Protoplasma 70, 73—86 (1970)

The Structure and Formation of the Test of *Pyura stolonifera (Tunicata)*

A. B. WARDROP

Department of Botany, La Trobe University, Melbourne, Australia

With 17 Figures

Received December 29, 1969

Revised February 20, 1970

Summary

Previous X-ray diffraction studies have established the crystallographic identity of tunicin with cellulose from plant cell walls. Enzymatic degradation of the tunicin results in the isolation of microfibrils 120–130 Å in width (which appear to consist of two sub-units). Using staining and freeze-etching techniques it has been demonstrated that the microfibrils occur in the test as aggregates measuring 2000–4000 Å in diameter. In this respect the physical texture of the cellulose of *Pyura* resembles that of collagen in animal connective tissue and does not resemble the texture of cellulose in either the primary or secondary cell walls of plants.

Examination of fixed and frozen etched specimens showed that ferrocytes of the test and vesicles derived from them are closely associated with the cellulose microfibril bundles. However, at the optical level, autoradiographs of animals treated with C^{14} glucose showed greatest radioactivity to be in the epidermal cells of the mantle and of the blood vessels, but not in the ferrocytes. These cells also showed a considerable development of rough ER and of Golgi bodies. On the evidence obtained it is considered that the sites of cellulose synthesis are the epidermal cells of the mantle and of the blood vessels. The function of the test cells is unknown. The migration of ferrocytes to areas of wounding in the test suggests that they may have some lytic function associated with wound repair.

1. Introduction

Although cellulose has been reported to occur in a number of animal structures (ENDEAN 1961, HALL and SAXL 1961), it is in the tests of the *Tunicata* that it occurs in greatest abundance. References to earlier literature are given by BERRILL (1950) and ENDEAN (1955 a–c, 1961). Summarizing earlier literature, BERRILL (1950) states that the test contains approximately 60 per cent cellulose, 27 per cent nitrogen-containing components and, in the fresh condition, ca. 90 per cent water. The nature of the protein in the test has been discussed by HALL and SAXL (1961). In different groups of the *Tunicata*

the extent of development of the test varies greatly. In colonial forms it may be thin and translucent or highly coloured, whereas in the *Pyuridae* it reaches its greatest development and contains numerous test cells originating in the epidermis or from the blood vessels which ramify through it.

The crystallographic identity of tunicin with cellulose I of plants was established by the studies of MARK and SUSICH (1929), HERZOG and GONELL (1924), and later by RÄNBY (1952). It was also demonstrated by SUTRA (1932) and by RÄNBY (1952) that tunicin may be mercerized to yield cellulose II identical with that obtained from plant cellulose. That tunicin is aggregated in the form of microfibrils similar to those of plant cellulose was shown by the electron microscopic studies of FREY-WYSSLING and FREY (1950) and of RÄNBY 1952. RÄNBY estimated the microfibrils to be 120 ± 20 Å in width.

Other investigations of the test have been directed to the study of the manner of aggregation of the microfibrils and to determine their orientation in relation to the shape of the animal. ENDEAN noted the presence of a network of fibres in the test of *Pyura* (1955 b) and of *Phallusia* (1961). He observed further that the microfibrils were often oriented parallel to the length of the blood vessels. In *Boltenia* and *Molgula* MISHRA and COLVIN (1969) demonstrated bands of microfibrils randomly arranged in the plane of the test surface, and in transverse section no lamellation of the test was observed. These investigators concluded that the organization of cellulose in the test differed from that in plant cell walls in that the microfibrils are not arranged in lamellae, but there was some analogy with the randomly arranged microfibrils of the primary walls of plant cells. DECK *et al.* (1966) also noted some similarities between the texture of cellulose in the tunic of *Perophora* and that of plant cell walls.

The site of formation of cellulose has generally been accepted as being in the epidermal cells (BERRILL 1950, DECK *et al.* 1966). On the other hand, ENDEAN (1955 a), while admitting this possibility, has noted the identity of blood cells in the test vessels with the amoeboid cells of the test and concluded that these amoebocyte (ferrocytes) cells may be involved in the formation of cellulose.

The purpose of the present study was, first, to study the texture of the test of *Pyura stolonifera* and, secondly, to attempt to detect any cytological phenomena which may indicate the role of the amoebocyte cells in the formation of the test.

2. Material and Methods

Specimens of *Pyura stolonifera* were collected from San Remo Western Port Bay, Victoria. Under laboratory conditions, in aerated sea water, the specimens could be kept alive for 3–4 weeks.

2.1. Optical Microscopy

Slices were removed from the test and frozen using the "Frigistor" equipment of the de la Rue Company, Cambridge and mounted in glycerol for examination between crossed nicols.

2.2. Electron Microscopy

Fixation. Slices ca. 1 mm thick were cut from the test with a scalpel; any adhering sand grains were cut off, and the remaining test cut into pieces not greater than 1 mm^3. The specimens were fixed in potassium permanganate (2 per cent at room temperature for 2 hours) or glutaraldehyde (6 per cent in 0.1 M phosphate buffer for 2 hours).
Embedding. Permanganate fixed material was embedded in a mixture of methyl and butyl methacrylate (1 : 4) after dehydration in ethanol or acetone. Glutaraldehyde fixed material was similarly dehydrated and embedded in Araldite using the method of GLAUERT and GLAUERT (1958).
Sectioning. Sections were cut using either an LKB or Reichert ultramicrotome and examined in a Siemens Elmiskop 1 A electron microscope. Some sections, after Araldite embedding, were shadowcast using Pt.-Pd. following the method of MASER *et al.* (1967).
Freeze-etching. Freeze-etching of specimens was carried out using the method of MOOR (1964) employing the apparatus of Balzers. Specimens were impregnated with glycerol (20 per cent) before the etching process.

2.3. Autoradiography

Autoradiography studies were carried out only at the optical level. Small whole animals (ca. 1 cm tall) were immersed in sea water to which C^{14} labelled glucose had been added (0.05 mc per 10 cc). The specimens were dissected after 2, 16, or 24 hours treatment. Alternatively, a solution of C^{14} labelled glucose (0.05 mc per 5 cc) was placed in a hypodermic syringe and the needle was inserted into the test of mature animals (10–20 cm tall) which were dissected after 2, 16, or 24 hours. From dissected pieces of the tunic slices were cut, fixed and dehydrated in ethanol and embedded in paraffin. From the embedded slices sections ca. 20 microns thick were cut, mounted on glass slides and covered with Kodak AR. 10 stripping film or with NTB. 2 (Kodak emulsion). The preparations were developed after 7–20 days using D. 19 Kodak developer, dried, and examined microscopically.

3. Results

3.1. The Texture of the Test

Electron micrographs of microfibrils isolated by digestion first with pepsin and then by cellulase, showed these structures to have the form of flat ribbons, 120–130 Å in width (Fig. 1). In some instances the microfibrils appeared to consist of two sub-units (arrow, Fig. 1).
In sections embedded in Araldite and post-stained with lead citrate the individual microfibrils could be resolved measuring 120–130 Å in width (Fig. 2). These structures thus appear similar to those isolated by enzymatic digestion (Fig. 1).
Evidence that the microfibrils were aggregated into bundles was obtained by the examination of sections of fixed material (Fig. 2), of Araldite embedded sections after shadowcasting (Fig. 13), and by the examination of specimens subjected to freeze-etching (Fig. 3). Near the surface of blood vessels the microfibril bundles tended to be aligned parallel to the vessel length. This could be seen both in electron micrographs and in sections when examined between crossed nicols (Fig. 14). In agreement with ENDEAN (1955 b), there was no other evidence of any preferred alignment of microfibrils in the test.

Thus, it is apparent that the microfibrils in the test of *Pyura* occur in aggregates resembling aggregates of collagen fibrils occurring in connective tissue of other animals. This is in marked contrast to the woven texture of primary walls and to the lamellate texture of secondary walls of plant cells.

3.2. The Cytology of the Test Cells

ENDEAN (1955 a) recognized seven types of cells in the blood of *Pyura stolonifera*. Of these, ca. 70 per cent consisted of lymphocytes and the so-

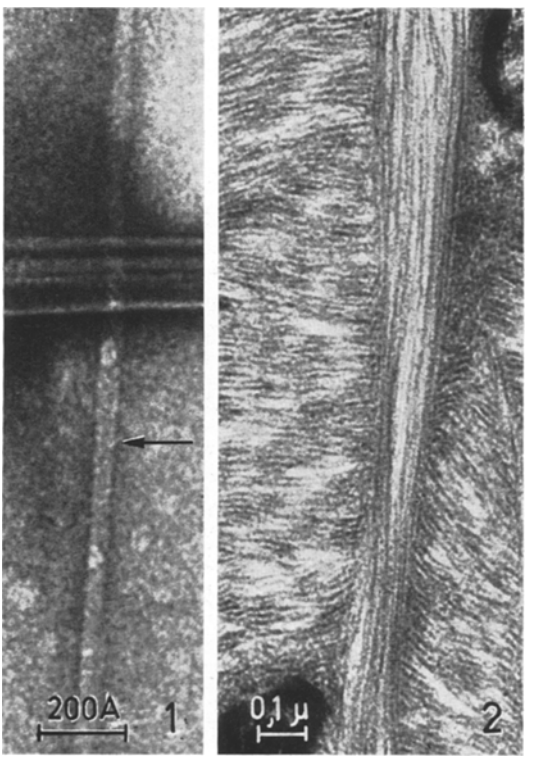


Fig. 1. Microfibrils of the test isolated by digestion with pepsin (1 per cent aqueous for 16 hours) followed by cellulase (1 per cent aqueous for 16 hours) and negatively stained with P.T.A. The arrow points to regions where there appear to be two sub-units

Fig. 2. A section of the test fixed in glutaraldehyde and stained with lead citrate and uranyl acetate; showing microfibril bundles

called "morula" cells or of cells intermediate between these types. The lymphocytes are characterized by a large nucleus and granular cytoplasm. The morula cells are ca. 8 microns in diameter and have a small nucleus and numerous cytoplasmic vesicles. In the present study the different cell types noted by ENDEAN were found in the test as well as in the blood vessels. The cells observed in the test were either lymphocytes, morula cells or cells of intermediate characteristics. The cells of intermediate structure were between 5 and 16 μ in length and were characterized by a very large nucleus and numerous vacuoles (Figs. 4–7).

Golgi bodies, endoplasmic reticulum and mitochondria could be recognized (Figs. 5 and 6). Also many cells such as those in Fig. 6 appeared to be at

various stages of degeneration. In some cells few organelles other than mitochondria could be seen, and the vacuoles were either filled or appeared empty (*A*, Fig. 6). Other cells appeared to be at a more advanced stage of degeneration (cell *B*, Fig. 6) and fragments of them could often be recognized among the test fibrils (Fig. 2). In Fig. 4 the similarity in appearance of vacuoles within the cell and vesicles external to it, can be seen. A cell at a similar stage of development is shown in the shadowcast section of fixed test shown in Fig. 13.

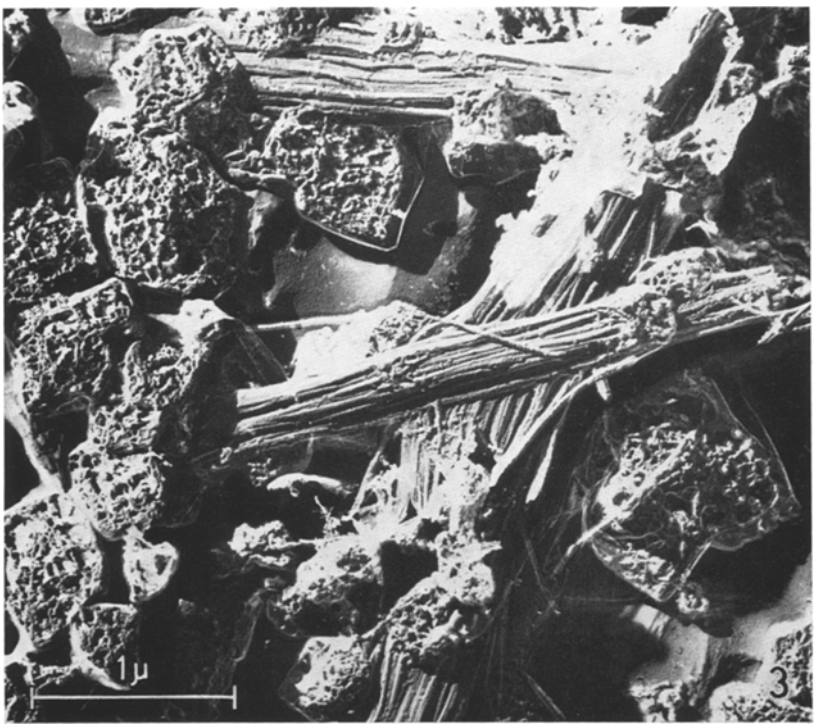


Fig. 3. A frozen-etched preparation of the test showing bundles of cellulose microfibrils

Cells of the morula type or at an advanced stage of differentiation towards this type of cell were characterized by the presence of numerous vacuoles and few other organelles could be recognized. With glutaraldehyde fixation fibrils could be seen closely associated with the plasmalemma (Fig. 7). This was also seen in frozen-etched preparations (Fig. 8). The vacuoles of the morula cells had thick walls (Figs. 9 and 11) which appeared in some instances to be lamellated. At one point (arrows, inset Fig. 9) the plasmalemma was cut almost normal to its surface and at higher magnification could be seen to consist of two layers separated by a depression. Vesicles similar to the vacuoles of the morula cells could be seen lying free in the test (Fig. 4) and

in some specimens, such as that shown in Fig. 10, it appeared that the vacuoles had passed through the surface of the plasmalemma. Evidence of vesicle secretion by both the developing cell and mature morula cells was also obtained by the examination of shadowcast glutaraldehyde fixed sections (Fig. 13) in which the ends of microfibril bundles in the test can be seen. Within the cell numerous vacuoles and the large nucleus were apparent. Some vesicle structures, some distance from the cell surface (arrows, Fig. 13), appeared similar to the vacuoles of the cell.

The vesicles lying free in the test in some instances appeared to be associated with microfibrils (Figs. 11 and 12). However, instances of this association were rarely seen and the possibility that they represent some artefact in preparation cannot be excluded. It was considered, however, sufficiently relevant to the discussion in this paper to include the observation.

3.3. The Cytology of the Epidermis and of the Epidermal Cells of Blood Vessels

The epidermal cells of the mantle contained extended areas of endoplasmic reticulum with associated ribosomes, Golgi bodies, and numerous vesicles possibly derived from either of these structures (Fig. 16). The epidermal cells of the blood vessels, so far as could be judged, contained fewer Golgi bodies than the epidermal cells of the mantle, but greatly inflated profiles of rough endoplasmic reticulum with granular contents were present (Fig. 17). At the margin of the test and the epidermal cells vesicles appeared to undergo dissolution (arrow, Fig. 17).

3.4. Autoradiography

As described, autoradiographs were prepared from young animals living in sea water to which C^{14} labelled glucose had been added and from mature specimens into the test of which hypodermic needles containing C^{14} glucose were inserted. In all specimens the epidermis and test vessels were heavily labelled and the test adjacent to the vessels was also labelled. The cells of the test did not show any radioactivity (Fig. 15).

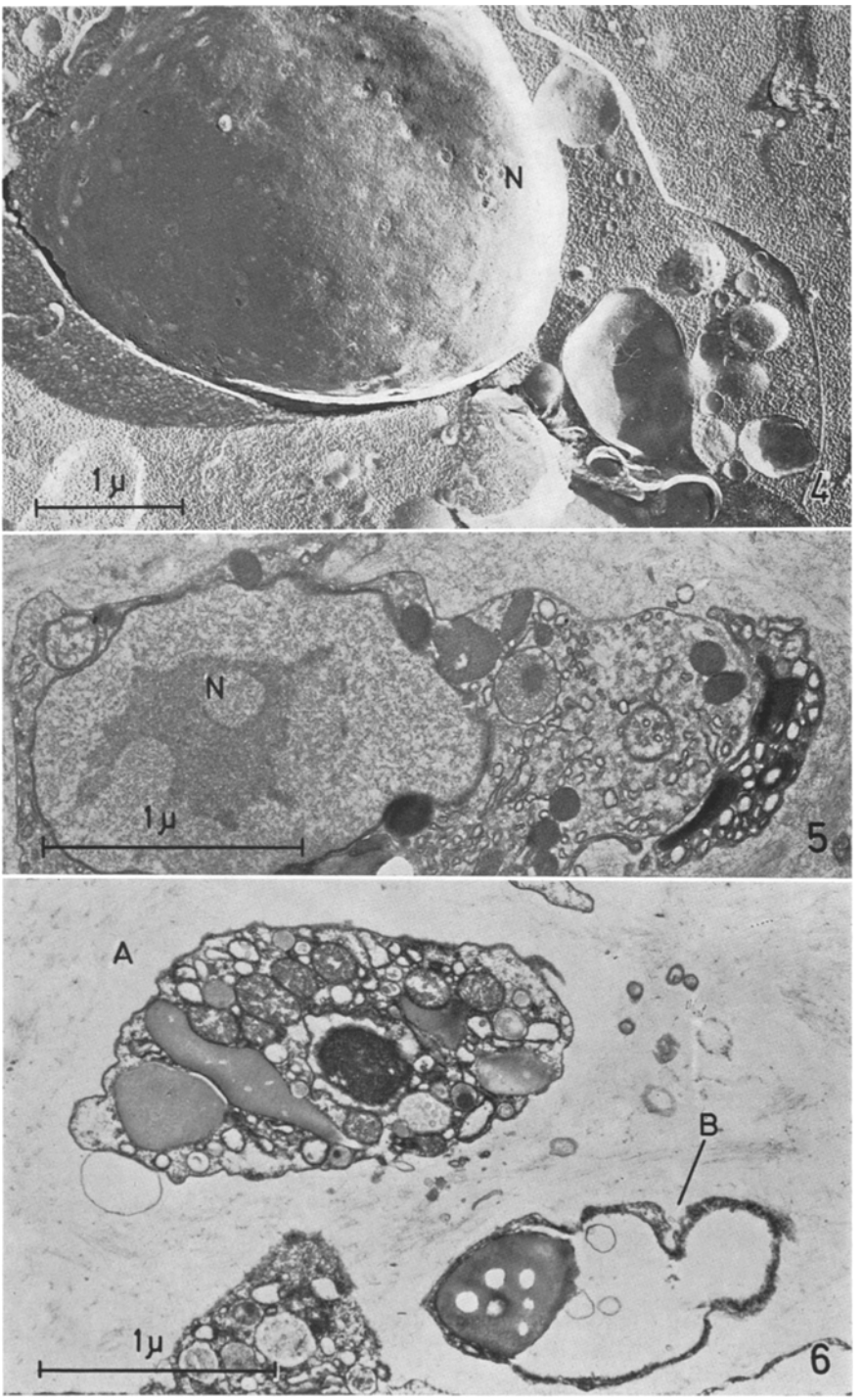
4. Discussion

From observations on preparations of enzymatically degraded tunicin it is apparent that the cellulose occurs in the test as microfibrils 120–130 Å in

Fig. 4. A frozen-etched preparation of a ferrocyte in the test. The similarity of the vesicles within the cell to those lying free in the test is apparent

Fig. 5. A section of a cell similar to that in Fig. 5. The cell is characterized by its large nucleus (*N*), and numerous vacuoles. Mitochondria and Golgi bodies can also be seen (permanganate fixation)

Fig. 6. A section of a degenerating ferrocyte with mitochondria and numerous vacuoles, some of which appear to possess dense contents (glutaraldehyde fixation)



Figs. 4–6

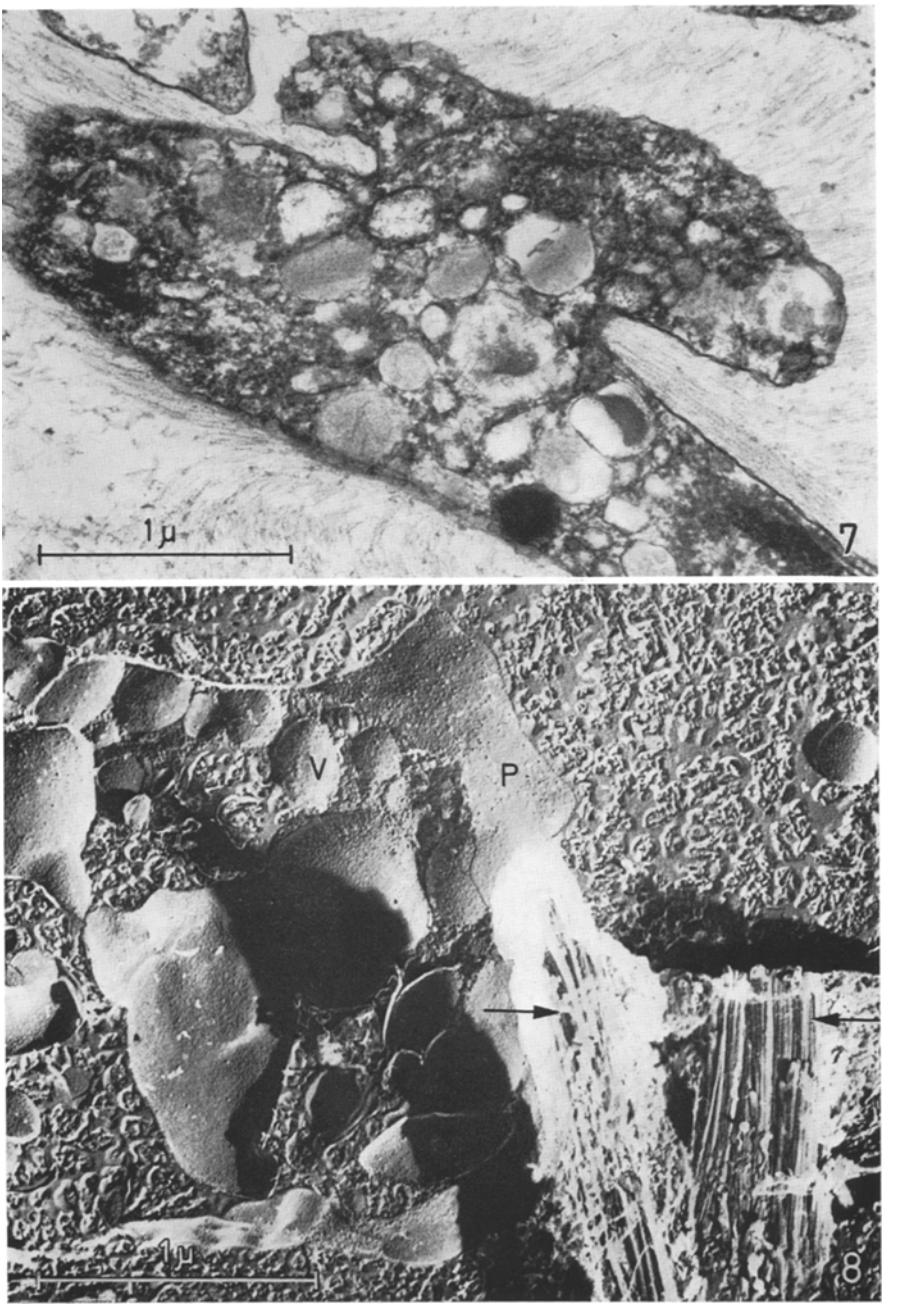


Fig. 7. A section of a ferrocyte showing numerous vacuoles and associated test microfibrils (glutaraldehyde fixation)

Fig. 8. A frozen-etched preparation of a ferrocyte showing numerous vacuoles (*V*), the plasmalemma (*P*), and associated microfibril bundles (arrows)

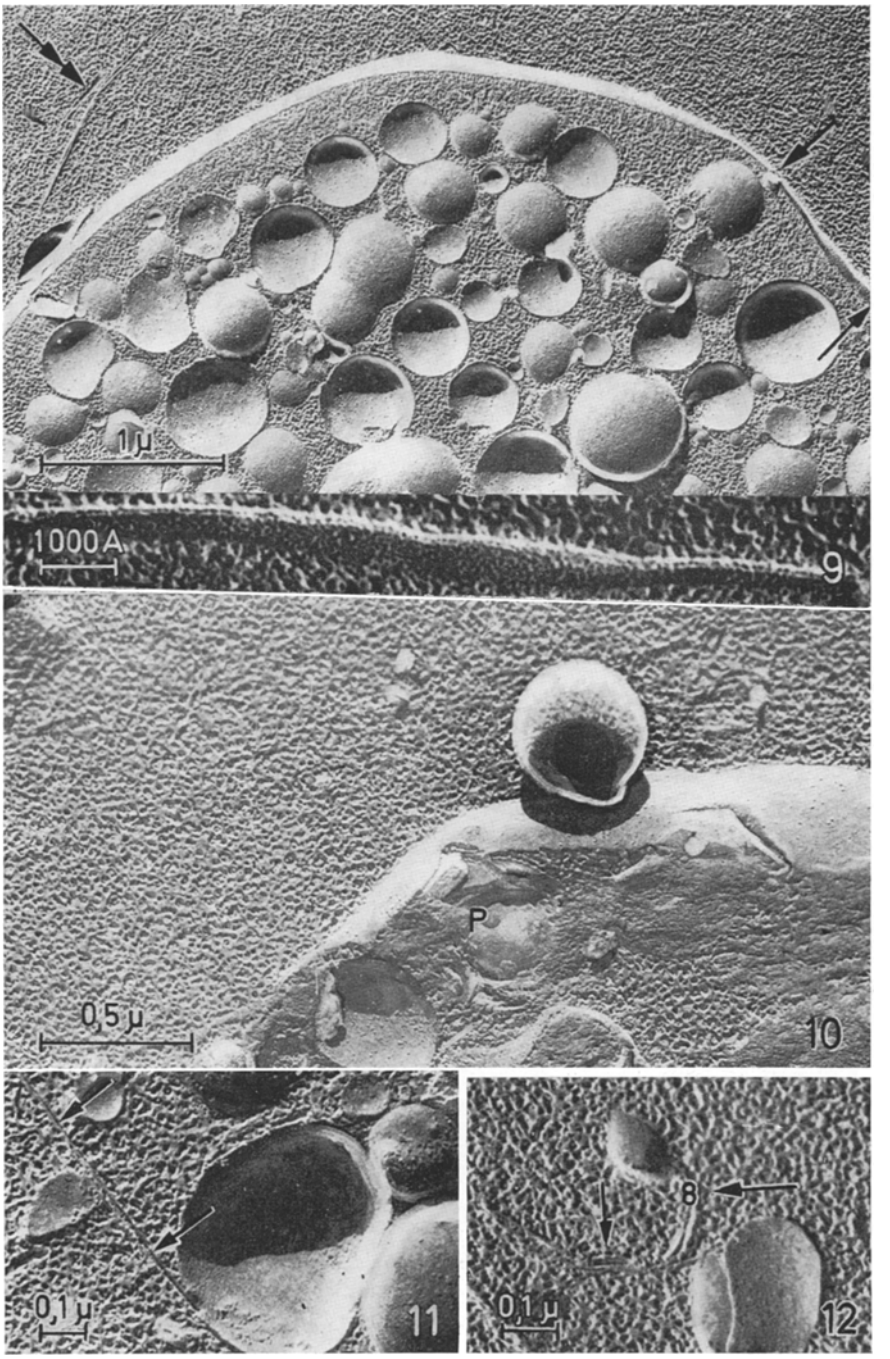


Fig. 9. A frozen-etched test cell at the "morula" stage showing numerous vacuoles, some microfibrils (double arrow). Inset = enlargement of the area between the single arrows, showing the structure of the plasmalemma in section

Fig. 10. Part of a preparation similar to Fig. 9 where a vesicle gives the appearance of having emerged through the plasmalemma

Figs. 11 and 12. Test vesicles similar to that shown in Fig. 10 and associated microfibrils (frozen-etched preparation)

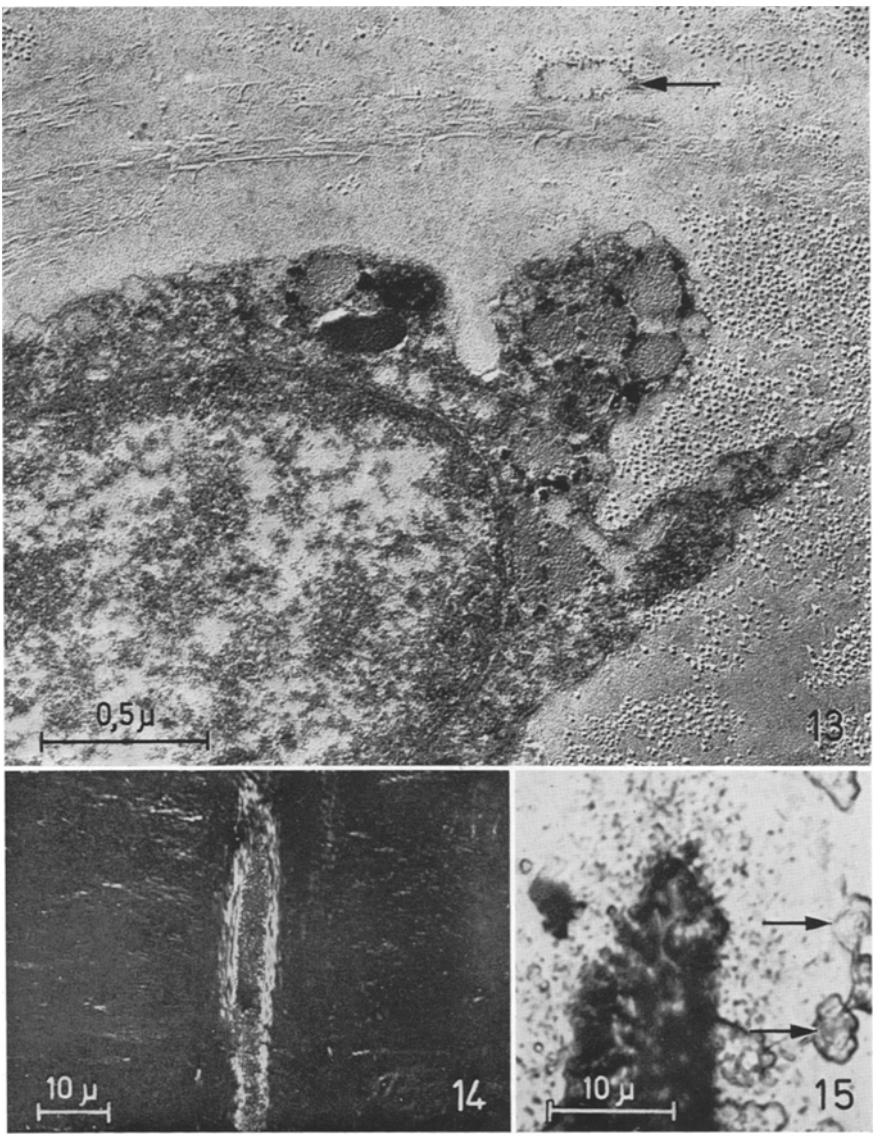


Fig. 13. A test ferrocyte, shadowcast after glutaraldehyde fixation, showing microfibril bundles in section and vesicles apparently derived from ferrocytes (arrow)

Fig. 14. A longitudinal section of test vessel photographed between crossed nicols, with the vessel at 45° to the plane of polarization. Note the patches of birefringence corresponding to regions of parallel microfibrils

Fig. 15. An autoradiograph of part of a test vessel from a specimen injected with C^{14} labelled glucose for 2 hours. Note the absence of radioactivity in the ferrocytes (arrows)

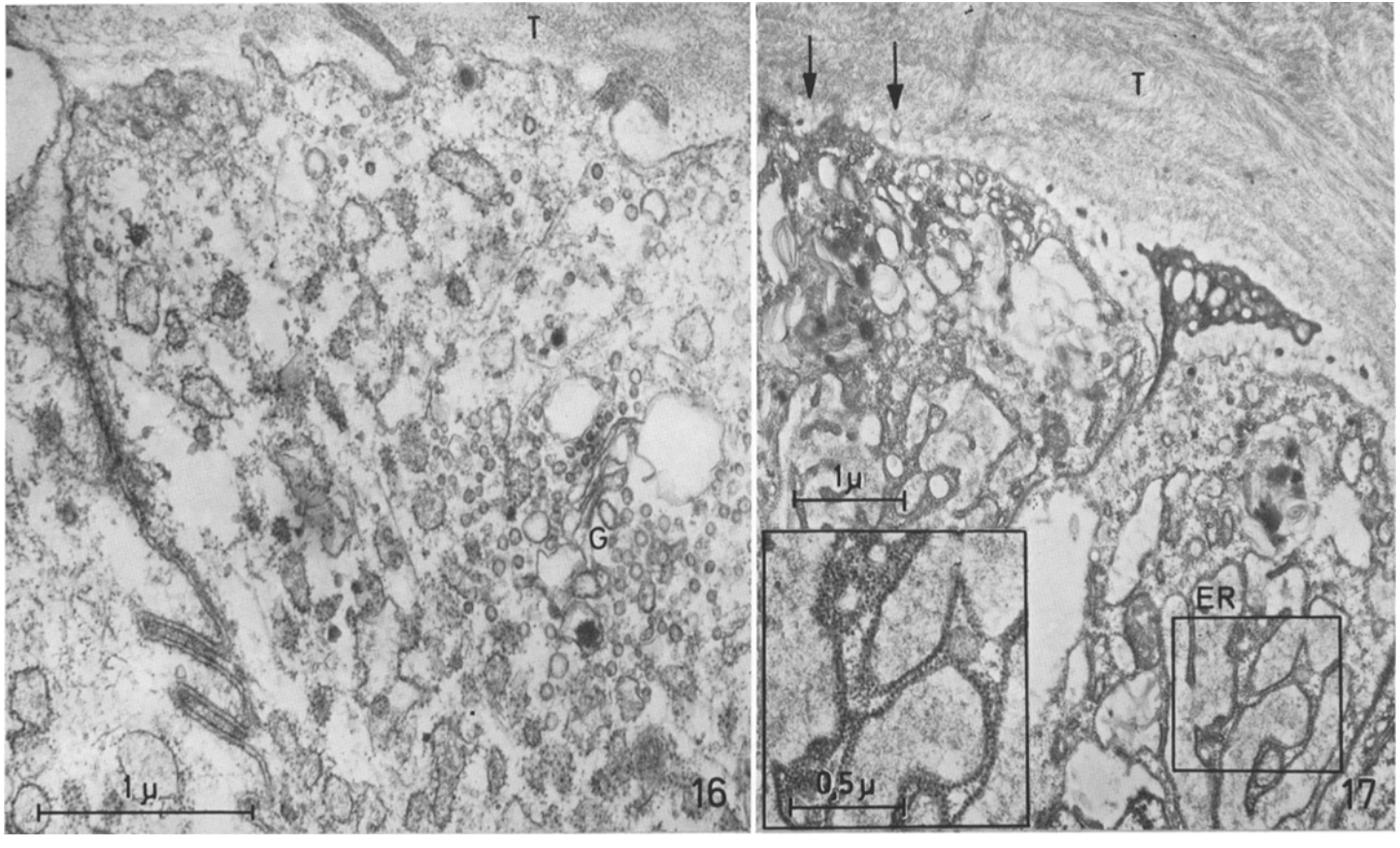


Fig. 16. A section of an epidermal cell of the mantle showing numerous vesicles derived from the Golgi bodies (*G*). *T* = test. (Glutaraldehyde and osmium tetroxide fixation—stained with lead citrate)

Fig. 17. A section of two adjacent epidermal cells of a test blood vessel showing greatly inflated profiles of rough ER and numerous derived vesicles. The arrows indicate some vesicles apparently breaking down in the test *T*. Inset = an enlargement of the marked area showing ribosomes on the ER and its granular contents. (Glutaraldehyde and osmium tetroxide fixation—stained with lead citrate)

thickness. Elementary fibrils 30–40 Å in their lateral dimensions, such as those demonstrated in plant cellulose by FREY-WYSSLING and MÜHLETHALER (1963) were not detected. Since elementary fibrils could be isolated from *Valonia* microfibrils by the same method as that used in the present study (WARDROP and JUTTE 1968) the query must be raised whether in fact tunicin microfibrils are composed of elementary fibrils as sub-units. However, in electron micrographs such as that in Fig. 1 there was some evidence that microfibrils may be composed of two smaller sub-units. From Figs. 2 and 3 it is apparent that in the test the microfibrils are aggregated into bundles 2000–4000 Å in width. Contrary to the conclusion of DECK *et al.* (1966) and of MISHRA and COLVIN (1968), this type of microfibril aggregation constitutes a texture quite different from the woven or lamellated textures of the walls of plant cells, but does resemble the manner of aggregation of collagen fibrils in connective tissue (PORTER 1959, ROSS 1968). In a previous X-ray study by Miss K. KELSEY (1963), in association with the author, it was shown by the critical point drying method of ANDERSON (1951) that the cellulose of the tunic existed in crystalline state. These observations are in agreement with the frozen-etched image shown in Fig. 3.

The question of the site of formation of the test has been reviewed by BERRILL (1950) and the consensus of opinion particularly resulting from the work of HECHT (1918) on *Ascidia* and of BRIEN (1930) on *Clavelina* is that test substances are secreted next to the epidermis and are lost from the surface of the test, so that this structure is in a state of dynamic equilibrium. BRIEN concluded that the test cells arise in the mesenchyme and migrate through the epidermis. In the *Pyuridae* the test contains numerous blood vessels which end in ampullae through which test cells pass from the blood.

In contrast to the conclusion of HECHT and of BRIEN, ENDEAN (1955 a–c) concluded that the test cells are involved in the formation of test substances. This conclusion was based on the observation that when wounded the test of *Pyura stolonifera* rapidly regenerates and the process of wound repair is characterized by the migration of numerous test cells to the site of test regeneration.

In the present study on the same species the test cells appeared to be closely associated with the fibril bundles (Figs. 7 and 8) and indeed the vesicles formed from the morula cells (Figs. 9–12) were seen to be scattered among the fibril bundles (Fig. 13) so that it appeared possible that the cellulose microfibrils could arise either from the plasmalemma of the test cells or from vesicles secreted from them. Indeed, some secreted vesicles appeared to be involved in fibril formation (Figs. 11 and 12). It may be noted that, in view of the migratory behaviour of the test cells, either site of origin would provide a ready explanation of the random, three-dimensional, distribution of the microfibril bundles.

The evidence of autoradiography, however, does not give any indication that cellulose may originate in the test cells or vesicles derived from them.

At the optical level it could be seen that, irrespective of the manner of administration of the C^{14} labelled glucose, the radioactivity was confined to the epidermis and the test immediately adjacent to it, or to the test vessels and the test adjacent to them (Fig. 15). The lack of any obvious association of the radioactivity and the test cells, even at the site of injection of the C^{14} labelled glucose, suggests that the mantle epidermis and epidermis of the vessels are the sites of cellulose synthesis. Evidence that the epidermis was involved in cellulose synthesis was obtained by DECK *et al.* (1966) for the colonial species *Perophora viridis*. It was further pointed out by these workers that the epidermal cells were rich in Golgi bodies and rough endoplasmic reticulum, but that the test cells generally lack these organelles. This was also found to be so for *Pyura* (*cf.* Figs. 5–7 and Figs. 16 and 17) but, in addition, the epidermal cells making up the walls of the blood vessels contained many profiles of endoplasmic reticulum with numerous ribosomes and was greatly inflated and contained granular contents (Fig. 17). Furthermore, there was some evidence of vesicular secretion within the test at the test-plasmalemma interface (arrow, Fig. 17). Thus, the autoradiographic evidence and the cytological features of the epidermis support the observation of DECK *et al.* (1966) that the epidermis is a site of the cellulose synthesis, but in *Pyura* the cells making up the walls of the test vessels (Figs. 15 and 17) are also involved.

Although there was no direct evidence as to what the function of the test cells may be, their accumulation near wounds in the test suggests that they may have some scavenging function by ingestion of wound debris or may achieve this result by the release of hydrolytic enzymes by the secretion of enzyme-containing vesicles.

Thus, generally, the observations made in the present study show that the cellulose of the test of *Pyura* is aggregated in bundles 2000–4000 Å in width, which is a form af aggregation analogous to the physical texture of collagen in connective tissue. Autoradiograph studies and cytological observations are considered with the view that the site of formation of cellulose is the epidermis of the animal or the epidermal cells of the test blood vessels.

Acknowledgements

The author wishes to express his appreciation to Mr. F. J. DANIELS for technical assistance, to Dr. R. ENDEAN of the Department of Zoology, University of Queensland, for helpful discussions and to Mr. R. PHILLIPS and Mr. F. COLLET for specimens of *Pyura stolonifera*.

Some of the preparations described (*viz.* those shown in Figs. 10, 12, and 13) were made in the laboratory of Professor K. MÜHLETHALER at the Eidg. Technische Hochschule, Zürich, Switzerland. The author wishes to thank Professor MÜHLETHALER for his generous help.

Grateful acknowledgement is also made to the Australian Research Grants Committee for equipment and funds, and also to Australian Paper Manufacturers of Melbourne, Associated Pulp and Paper Mills, Burnie, Tasmania, and Australian Newsprint Mills, Boyer, Tasmania, for a generous donation to assist the purchase of the freeze-etching equipment.

References

ANDERSON, T. F., 1951: Trans. N.Y. Acad. Sci. **13**, 130—134.
BERRILL, N. J., 1950: The *Tunicata* Ray Society, London.
BRIEN, P., 1930: Ann. Soc. Roy. Zool. Belg. LXI **61**, 19—112.
DECK, DAVID J., ELIZABETH D. HAY, and JEAN-PAUL REVEL, 1966: J. Morphology **120** (3), 267—280.
ENDEAN, R., 1955 a: Aust. J. Marine and Freshwater Res. **6**, 35—59.
— 1955 b: Aust. J. Marine and Freshwater Res. **6**, 139—156.
— 1955 c: Aust. J. Marine and Freshwater Res. **6**, 157—164.
— 1961: Quart. J. Microscopical Sci. **102**, 107—117.
FREY-WYSSLING, A., and R. FREY, 1950: Protoplasma **39**, 656—660.
— and K. MÜHLETHALER, 1963: Makromol. Chem. **62**, 25.
GLAUERT, A. M., and R. H. GLAUERT, 1958: J. biophys. biochem. Cytol. **4**, 191.
HALL, D. A., and H. SAXL, 1961: Proc. Roy. Soc. (Lond.) **B 155**, 202—217.
HECHT, S., 1918: J. Exp. Zool. **25**, 229—260.
HERZOG, R., and H. W. GONELL, 1924: Z. physiol. Chem. **141**, 63—67.
KELSEY, K. E., 1963: The structure and properties of tunicin with special reference to drying and shrinkage, Internal Report, C.S.I.R.O. Division of Forest Products, Melbourne, Australia.
MARK, H., and G. v. SUSICH, 1929: Z. physik. Chem. **4**, 431.
MASER, M. D., T. P. O'BRIEN, and MCCULLY, 1967: J. de Microscopie **6**, 305—312.
MISHRA, A. K., and J. R. COLVIN, 1969: Canad. J. Zoology (in press) (Private communication).
MOOR, H., 1964: Z. Zellforsch. **62**, 546—580.
PORTER, K. R., 1959: J. biophys. biochem. Cytol. **5**, 153—166.
RÄNBY, B. G., 1952: Arkiv. for Kemi **4** (13), 241—248.
ROSS, R., 1968: Biol. Rev. **43** (1), 51—96.
SUTRA, R., 1932: Compt. rend. **195**, 181.
WARDROP, A. B., and S. M. JUTTE, 1968: Wood Sci. and Technol. **2**, 105—114.

Author's address: Prof. A. B. WARDROP, Department of Botany, La Trobe University, Melbourne, Australia.