-4 and 6-7, 10 ml of macronutrient 5 and 3 ml of micronutrient solution to 981 ml of glass-distilled water. Adjust pH to 7.0 with 1 N NaOH.

Modified Volvox Medium (MVM)

Prepare all nutrient solutions separately.

1. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	11.8 g/100 ml H_2O	3 ml
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4.0 g/100 ml	1 ml
3. Na_2 glycerophosphate $\cdot 5\text{H}_2\text{O}$	5.0 g/100 ml	1 ml
4. KCl	5.0 g/100 ml	1 ml

- | | | |
|-------------------|-------------------|--------|
| 5. Vitamins | see below | 0.5 ml |
| 6. Micronutrients | see Volvox Medium | 3.0 ml |

Vitamin supplement (Guillard and Ryther, 1962)

Biotin primary stock (keep frozen)

10 mg biotin
100 ml distilled water

B₁₂ primary stock (keep frozen)

10 mg B₁₂
10 ml H₂O

To make final vitamin stock, bring 1.0 ml biotin primary stock and 0.1 ml of B₁₂ primary stock to 100 ml and add 20 mg of thiamine HCl. Keep frozen until use.

Add recommended amounts of stocks to 992 ml of glass distilled water. Adjust pH to 7.0 with 1 N NaOH.

TABLE XVIII
SOIL-WATER MINERAL SUPPLEMENTS

Supplement	Concentration Per 100 ml Media
1. $\text{Ca}(\text{NO}_3)_2$	0.0354 g
2. MgSO_4	0.004 g
3. Na_2 Glycerophosphate	0.005 g
4. HCl	0.005 g
5. MVM Vitamins	0.05 ml
6. MVM Trace	0.3 ml

TABLE XIX
MODIFICATION OF NITROGEN SOURCES

Nitrogen Source	Concentration Per Liter
1. None	0.0 mM
2. $\text{Ca}(\text{NO}_3)_2$	1.5 mM
3. NaNO_3	3.0 mM
4. NaNO_2	3.0 mM
5. KNO_3	3.0 mM
6. $(\text{NH}_4)_2\text{SO}_4$	1.5 mM
7. NH_4NO_3	1.5 mM
8. $(\text{NH}_4)_2\text{HPO}_4$	1.5 mM
9. Casein Hydrolysate	0.6 g
10. $\text{CO}(\text{NH}_2)_2$ (Urea)	1.5 mM
11. 2X Urea	3.0 mM
12. 5X Urea	7.5 mM
13. 10X Urea	15.0 mM
14. 25X Urea	37.5 mM
15. 50X Urea	75.0 mM

APPENDIX B

POST-STAINS USED IN TRANSMISSION ELECTRON MICROSCOPY

Potassium Permanganate (Bray and Wagenaar, 1978)

KMnO ₄	1 g
Distilled water	100 ml

Dissolve KMnO₄ in distilled water; store tightly stoppered in the dark at 4°C.

Reynolds' Lead Citrate (Reynolds, 1963)

Lead nitrate	0.665 g
Sodium citrate	0.88 g
Glass distilled water	15 ml

Add above ingredients to a 25 ml volumetric flask; shake well for one minute. Shake over a period of 30 minutes to complete the conversion of lead nitrate to lead citrate. Add 4 ml of 1 N NaOH; mix well. Add glass distilled water to 25 ml and shake until all precipitate disappears. Store refrigerated.

APPENDIX C

CYTOCHEMICAL STAINS

1. Methylene Blue (Johansen, 1940)

Methylene blue	1 g
Distilled water	100 ml

2. Potassium Iodide (Jensen, 1962)

Potassium iodide	3 g
Iodine	2 g
90% EtOH	100 ml

3. Potassium Ferrocyanide (Johansen, 1940)

1% aqueous HCl	25 ml
35% aqueous potassium ferrocyanide	0.5 ml

4. Ruthenium Red (Chambers, 1973)

a. 0.5% Ruthenium Red in 1.34 M sodium cacodylate

Ruthenium Red	0.5 g
1.34 M sodium cacodylate	99.5 ml

b. 1.34 M Sodium Cacodylate

Sodium cacodylate	57.35 g
Distilled water	200 ml

5. Alcian Blue (Ravetto, 1964; Parker and Diboll, 1966)

Stain with 0.5% aqueous Alcian Blue 8GS, adjusted to pH 0.5 with 1 N HCl for 30 to 60 minutes. Wash 10 seconds in distilled water adjusted to pH 0.5. Wash well in distilled water.

6. Alcian Yellow (Ravetto, 1964; Parker and Diboll, 1966)

Stain with 0.5% aqueous Alcian Yellow adjusted to pH 2.5 with 1 N HCl for 30 to 60 minutes. Wash with distilled water.

7. Toluidine Blue Method for Basophila (Chayan et al., 1969)

Solutions:

1. Toluidine Blue

Toluidine blue	0.025 g
Acetate buffer (see below)	25 ml

2. Acetate Buffer(s)

Solution A: 2.85 ml glacial acetic acid made up to 500 ml with distilled water.

Solution B: 6.8 g sodium acetate, made up to 500 ml with distilled water.

For pH 4.2: Add 28 ml A to 12 ml B.

5.0: Add 12 ml A to 28 ml B.

5.6: Add 4 ml A to 36 ml B.

Stain in aqueous toluidine blue in appropriate buffer for 30 minutes. Wash in acetate buffer. Normal basophila will stain blue to blue-purple. If a red, pink or red-purple stain results, this is an indication of metachromasia (see following section).

8. Metachromatic Staining with Toluidine Blue (Chayan et al., 1969)

1. Immerse material in 0.1% solution of toluidine blue at selected pH* for 30 minutes.

2. Rinse in buffer at the same pH.

3. Air dry; mount with Permount.

*For pH 4.2, 5.0 and 5.6, use acetate buffer. For a lower pH, use distilled water and 1 N HCl for the desired pH.

Acid polymers will stain metachromatically at pH 5.6. If the acidity is due to carboxyl groups, the staining will be negative at a lower pH (e.g., 4.2). Sulfate and polyphosphate groups will stain metachromatically at pH 1.0.

9. Periodic Acid-Schiff's Reaction (PAS) (Mowry, 1963)

Solutions:

1. Periodic Acid

Periodic acid	0.5 g
Distilled water	100 ml

2. Stock Sodium Bisulfite

NaHSO ₃	10.4 g
Distilled water	100 ml

3. Sodium Bisulfite Rinse

1 volume stock sodium bisulfite
19 volumes distilled water

4. Schiff's Reagent

Distilled water	96 ml
HCl (conc.)	4 ml
Sodium sulfite	2.5 g
Basic fuchsin	0.25 g

Shake at 20 to 30 minute intervals until solution is clear and brown to amber in color. Add 0.5 g active, decolorizing carbon. Shake 2 minutes and filter; solution should be colorless. Store in a dark bottle at 5°C.

Procedure:

1. Oxidize material for 10 minutes in periodic acid.
2. Wash in running tap H₂O for 5 minutes; rinse in distilled water.
3. Immerse in Schiff's reagent for 10 minutes.
4. Wash with sodium bisulfite rinse; 3 changes, 2 minutes each.
5. Wash 5 minutes in running tap water; rinse in distilled water. Air dry and mount with permount.

10. Mercuric Bromphenol Blue (Mazia et al., 1963)

Mercuric chloride	10 g
Bromphenol Blue	100 mg
95% EtOH or distilled water	100 ml

Stain material with mercuric bromphenol blue solution for 15 minutes. Wash 20 minutes in 0.5% acetic acid. Immerse material in water or a buffer of pH 6-7 for 3 minutes to convert the dye to its blue alkaline form.

Cytochemical Staining of Loricas

Circle a 1-2 cm area in the middle of a clean glass slide with a wax crayon. Pipet enough poly-D-lysine onto the slide to cover the circled area; shake off extra and air-dry. Apply several drops of a lorica suspension to the circled area and allow loricas to settle for 5-10 minutes. Draw off excess liquid with a pipette, leaving enough to keep the surface wet; add stain to circled area. After staining, rinse and make wet mount or dry and mount with Permount.

TABLE XX
REACTIONS OF CYTOCHEMICAL STAINS

Stain	Positive Reaction/General Use	Reference
Methylene Blue	Pectins and cellulose turn blue.	Johansen, 1940
Potassium Iodide	Starch turns blue-black.	Jensen, 1962
Potassium Ferrocyanoide	Iron compounds turn blue.	Johansen, 1940
Ruthenium Red	Pectins and acid mucopolysaccharides turn red.	Chambers, 1973
Alcian Blue	Mucins and acidic polysaccharides turn blue. At pH 0.5, AB complexes exclusively with sulfate groups.	Ravetto, 1964; Parker and Diboll, 1966; Chayan et al., 1969
Alcian Yellow	At pH 2.5, AY complexes with carboxyl groups.	Ravetto, 1964
Toluidine Blue	Basophila turn blue or blue-purple. Acidic polymers cause metachromasia at pH 5.6. Sulfate or polyphosphate groups cause metachromasia at pH 1.0.	Chayan et al., 1969
Periodic Acid- Schiff's Reaction	Vicinal hydroxyl groups turn pink.	Mowry, 1963
Mercuric Bromphenol Blue	Proteins turn blue.	Mazia et al., 1953

APPENDIX D

PROCEDURES FOR EXTRACELLULAR MUCILAGE ISOLATION

Ethanol Precipitation (Lewin, 1956)

1. Centrifuge cells in a clinical centrifuge at medium speed.
2. Combine all supernatants.
3. Millipore filter supernatant with RA type filters (mean pore size: 1.2 μm).
4. Re-filter supernatant with HA type filters (mean pore size: 0.45 μm).
5. Concentrate to 50 ml.
6. Add concentrated supernatant to 150 ml of cold 95% ethanol.
7. Allow precipitate to settle for one hour.
8. Collect precipitate by centrifugation. Extract supernatant again; collect precipitate.
9. Wash combined precipitates with absolute ethanol and dry in a dust-free area at room temperature.
10. Dry overnight in a vacuum desiccator before use.

Ethanol Precipitation (modified from Evans et al., 1974)

1. Collect cells by centrifugation at 10,000 g for 15 minutes.
2. Filter supernatant with Millipore RA type filters.
3. Dialyze for 48 hours against running tap water and 24 hours against deionized water (2 changes).
4. Concentrate medium under reduced pressure.
5. Precipitate mucilage with 4 volumes of absolute ethanol and recover by centrifugation at 40,000 g for 30 minutes.
6. Air-dry and store as in preceding protocol.

Cetylpyridinium Chloride Precipitation (Scott, 1960; Ramus, 1972)

1. Collect cells by centrifugation at 10,000 g for 10 minutes; save supernatant.
2. Filter supernatant with 1.2 μm Millipore filters, followed by 0.45 μm filters.
3. Add NaCl to 0.05 M and CPC to 0.1%. Heat solution to 40°C.
4. Centrifuge and collect precipitate.
5. Wash CPC-precipitate with 0.05 M NaCl + 0.1% CPC at 40°C for 24 hours.
6. Wash CPC-precipitate with distilled water for 24 hours.
7. Solubilize in 2 M CaCl_2 at 40°C.
8. Precipitate in 3 volumes of cold ethanol, wash in absolute ethanol and dry.

APPENDIX E

HYDROLYSIS PROCEDURES

Hydrolysis of Polysaccharides

1. Trifluoroacetic Acid (TFA) (Jones and Albersheim, 1972)

Suspend 3 to 10 mg of material in 2 ml of 2N TFA and hydrolyze at 121°C in an autoclave for one hour. Remove TFA by evaporating to dryness in a stream of filtered air. Store hydrolysate at least 12 hours in a vacuum desiccator over KOH pellets to remove any remainder of TFA.

2. Sulfuric Acid (H₂SO₄) (Ramus, 1972)

Suspend 3 to 10 mg of material in 10 ml of 1 N H₂SO₄ and hydrolyzed for one hour at 100°C. Neutralize hydrolysate with solid barium carbonate, using Congo Red as an indicator. Centrifuge, filter and dry in a vacuum desiccator at least 12 hours over CaSO₄.

Hydrolysis of Proteins

Suspend 10 mg of material in 2 ml of 6 N HCl. Evacuate and seal tube; hydrolyze for 6 hours at 138°C. Neutralize hydrolysate with 3 N and 0.3 N NaOH, using Congo Red as an indicator. Filter and store frozen.

Hydrolysis and Deionization of Polysaccharides for Sulfate Determination (Jones and Albersheim, 1972; Ramus, 1972)

1. Hydrolysis

Suspend 20 mg of material in 1 ml 6 N HCl and hydrolyze for 3 hours at 100°C. Evaporate over CaCl₂ and NaOH pellets in a vacuum desiccator.

2. Deionization

Add 2 to 3 ml of hot water to the hydrolyzed sample to solubilize. Add sample to a test tube containing 0.3 g of Rexyn 101 or Dowex 50 cation exchange resin. Stir one hour. Decant liquid and transfer to a new tube containing 0.2 g of resin. Add 3 ml distilled water to resin in the first tube. Stir both tubes for one hour. Decant liquid from the second tube and save. Wash resin in the second tube with 3 ml of distilled water. Combine all liquid and evaporate in a vacuum desiccator.

APPENDIX F

PROCEDURES FOR THIN-LAYER CHROMATOGRAPHY

TABLE XXI
SOLVENT SYSTEMS FOR THIN LAYER CHROMATOGRAPHY

Solvent System	Components	Development	Major Use	Reference
I	Butanol/Pyridine/Water; 6/4/3	Develop for 90 minutes, twice	General separation of carbohydrates	J. T. Baker Chemical Co. Bulletin
II	Ethyl Acetate/Pyridine/Water; 120/25/20	Develop 10 cm	Used to separate xylose and ribose	Jones, 1962
III	Isopropanol/Water 80/20	Develop 10 cm	Used to separate glucose and galactose	Jones, 1962
IV	Ethyl Acetate/Pyridine/Water/Acetic Acid; 5/5/3/1	Develop 10 cm	Separation of uronic acids	Gee and McCready, 1957
V	Chloroform/Methanol/Acetone/Butanone/NH ₄ OH 80/80/20/10/30	Develop 15 cm (1st dimension)	Separation of amino acids	Tautvydas, 1978
VI	Butanol/Acetic Acid/Water 4/1/1	Develop 15 cm (2nd dimension)	Separation of amino acids	Tautvydas, 1978

TABLE XXII
SPRAY REAGENTS FOR THIN LAYER CHROMATOGRAPHY

Reagent	Visualization	Appearance of Compound	Major Use	Reference
Anthrone	Heat plate at 110 C for 5 to 6 min.	Yellow spots	Ketoses and oligosaccharides	Kodak Technical Bulletin JJ-6
Aniline Phosphate	Heat plate at 100 C for 5 minutes.	Pentoses, red-brown; aldoses and sorbose, yellow or yellow-brown	Carbohydrates	Kirchner, 1978
Anisidine Hydrochloride	Heat plate at 100 C for 5 to 7 min.	Aldohexoses, green-brown; ketohexoses, yellow; aldopentoses, green; uronic acids, red; 2-deoxy aldoses, gray-brown	Carbohydrates	Kodak Technical Bulletin JJ-6
Ethyl Acetate-Aniline-TCA	Spray plate, air-dry; heat at 95° C for 5 minutes.	Tan spots	Uronic acids	Gee and McCreedy; 1957
Ninhydrin	Dry plate at 105 C for 1 to 2 min.	Yellow, orange or purple spots	Amino acids	Jones, 1962

Preparation of Spray Reagents for Thin Layer Chromatography

1. Anthrone

Dissolve 300 mg anthrone reagent in 10 ml boiling glacial acetic acid. Add 20 ml ethanol, 3 ml H_3PO_4 and 1 ml H_2O . Spray and heat for 5-6 minutes at 110°C .

2. Aniline Phosphate

Add 20 ml aniline to 200 ml of water, then add 180 ml of acetic acid followed by 10 ml H_3PO_4 . Store at 4°C .

Dilute 2 parts of stock reagent with 3 parts of acetone for spraying. Heat plate at 100°C for 2 to 5 minutes.

3. p-Anisidine Hydrochloride

Spray with 3% p-anisidine hydrochloride in 1-butanol. Heat for 2 to 10 minutes at 100°C .

4. Ethyl Acetate--Aniline--TCA

Dissolve 2 g each of aniline and crystalline trichloroacetic acid hydrate in 100 ml of ethyl acetate. Spray plate and air dry for 15 minutes. Heat for 5 minutes at 95°C .

5. Basic Lead Acetate

Prepare a saturated, aqueous solution of basic lead acetate and filter. Spray plate and blot to remove excess solution. Heat plate over steam for 1 minute.

6. Ninhydrin

Spray with 0.2% ninhydrin in acetone. Heat at 105°C for 1 to 2 minutes. Do not overheat.

APPENDIX G

PROCEDURES FOR GAS-LIQUID CHROMATOGRAPHY

Derivative Formation

1. Dissolve hydrolyzate in 0.5 to 1.0 ml of TRI-SIL (Pierce Chemical Company); stopper tightly.
2. Shake for 30 seconds; let stand at room temperature for at least 10 minutes.
3. If all sugars do not dissolve, warm to 60°C for 2 to 3 minutes.
4. Inject 1 μ L or amount desired into the gas chromatograph.

Standard Solutions

Authentic carbohydrates were used to determine standard retention times for comparative purposes. Standards were prepared at a concentration of 2.0 to 3.0 mg/ml TRI-SIL for routine analyses or were partially derivitized by saturating 1.0 ml TRI-SIL with the standard. The latter procedure produced characteristic peaks for the various anomer and ring isomer forms of individual sugars.

APPENDIX H

PROCEDURES FOR COLORIMETRIC ANALYSES

Phenol-Sulfuric Acid Method of Carbohydrate Determination (Dubois et al., 1956)

Solutions:

1. Phenol Reagent

90% Phenol solution	90 ml
Distilled water	10 ml

2. Reagent Grade H₂SO₄

3. Sugar Standard Solution

Sugar standard	50 mg
Distilled water	100 mg

Procedure:

Pipette 20 to 140 μ g of standard sugar solutions into a series of test tubes. Adjust volume to 2 ml. Pipette 2 ml of unknowns and a reagent blank into another series of tubes. Add 50 μ L of phenol reagent to each tube; mix well. Rapidly add 5 ml of H₂SO₄ to each tube (do in a fume hood, as spattering of hot acid may occur). Mix each sample well and allow samples to stand at room temperature for 30 minutes. Read absorbance at 485 nm.

Uronic Acid Determination (Ahmed and Labavitch, 1977)

Reagents:

1. 0.15% m-hydroxydiphenyl (m-phenylphenol) in 0.5% NaOH. Foil wrap to exclude light and store at 4°C.
2. 0.0125 M sodium tetraborate in concentrated sulfuric acid. Store at 4°C.

Procedure:

Weigh 5 mg of isolated unhydrolyzed ECM into a 20 ml beaker. Add 2 ml of chilled, concentrated H₂SO₄ to the beaker and swirl gently. Place the

beaker in an ice bath on a stir plate. Add 0.5 ml distilled water to the beaker while stirring slowly. Allow the preparation to stir for approximately 5 minutes. Add another 0.5 ml distilled water dropwise to the sample and stir until the sample dissolves. Transfer sample to a 10 ml volumetric flask; wash beaker several times and use these washings to bring volume to 10 ml.

Add 0.6 ml of sample (or standard) to chilled test tubes. To this add 3.6 ml of sodium tetraborate reagent and mix well. Heat the tubes in a boiling water bath for 5 minutes; cool in tap water. Add 60 μ L of m-hydroxydiphenyl and mix promptly. Read absorbance at 520 nm.

When solutions containing neutral sugars are heated in H_2SO_4 -tetraborate, a slight pink color is produced. To correct for this, blanks were used in which 60 μ L of 0.5% NaOH was added instead of m-hydroxydiphenyl.

Hydroxyproline Determination (Leech, 1960)

Reagents:

1. 0.05 M Copper sulfate in water
2. 2.5 N NaOH
3. 6% Hydrogen peroxide. This solution must be made and used within 24 hours.
4. 3 N H_2SO_4
5. 5% p-Dimethylaminobenzaldehyde in 1-propanol. Store in the dark.
6. Standard hydroxyproline solution. Dissolve 0.05 g of hydroxyproline in 400 ml distilled water; add 20 ml concentrated HCl and bring volume to 500 ml with distilled water. This 100 μ g/ml solution is diluted to give standards of 5, 10, 15 and 20 μ g/ml.

Procedure:

Add 1 ml of hydrolyzed sample or standard to a Pyrex test tube. To this add 1 ml of CuSO_4 followed by 1 ml of 2.5 N NaOH. Mix all contents by gentle swirling. Heat the test tubes in a water bath until contents reach 40°C (about 3 to 5 minutes). Add 1 ml of 6% H_2O_2 to each tube, mixing each thoroughly before the addition of H_2O_2 to next tube. Incubate tubes at 40°C for 10 minutes, with gentle agitation every 2 to 3 minutes. Cool test tubes with tap water; add 4 ml of 3 N H_2SO_4 , followed by 2 ml of 5% p-dimethylaminobenzaldehyde. Mix well. Cap all test tubes and heat to 70°C for 16 minutes. Cool with tap water and mix well. Read absorbance at 555 nm.

Sulfate Determination (Lloyd, 1959)

Reagents:

1. 0.05 M Potassium hydrogen phthalate, pH 4.0
2. Barium Chloranilate
3. H_2SO_4 standards; 80-200 $\mu\text{g}/\text{m}$

Procedure:

Add a 4 ml aliquot of a hydrolyzed, deionized sample or K_2SO_4 standard to a test tube. Mix with 1 ml of potassium hydrogen phthalate, pH 4.0, and 5 ml of 95% ethyl alcohol. Add approximately 0.3 g of barium chloranilate, cap with parafilm and agitate at 1 minute intervals for 20 minutes. Barium chloranilate is only slightly soluble and will be present in excess, therefore the addition of an exact amount is not critical. After 20 minutes, excess barium chloranilate and precipitated barium sulfate are removed by centrifugation at 4000 g for 5 to 10 minutes. Read absorbance at 530 nm.

BIO-RAD Protein Assay (Bio-Rad Laboratories)

Reagent:

Dilute 1 volume of Bio-Rad dye reagent with 4 volumes of deionized water. Filter with Whatman #1 paper. Store at room temperature no longer than two weeks.

Standard Assay Procedure (20-140 μg protein; 200-1400 $\mu\text{g}/\text{ml}$):

Prepare BSA protein standards of from 0.2 to 1.4 mg/ml. Place 0.1 ml of standards and samples in test tubes. Add 5 ml of diluted dye reagent. Mix by inversion. After a period of from 5 minutes to 1 hour, measure absorbance at 595 nm.

Microassay Procedure (1-20 μg protein; $\leq 25 \mu\text{g}/\text{ml}$):

Prepare protein standards of from 1 to 25 $\mu\text{g}/\text{ml}$. Place 0.8 ml of standards or samples in test tubes. Add 0.2 ml of dye reagent concentrate. Mix by inversion. Measure absorbance as above.

Estimation of Carbohydrates by Anthrone (Yemm and Willis, 1954)

Anthrone Reagent:

Dissolve 0.2 g of anthrone in 100 ml of H_2SO_4 , made by adding 500 ml of concentrated acid to 200 ml of distilled water.

Procedure:

Pipette 5 ml anthrone reagent into Pyrex test tubes and chill in an ice bath. Layer 1 ml of sample on the acid; cool for 5 minutes. Mix while still cooling in the ice bath. Fit tubes loosely with corks and boil for 5 minutes and measure absorbance at 620 nm.

APPENDIX I

PROCEDURES FOR SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Preparation of Gels (Weber and Osborn, 1969)

Solutions:

1. Gel Buffer

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	7.8 g
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	38.6 g
SDS	2.0 g
Distilled water	to 1 liter

2. 10% Acrylamide Solution

Acrylamide	22.2 g
N, N'-Methylenebis-acrylamide	0.6 g
Distilled water	to 100 ml

Store at 4°C in the dark.

3. Ammonium Persulfate

Ammonium persulfate	0.15 g
Distilled water	10 ml

Procedure:

Mix 15 ml deaerated gel buffer with 13.5 ml acrylamide solution. After further deaeration, add 1.5 ml ammonium persulfate and 0.045 ml of N,N,N', N'-tetramethylethylenediamine. Mix quickly and deaerate; fill gel tubes to a level of 10 cm with a syringe. Before gels harden, add several drops of water to the top to the gel solution. Within 15 minutes, gels will have solidified and water may be removed.

Preparation of Samples

For each gel, the following were mixed in a small test tube:

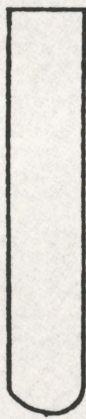
Protein digest	150 μ L
β -mercaptoethanol	15 μ L
Glycerol	2 drops
Bromphenol Blue (0.05% in water)	15 μ L

From 50 to 150 μ L of the sample were applied to the tops of each gel. The gel buffer was diluted 1:1 or 1:2 for use as a running buffer. Gel running time is decreased by lowering the molarity of the buffer. The two compartments of the apparatus were filled with the running buffer and electrophoresis was performed with the positive electrode in the lower chamber.

APPENDIX J

CRITERIA FOR VISUAL ESTIMATION OF GROWTH

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VITA

Linda Ann Pocratsky was born to Stanley and Irene Pocratsky on February 10, 1954 in Connellsville, Pennsylvania. She attended Immaculate Conception Grade School in Connellsville through Grade 4 and subsequently finished her elementary school education at Transfiguration Grade School in Monongahela, Pennsylvania. She attended Mon Valley Catholic High School and was graduated second in her class of 1971. She received a scholarship to Washington and Jefferson College in Washington, Pennsylvania, and graduated in 1975 with a Bachelor of Arts degree and a major in biology. In September, 1975, she began graduate work at Kent State University, Kent, Ohio, in the Department of Biological Sciences. She was employed as a laboratory technician in 1975 and a Graduate Teaching Assistant in 1976-1977. She participated in the Marine Botany program at the Marine Biological Laboratory, Woods Hole, Massachusetts during the summer of 1976. She completed the requirements for the Master of Science degree in Botany in August, 1977.

In September, 1977, she entered The Graduate School of The University of Tennessee, Knoxville, in the Department of Botany. She served as a Graduate Teaching Assistant in 1977-79 and 1980-82. She was the recipient of a Hilton A. Smith Non-Service Fellowship from The University of Tennessee, Knoxville, during the 1979-80 academic year. She was also awarded a Sigma Xi Grant-in-Aid in 1981. She received the Doctor of Philosophy degree in March, 1982.

She is a member of Phi Kappa Phi, an associate member of Sigma Xi and has been listed in the 1982 edition of Who's Who in the Southeast.