The Test of the Ascidian, *Phallusia mammillata*

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With three plates (figs. 4, 7, and 8)

SUMMARY

A mucopolysaccharide, readily removed by mild acid hydrolysis, is associated with cellulose in the test of *Phallusia mammillata* (Cuvier). The mucopolysaccharide is produced in the vacuolated cytoplasm of epidermal cells covering the blood-vessels of the test.

The cellulose of the test appears to be produced by vanadocytes which have migrated into the test matrix through the walls of the test blood-vessels. In the test, the vanadocytes lose their acid and intraglobular osmiophil material and produce processes which extend into the surrounding test matrix. Minute globules and submicroscopic vesicles are present in the cytoplasm surrounding the large polysaccharide-containing globules of such vanadocytes, and also in the processes arising from such cells. The small globules and vesicles appear to arise from the large globules and to be involved in the production of micro-fibrils observed in electron micrographs of the test. The micro-fibrils, which vary from 20 to 40 mμ in diameter and are arranged in the form of an open network, may constitute a cellulose framework within the test.

An area devoid of test matrix occurs usually near each disintegrating vanadocyte. The remnants of each vanadocyte often come to lie in such an area. Capsules—the so-called 'bladder cells' of the test are thereby formed.

Phagocytes occur throughout the test and melanin-containing pigment cells are aggregated around its periphery.

INTRODUCTION

BEFORE the recent discoveries of cellulose fibres in mammalian connective tissue (Hall & others, 1958; Cruise & Jeffery, 1959) and of 'tunicine' in the Pogonophora (Ivanov, 1959), ascidians and their kin comprising the Sub-Phylum Tunicata were believed to be the only animals that synthesized cellulose. Consequently, the ascidian test has been the subject of numerous investigations. For an introduction to the literature on the subject the reader is referred to reviews by Saint-Hilaire (1931) and Pruvot-Fol (1951).

As pointed out by these authors, nitrogenous material, as well as cellulose, occurs in the ascidian test. In the test of the ascidian *Pyura stolonifera* (Heller), a mucopolysaccharide is associated with cellulosic material (Endean, 1955*a*). One of the aims of the present investigation was to ascertain whether the test of *Phallusia mammillata* has a similar chemical constitution.

Several modes of formation of the ascidian test have been postulated (Brien, 1930; Saint-Hilaire, 1931; Pérès, 1945, 1948; Millar, 1951; Pruvot-Fol, 1951). Apart from Pruvot-Fol's suggestion that symbiotic algae may be responsible for cellulose production in ascidians, the concensus of opinion seems to be that epidermal cells play the principal role in test formation.

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However, Brien (1948) points out that the formation of the test coincides with a migration of cells from the haemocoele, and Endean (1955*b*) has shown that certain blood-cells participate in the formation of the test of *Pyura stolonifera*. In view of this, it was decided to investigate the nature and roles of the cells found in the test of *Phallusia mammillata*.

Material and Methods

Adult specimens of *P. mammillata* were obtained from Salcombe, South Devon. They were kept in well-aerated sea-water until required.

Pieces of fresh test were sectioned with a razor and examined by direct, phase-contrast, and polarized-light microscopy. Some sections were dyed supervitally with 0·001% neutral red, 0·001% methylene blue, or 0·1% toluidine blue. Others were exposed to osmium tetroxide vapour before examination.

Frozen sections of material fixed in 5% formalin in sea-water were also examined. Some were dyed with Weigert's haematoxylin and eosin, others with Mann's methyl blue / eosin. Some were subjected to histochemical tests for polysaccharide (PAS reaction), mucopolysaccharide (0·1% toluidine blue, alcian blue 8GS, Hale's 1946 method), protein ($HgCl_2$/BTB), and lipid (Sudan black B).

Paraffin sections of pieces of test fixed in Bouin and Susa were also prepared and dyed with Mallory's or the Azan mixtures, or with pyronin or orcein.

Small pieces of fresh test were fixed with buffered 1% osmium tetroxide (Palade, 1952), embedded in araldite (Glauert & Glauert, 1958) and sectioned on an ultramicrotome of the A. F. Huxley pattern. Sections showing gold interference colours were selected, mounted on formvar-coated grids, and examined in a Phillips electron microscope.

The Structure of the Test

The test consists of translucent, gelatinous material bounded externally by a thin brownish layer.

Sections of the test, viewed by direct microscopy, showed the presence of blood-vessels with contained blood-cells, large so-called 'bladder cells', and an apparent variety of smaller cells, all surrounded by hyaline test material (fig. 1). This hyaline material was dyed diffusely by orcein and pyronin but not by Mallory or Azan mixtures. As noted by Saint-Hilaire (1931), no fibrillar structure is apparent in this material when it is viewed by the optical microscope. However, some electron micrographs revealed the presence of a fibrillar network (fig. 4, A). The coarser fibres in this network were about 40 mμ thick, and were apparently oriented. Smaller fibres about 20 mμ thick were also present.

The blood-vessels, which ramified through the test, branched extensively and terminated in knob-like bulbils near the outer border of the test. Each blood-vessel possessed a peripheral layer, one cell thick, of epidermal cells.

These cells were, for the most part, disposed in rows running parallel to the long axis of the blood-vessel. In fresh test slices, the cells were observed to possess short conical projections (fig. 2) extending into the surrounding test material, which, in the immediate vicinity of the blood-vessels, was more transparent than elsewhere. Each cell possessed a large ellipsoidal nucleus.

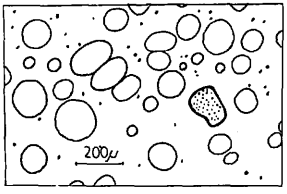

FIG. 1. Section of test of *P. mammillata* showing large 'bladder cells', small test-cells, and a blood-vessel.

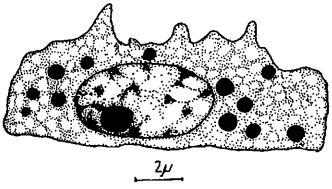

FIG. 2. An epidermal cell from a test blood-vessel fixed with Bouin's fluid and dyed with Azan.

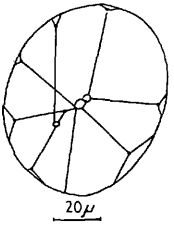

FIG. 3. A 'bladder cell' in optical section, viewed by phase contrast.

The vacuolated cytoplasm of each cell was dyed faintly by the aniline blue component of Mallory and Azan. A few granules, which appeared to be mitochondria, as evidenced by their behaviour to Janus green B used supervitally, were observed in the cytoplasm, particularly near the nucleus. The nucleus possessed a prominent nucleolus, and, in fixed material, a meshwork of basiphil material was apparent.

From the outer border of the test to about three-quarters of the distance to the inner edge of the test, bladder cells were prominent (figs. 1; 3). These cells were circular or oval in cross-section. Just below the outer boundary of the test the spherical cells averaged about 60 μ in diameter but elsewhere they averaged about 120 μ.

A meshwork of very fine fibres or strands which radiated to the periphery of the cells from colourless, poorly refractive, spherical bodies was present in the bladder cells (fig. 3). Some of these bodies were at the limit of resolution,

others were as large as 4·5 μ in diameter. The majority were between 1 and 3 μ. At the periphery of the bladder cells the fibres merged with hyaline material, which appeared to be continuous with the substance of the test. Bladder cells that abutted on one another were mutually compressed, presumably because of the turgor of their contained fluid. No nuclei or typical cell organellae were observed. Effectively, the bladder cells are not cells but fluid-filled capsules in each of which there is a taut meshwork of fibre-like processes.

Certain of the cells found in the blood of *P. mammillata* (Endean, 1960) were found in the test. Intact and fragmented pigment cells were prominent in the outermost regions of the test and probably the brownish appearance of this region is due to their presence. Pigment cells were also aggregated in large numbers against the walls of the bulbils. Phagocytes were common in the outer regions of the test and occasionally cells with acidic vacuoles were observed in this region.

Close study of the variously shaped cells found in the hyaline material comprising the bulk of the test revealed that the majority were vanadocytes in varying stages of disintegration.

Many of these test vanadocytes, although giving typical blood vanadocyte reactions to OsO_4, neutral red, and methylene blue, were flattened and often up to 16 μ in diameter (fig. 4, B). Some were irregularly shaped, being branched, with their contained globules disposed along the branches. Also, some had more globules than the vanadocytes of blood were observed to possess. Since many of these globules were small, it seems likely that some globules had fragmented to give smaller ones.

Other vanadocytes were similarly shaped to those just described but only the peripheries of some of their contained globules (or possibly the cytoplasm surrounding the globules) blackened with OsO_4. In other vanadocytes, of similar structural appearance and dimensions, none of the cores of the globules blackened with OsO_4. From the borders of such cells, processes containing granules had arisen and extended out into the surrounding hyaline material (fig. 4, C, D). A further stage was reached in the disintegration of vanadocytes when these were surrounded by a meshwork of fibre-like processes which branched repeatedly. Some of the processes were traced to distances between 70 and 80 μ from the main cell mass before they became so fine that they were lost to view in the hyaline substance of the test. Along the length of the processes, and particularly at the points of branching, minute granules, some

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Fig. 4 (plate). A, electron micrograph of section through test showing network of microfibrils. Part of the distal region of a fibre-like process (*p*) is visible.

B, photomicrograph of a test vanadocyte.

C, photomicrograph of a test vanadocyte which has begun to produce fibre-like processes.

D, photomicrograph of a partially disintegrated test vanadocyte showing some of the fibre-like processes produced. All photomicrographs are of cells in test sections fixed with OsO_4 vapour.

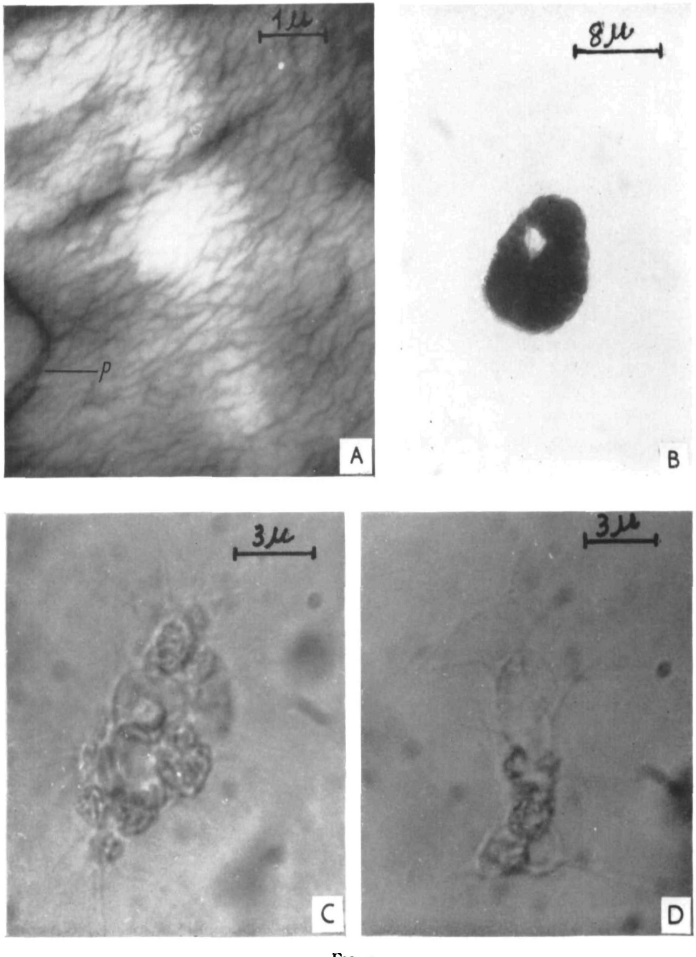

FIG. 4

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at the limit of resolution, others ranging up to 0·5 μ in diameter, were in evidence (fig. 5).

There was a progressive decrease in the size of the central mass and a reduction in the number of globules as the vanadocytes disintegrated further. As the central mass of each disintegrating vanadocyte decreased in volume the nucleus of each cell became progressively more indistinct and was usually no

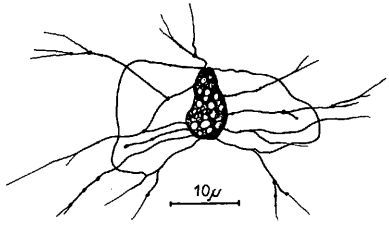

FIG. 5. A test vanadocyte in optical section, showing granule-containing processes crossing areas devoid of test substance in the immediate vicinity of the cell. The cell has been fixed with OsO_4 vapour.

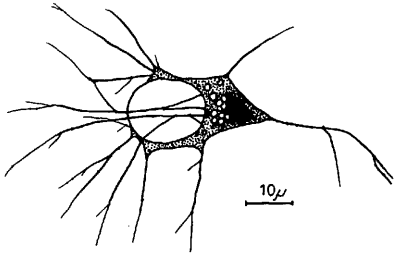

FIG. 6. A partially disintegrated test vanadocyte in optical section. An area, oval in outline and devoid of test material, is almost enclosed by cytoplasmic projections. Granule-containing processes have arisen from the cytoplasm of the cell, which has been dyed supervitally with 0·1% toluidine blue. The central mass consists of globules dyed blue with the dye and of other globules which are not dyed, and which appear to be 'ghosts'.

longer visible when the central mass was less than 5 μ in diameter. The globules left in the reduced central mass were often refractory to dyes and appeared as mere 'ghosts'.

Frequently an area (or areas) devoid of test matrix was observed in the immediate vicinity of a disintegrating vanadocyte. Sometimes the clear area surrounded the central mass of such a vanadocyte, sometimes it was to one side (fig. 6). Processes containing granules crossed the clear areas and extended into the surrounding test matrix. It was evident that small test capsules arise

from such disintegrating vanadocytes and that these capsules each contain the remnants of a vanadocyte enclosed in a fluid-filled cavity. The taut fibres inside these capsules are the stretched proximal regions of fibre-like processes. Larger capsules presumably result from a further accumulation of fluid.

In some cases vanadocytes had been disrupted and their contained globules had separated one from another. Each globule had acted as a centre from which granule-containing processes had originated. There was no dissolution of test material in the immediate vicinity of these isolated globules.

Small wedge-shaped pieces were cut from the peripheral region of the test of living specimens of *P. mammillata* and the animals were then returned to a tank of sea-water. After 72 h noticeable regeneration of the test had occurred. Large numbers of cells appeared to have aggregated in the regenerated regions. Some were typical vanadocytes and many were disintegrating vanadocytes surrounded by processes. Most, however, proved to be globules which had separated from their parent vanadocytes and were surrounded by interlacing granule-containing processes. These globules gave a purplish-red colour with Mann's methyl blue / eosin which contrasted with the yellow colour given by the globules of typical vanadocytes in the test and in adjacent blood-vessels. Some vanadocytes appeared to have been fixed while in transit through the walls of these blood-vessels.

In electron micrographs of sections of vanadocytes from the test, globules were easily recognized. Some globules (fig. 7, A) contained electron-dense material which appeared irregularly dispersed. Such globules probably correspond with those observed, under the optical microscope, to have blackened with OsO_4. The globules of other vanadocytes (fig. 7, B) contained only small amounts of material of high electron density, and, in still other vanadocytes (figs. 7, C; 8, A), only material of low electron density was present in the globules. However, electron-dense material was present around the peripheries of the globules and these vanadocytes correspond with those observed under the optical microscope to have blackened with OsO_4 only around the peripheries of the globules. In electron micrographs of sections of these cells minute vesicles (averaging about 80 mμ in diameter) were present in the cytoplasm, particularly near the cell boundaries. Similar vesicles, some only partially differentiated, were apparent in and around the edges of some globules. This suggests that the vesicles are derived from the globules and that each vesicle encloses part of the material of low electron density found in the globules.

FIG. 7 (plate). A, electron micrograph of a section through part of a test vanadocyte showing two large globules and portions of others containing electron-dense material.

B, electron micrograph of a section through part of a test vanadocyte which shows little electron-dense material within its globules. Vesicles (*v*) are present below the cell surface and around the periphery of a globule.

C, electron micrograph of a section through a test vanadocyte which possesses globules almost devoid of electron-dense material.

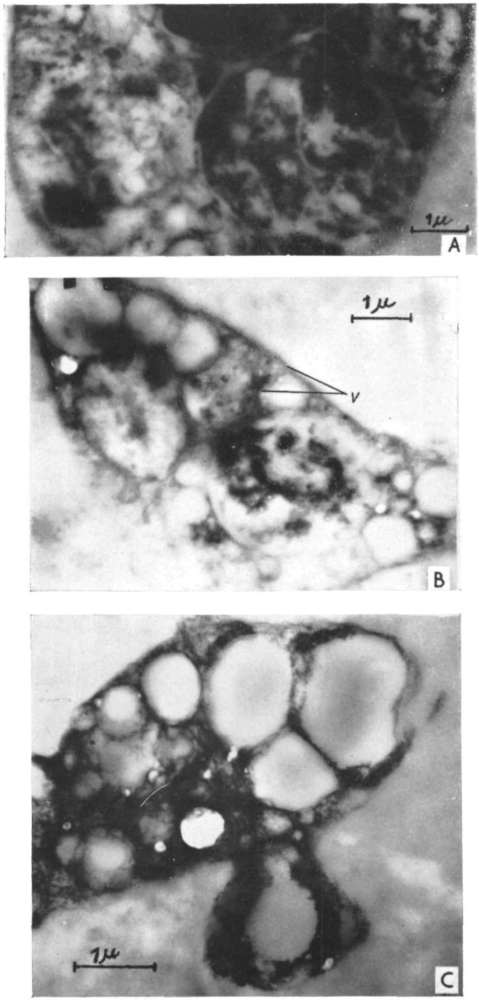

FIG. 7

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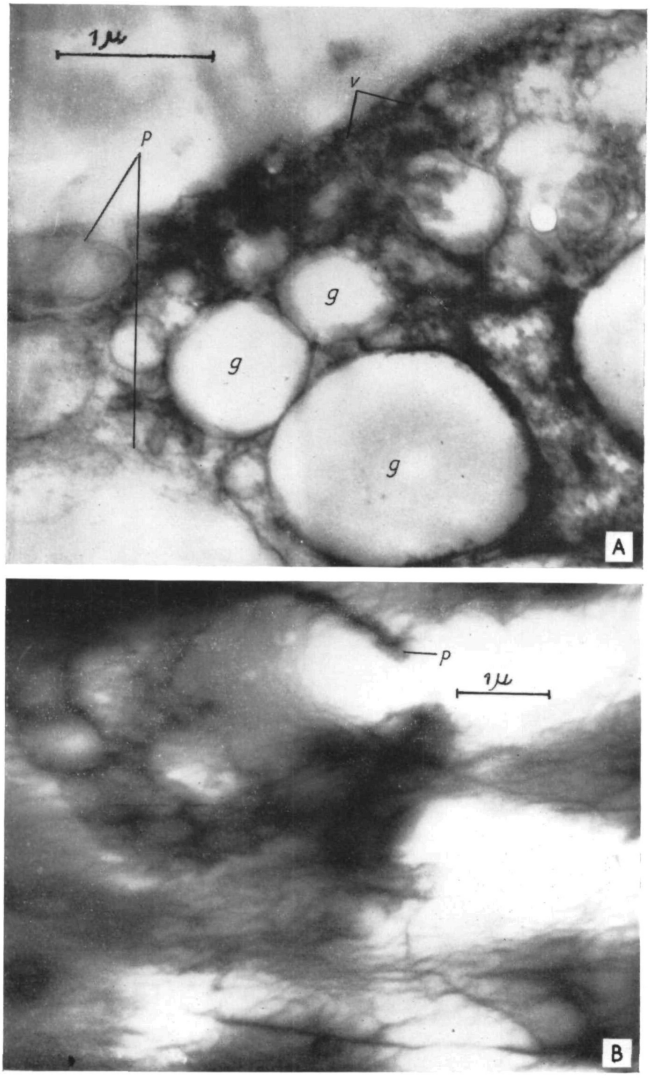

FIG. 8

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Some of the larger globules appeared to be in the process of breaking up to give smaller globules.

In electron micrographs of some sections of test vanadocytes, projections of cytoplasm containing globules of various sizes, and vesicles, were noted. Many of the cytoplasmic projections were attenuated distally. Although the vesicles and most of the globules present in the processes would be too small to be visible with the optical microscope, a few larger globules, spaced at irregular intervals along the processes, and situated at or near points of branching of the processes, would be visible. These larger globules probably correspond with the 'granules' of 'granule-containing' fibre-like processes seen with the optical microscope and described on p. 110.

In those electron micrographs where micro-fibrils were apparent, the fibrils were continuous with the surfaces of vanadocytes and their processes. This suggests that the micro-fibrils are derived from vanadocytes. Indeed, micro-fibrils from 20 to 40 mμ in diameter appeared to arise directly from the surfaces of cytoplasmic projections of vanadocytes (fig. 8, B) and to have shredded from the surface of each fibre-like process (figs. 4, A; 8, B).

Areas which seemed devoid of fibrillar material were often noted in electron micrographs of sections of vanadocytes in the immediate vicinity of vanadocytes. These areas were sometimes crossed by processes from vanadocytes and these areas seemed to correspond with the clear areas observed with the optical microscope near disintegrating test vanadocytes (p. 111).

Histochemistry of the Test

The intercellular matrix. This gave a positive PAS reaction but did not contain protein detectable by the $HgCl_2$/BTB reagent. With toluidine blue the matrix gave strong γ-metachromasy which was most intense in the immediate vicinity of blood-vessels. It gave a deep blue colour with alcian blue 8GS and a strong blue coloration with Hale's acid mucopolysaccharide test.

Testicular hyaluronidase at 0·1% did not remove the PAS-positive metachromatic material. However, if formalin-fixed sections were heated at 98° C with N H_2SO_4 or N HCl for 10 min the metachromatic material was completely removed. The test sections were not visibly affected by the treatment with acid. Subsequently they gave a faint PAS reaction and no reaction for protein.

The above results indicate that the intercellular matrix of the test contains acid mucopolysaccharide which is not hyaluronic acid and a frame substance

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Fig. 8 (plate). A, electron micrograph of a section through part of a test vanadocyte, showing globules (*g*), vesicles (*v*), and the base of a granule-containing process (*p*), the cytoplasm of which is continuous with that of the cell.

B, electron micrograph of a section through a portion of a disintegrating vanadocyte containing globules and vesicles. Part of a fibre-like process (*p*) is shown. Micro-fibrils appear to arise directly from the surfaces of the cell and process.

which does not appear to be proteinaceous but which may contain polysaccharide.

The epidermal cells of the blood-vessels. The vacuolated cytoplasm of these cells was PAS-positive and showed strong γ-metachromasy with toluidine blue. A positive result was obtained for acid mucopolysaccharide with Hale's (1946) method. Treatment with N HCl for 10 min at 98° C removed the mucopolysaccharide from the cells. No PAS reaction was then given by these cells.

The test cells. The histochemistry of pigment cells, phagocytes, cells with acidic vacuoles, and vanadocytes, has been described in an earlier paper (Endean, 1960).

Some of the flattened vanadocytes of the test reacted with the dyes and histochemical reagents used in this investigation in the same way as typical blood vanadocytes. The globules of such vanadocytes blackened with OsO_4. However, as noted earlier, the globules of vanadocytes which were producing processes had lost the ability to blacken with OsO_4. Such globules appeared to have lost part or all of their acid also, for they became deep blue (instead of yellow) with $HgCl_2$/BTB and pink (instead of deep red) with neutral red. In fixed material, they were amphoteric in their behaviour towards dyes. Thus they were dyed purple or red by Mann's methyl blue / eosin, red by Azan, orange by Mallory, and red by pyronin. It was thought that the fixatives used may have caused a release of acid from the globules, but vanadocytes fixed in the blood-vessels of the same sections were strongly basiphil, as are the typical vanadocytes of the blood.

Vanadocytes that were producing fibrous processes were strongly PAS-positive, as were their processes and the granules on these processes. Although the vanadocytes gave a deep blue colour with toluidine blue, the processes and their granules were blue to purplish, near the vanadocytes, and purplish red to red distally. If a vacuole or vacuoles were present in the vicinity of a disintegrating vanadocyte they were coloured a purplish red by the dye.

The aniline blue component of Mallory and Azan dyed the granule-containing processes blue. These processes were dyed a faint blue by $HgCl_2$/BTB.

Proteinaceous material detectable with $HgCl_2$/BTB gradually disappeared from the disintegrating vanadocytes and none was present in the test capsules. Acid was absent from the capsules, but a mucopolysaccharide, which gave similar histochemical reactions to the mucopolysaccharide of the test, was present.

Chemistry of the Test

Weighed samples of gelatinous test were heated at 104° C in an oven until constant weight was obtained. The percentage loss in weight averaged 94·1%, indicating that the test has a remarkably high water-content.

Samples of the test after water, water-soluble, and acetone-soluble material had been removed were subjected to hydrolysis with 2 N H_2SO_4 for 8 h at

100° C. There was little apparent disintegration of the test material and the latter was filtered off and retained. The hydrolysate, in each case, was neutralized with $BaCO_3$ and centrifuged, and the volume of the supernatants reduced by evaporation under reduced pressure. The concentrated supernatants gave a strong Molisch reaction and reduced Benedict's solution. They gave a positive result when tested for hexosamine following the procedure of Elson and Morgan (1933).

Samples of the test residue, washed with distilled water, were placed in contact with 80% H_2SO_4 for 3 days. The acid was then diluted to 2 N H_2SO_4 and the material hydrolysed by heating at 100° C for 16 h. The hydrolysates were then neutralized with $BaCO_3$ and centrifuged, and the volume of the supernatants reduced in each case by evaporation under reduced pressure. A strong Molisch reaction was given by the supernatants and these also reduced Benedict's solution.

A drop of each supernatant was placed on a filter paper strip and chromatograms run with ethyl acetate / pyridine / water as solvent. The papers were dried and sprayed with *p*-anisidine. A single spot was given in each case and these spots corresponded with those given by drops of pure glucose run at the same time as reference markers.

Discussion

Over 90% of the test of *P. mammillata* is composed of water. Part of the organic material present gives histochemical reactions indicative of acid mucopolysaccharide. This material can be readily removed by subjecting the test to mild hydrolysis, and hexosamine is present in the hydrolysate. The residual insoluble material contains no protein and, on hydrolysis, yields glucose as the only sugar. It appears to be cellulose, which has been found repeatedly in the tests of ascidians since its occurrence there was first reported by Schmidt in 1845.

Some electron micrographs of the test revealed the presence of an open meshwork of micro-fibrils. It is perhaps significant that the diameters of the micro-fibrils correspond with those recorded for cellulose micro-fibrils in plant fibres (Mühlethaler, 1949). Other sections of the test revealed no traces of micro-fibrils and it is possible that those observed were artifacts or that micro-fibrils were present only in certain regions of the test. However, it is also possible that the apparent absence of micro-fibrils in some test sections was due to poor penetration of the OsO_4 fixative. Prolonged periods in histological fixatives were necessary in order to ensure that pieces of the test were suitably fixed for examination with the optical microscope.

In a previous paper (Endean, 1960) it was shown that the spherical blood vanadocytes possess conspicuous globules in which polysaccharide is synthesized in an acid medium in the presence of a vanadium chromogen and that these cells must leave the blood at some stage. It has now been shown that fully developed vanadocytes migrate into the test substance, where they give rise to fibre-like processes. Acid as well as osmiophil material (believed to be

the vanadium chromogen) are soon lost from the globules, and the cells disintegrate. Study of electron micrographs suggests that the residual intraglobular material is released into the surrounding cytoplasm in small vesicles. Since this residual material, as evidenced by histochemical tests, is composed chiefly of polysaccharide, it is probable that the vesicles contain polysaccharide. The whole cell, including processes, is strongly PAS-positive at this stage but only the distal parts of the fibre-like processes exhibit metachromasy with toluidine blue. Probably this is due to the processes becoming associated with the mucopolysaccharide of the test.

The test mucopolysaccharide appears to be produced in the vacuolated cytoplasm of the epidermal cells lining the blood-vessels of the test. Histochemical tests show that mucopolysaccharide is present in the cytoplasm of these cells and is more concentrated in the test matrix in the immediate vicinity of these cells than elsewhere in the test. After treatment with dilute acid no residual mucopolysaccharide could be detected in these cells, whereas the polysaccharide material in vanadocytes resists the action of dilute acid (Endean, 1960). These observations militate against the epidermal cells themselves being involved in the production of cellulosic material but suggest strongly that the vanadocytes are so involved. Also, the micro-fibrils of the test appear to arise directly from the surfaces of vanadocytes and their processes. It seems likely that the sub-microscopic vesicles found just below these surfaces are involved in the formation of fibrils.

Growth of the test appears to be by intussusception—newly formed micro-fibrils interlacing with those already present. An increase in test volume might also result from an increase in the number of fluid-filled capsules in the test. With respect to the formation of these capsules, it may be of importance that the globules of vanadocytes contain free H_2SO_4, the concentration of which has been calculated by Webb (1939) to be about 1·8 N. As the test mucopolysaccharide is readily removed from sections of the test by N H_2SO_4, mucopolysaccharide molecules in the neighbourhood of disintegrating vanadocytes would be affected by acid released from vanadocyte globules. This may affect the stability of the test matrix in the immediate vicinity of such vanadocytes and result in the formation of the clear areas frequently observed in such regions. Why these areas devoid of test substance should subsequently become spherical, accumulate test fluid, and increase in size, thereby forming typical test capsules, is not clear. However, the dissolution of the test matrix in the immediate vicinity of vanadocytes would facilitate the penetration of vanadocyte processes into this matrix.

As well as an outflow of vanadocytes from the blood-stream there must be an outflow of phagocytes and fully differentiated pigment cells (Endean, 1960). Phagocytes were common in the test, where they appear to be responsible for conveying necrotic cells and foreign material to the exterior. Pigment cells formed a covering around the periphery of the test, and, since they contain melanin, it is probable that they afford a protection against ultra-violet light.

This paper embodies the results of part of the research work carried out in 1958–9 during the tenure of a Nuffield Dominion Travelling Fellowship. The assistance, financial and otherwise, received from the Nuffield Foundation is gratefully acknowledged. The author wishes to thank Professor J. E. Harris of the Zoology Department, University of Bristol (where the bulk of this research was carried out), for providing research facilities and for reading and discussing the manuscript. The author also wishes to thank Dr. F. S. Russell of the Marine Laboratory, Plymouth, for providing research facilities at Plymouth. The assistance in the use of the electron microscope provided by Miss E. Harris of the Physics Department, University of Bristol, is gratefully acknowledged.

REFERENCES

BRIEN, P., 1930. Ann. Soc. zool. Belg., **61**, 19.
—— 1948. *Traité de zoologie.* Tome XI. Paris (Masson).
CRUISE, A. J., & JEFFERY, J. W., 1959. Nature, **183**, 678.
ELSON, L. A., & MORGAN, W. T. J., 1933. Biochem. J., **27**, 1824.
ENDEAN, R., 1955*a*. Aust. J. mar. freshw. Res., **6**, 139.
—— 1955*b*. Ibid., **6**, 157.
—— 1960. Quart. J. micr. Sci., **101**, 177.
GLAUERT, A. M., & GLAUERT, R. H., 1958. J. biophys. biochem. Cytol., **4**, 191.
HALE, C. W., 1946. Nature, **147**, 1254.
HALL, D. A., LLOYD, P. F., SAXL, H., & HAPPEY, F., 1958. Nature, **181**, 470.
IVANOV, A. V., 1959. Proc. XVth Int. Congr. Zool. London, 1958, 345.
MILLAR, R. H., 1951. Quart. J. micr. Sci., **92**, 249.
MÜHLETHALER, K., 1949. Biochem. biophys. Acta, **3**, 15.
PALADE, G. E., 1952. J. exp. Med., **95**, 285.
PÉRÈS, J. M., 1945. Bull. Inst. océanogr. Monaco, No. **882.**
—— 1948. Ibid., No. **936.**
PRUVOT-FOL, A., 1951. Rev. suisse Zool., **58**, 605.
SAINT-HILAIRE, K., 1931. Zool. Jahrb. Jena (Anat.), **54**, 435.
SCHMIDT, C., 1845. Liebig's Annalen der Chemie, **54**, 284.
WEBB, D. A., 1939. J. exp. Biol., **16**, 449.