Adv. Mar. Biol., Vol. 12, 1974, pp. 1–149.

THE PHYSIOLOGY OF ASCIDIANS

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I. Introduction

The literature on ascidians is extensive but no comprehensive review of their physiology seems ever to have been attempted. Following Kowalewsky's (1867) discovery of their true systematic position much of the early research on ascidians was directed towards elucidation of their relationship with the chordates and the first serious attempt to investigate their physiology seems to be the work of S. Hecht (1916, 1918a, b, c, d, 1926) on *Ascidia nigra* (Savigny) and *Ciona intestinalis* (Linnaeus). Subsequent workers concentrated on specific aspects of physiology in the group often with emphasis on those functional aspects which are unusual or which particularly illuminate problems of chordate physiology. This applies especially to the universal occurrence of heart beat reversal in all Tunicata and the existence of reducing compounds in the blood often containing vanadium, both of which have been the subject of much study. At the same time, because of the simplicity of their organization, they provide a useful material for certain types of research, particularly developmental physiology, heart function and the possible endocrinological role of the neural gland. With the exception of the nervous system (Bullock and Horridge, 1965) there have been few recent attempts to review any aspects of the literature on ascidian physiology and hence it is often necessary to refer back quite far for source material.

It is difficult for a single person to encompass in detail all of the many facets of physiology contained in the literature on ascidians and I have been deliberately selective in the topics chosen for discussion. The literature on ascidian embryology has been reviewed recently by Reverberi (1961, 1971) and I have made no attempt to discuss developmental physiology. Nor have I discussed larval physiology, which presents special problems associated with a free swimming mode of life, and would be better included in a separate review of larval biology as a whole. Finally, I have not attempted to include a discussion of any special problems of the pelagic Tunicata although, where appropriate, reference is made to them.

In preparing this review I have been guided in the selection of material and the mode of presentation by an attempt to consider problems in the light of their relevance to ascidian biology and not their specific relevance to comparative physiology. In the process some papers and some problems have been omitted or passed over rather briefly. Structure and function are inseparable aspects of biology and in order to elaborate many points in the animals' physiology it has been necessary to include some material which is essentially descriptive

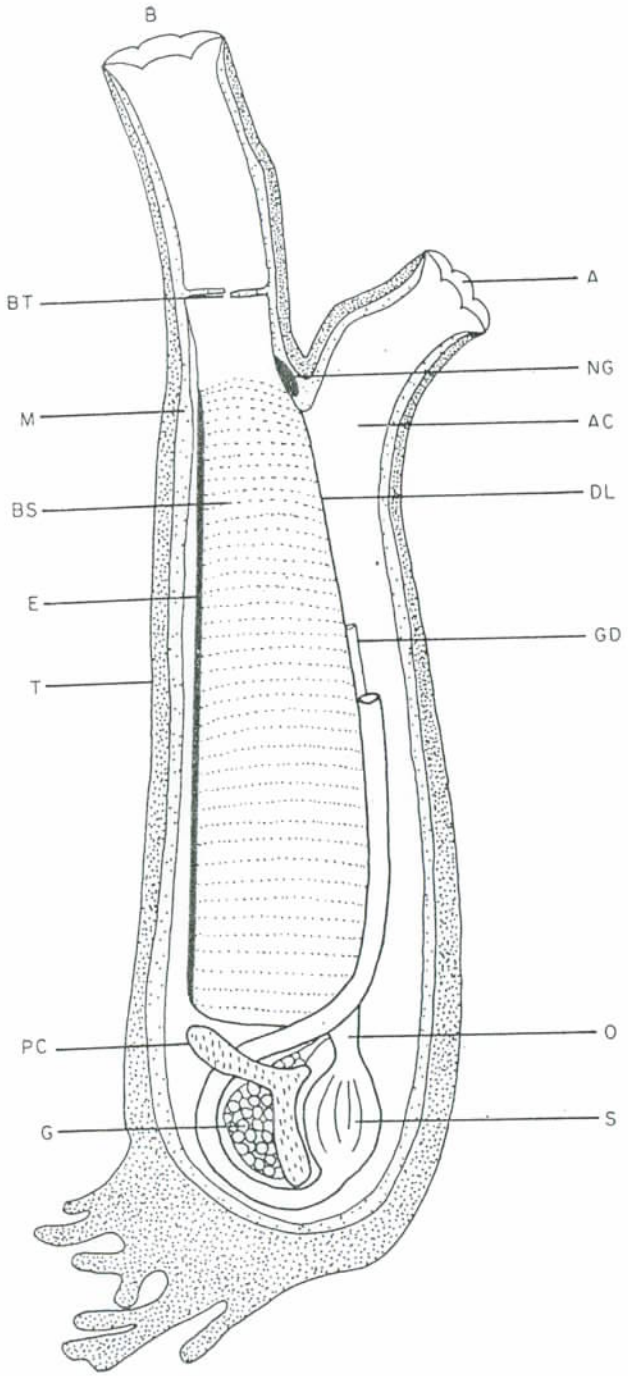
morphology. Indeed some of the most striking advances in our understanding of ascidian function have been made possible only through recent work on the fine structure of such features as the test, the endostyle, the blood and the neural gland.

Because of their abundance and accessibility much of the work which has been carried out on ascidian physiology has been on *Ciona intestinalis*, *Phallusia mammillata* (Cuvier) or *Ascidia nigra*. The first two are frequently referred to in the text simply as *Ciona* and *Phallusia*. *Ascidia nigra* is a warm water species which strictly speaking belongs to the sub-genus *Phallusia* and is frequently referred to in the literature as *Phallusia nigra*, *Phallusia atra* or *Ascidia atra*; the name *Ascidia nigra* is now more widely accepted and is used throughout this text. The use of biological nomenclature by physiologists leaves much to be desired, and those working with ascidians are no exception. Throughout the text of this review I have endeavoured to use the correct nomenclature and have, in a few cases, altered the names as used by original authors in their papers so as to make cross reference possible. A wide selection of taxonomic literature has been used in order to check names, but in general the nomenclature of most of the animals referred to in this text is to be found in either Van Name (1945), Berrill (1950), Tokioka (1953) or Tokioka (1967).

For ease of reference the principal structures discussed in the text are illustrated in Figs 1 and 2.

II. The Ascidian Test

The test is an outer protective covering which completely surrounds the individual zooid in solitary ascidians or forms a common groundwork in which the zooids are embedded in colonial species. It is soft and flexible and varies in consistency from gelatinous to fibrous. In the colonial aplousobranchs it is gelatinous, in the phlebobranchs and particularly the Ascidiidae it is thick and slightly cartilaginous while in some solitary stolidobranchs it is tough and almost leathery in consistency. In many solitary forms there are blood vessels ramifying throughout the test; these arise from two test vessels one at either end of the heart which cross the mantle and form vessels lined by a single layer of epithelium (Das, 1936; Endean, 1955a). They form a network of vessels with lateral branches ending in terminal bulbs particularly toward the periphery (Fig. 3). In some colonial ascidians similar stolonic vessels arising as epithelial outpushings penetrate into the test substance. Blood cells, particularly morula cells, and in *Halocynthia aurantium* (Pallas) dispersed vesicular cells (Smith, 1970a), are found throughout the test substance and apparently wander in there through



the mantle wall or through the epithelial walls of the test vessels. For a fuller review of the cell types invading the matrix of the test see Godeaux (1964b). The early literature on the nature and formation of the test has been reviewed by Saint-Hilaire (1931), Berrill (1950), Pruvot-Fol (1951), Ranby (1952), Endean (1955b, 1961), Godeaux (1964b), Wardrop (1970), Smith (1970a).

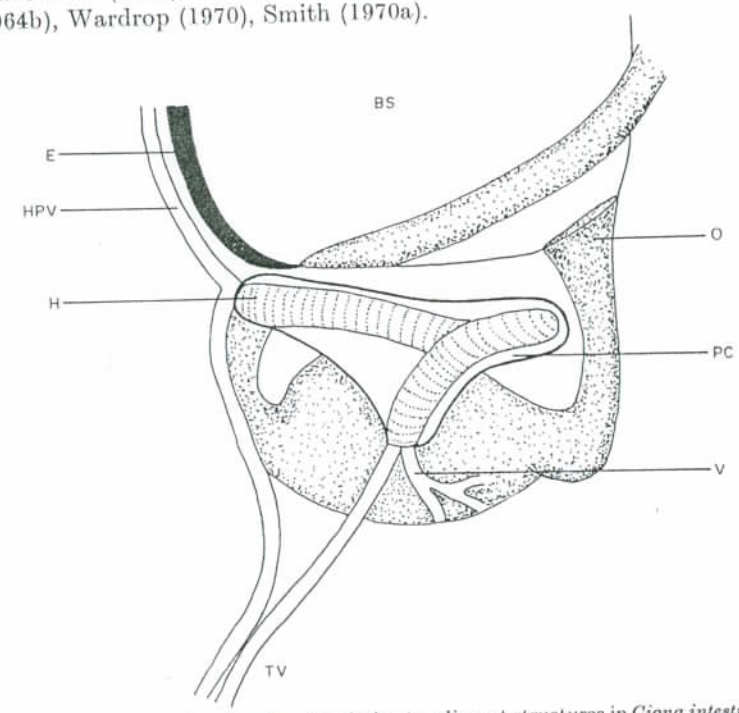


FIG. 2. The heart and pericardium in relation to adjacent structures in *Ciona intestinalis*. (After Berrill, 1950.) BS, branchial sac; E, endostyle; H, heart; HPV, hypobranchial vessel; O, oesophagus; PC, pericardium; TV, test vessels; V, visceral vessels.

It has been known for over 100 years that the ascidian test contains carbohydrate and that some of this is a cellulose-like compound called tunicin (for references see Berrill, 1950; Hunt, 1970). The organic matter, however, is not pure carbohydrate and there is usually a high proportion of protein in the test matrix (Table I). The outer surface of the test forms a thin cuticle composed almost entirely of protein (Pérès, 1948a; Barrington, 1957; Barrington and Barron, 1960; Barrington

FIG. 1. The general disposition of structures in an adult ascidian, *Ciona intestinalis*. (Modified from Seeliger and Hartmeyer, 1893–1911.) A, atrial siphon; AC, atrial cavity; B, branchial siphon; BS, branchial sac; DL, dorsal lamina; E, endostyle; G, gonad; GD, genital duct; M, mantle; NG, neural gland and ganglion; O, oesophagus; PC, pericardium; S, stomach; T, test.

and Thorpe, 1968; Stiévenart, 1970). Since Berrill's summary of the literature there have been considerable advances in our knowledge of the composition and formation of the test in various species which has been enhanced by the use of tracer techniques and electron-microscopy. As the structure and processes involved appear to be different in different species, some of these are individually summarized below.

TABLE I. COMPOSITION OF THE TEST IN VARIOUS ASCIDIAN SPECIES

SPECIES	% Water	Nitrogenous substances (dry wt)	% Carbohydrate (dry wt)	Reference
Ciona intestinalis	—	50	50	Godeaux, 1963
Ascidia mentula Müller	90	—	—	Saint-Hilaire, 1931
Ascidiella aspersa	95	65	35	Stiévenart, 1970
Phallusia mammillata	95	60	40	Endean, 1961
Pyura stolonifera	94	—	—	Endean, 1955b
Halocynthia roretzi (Von Drasche)	—	42	58	Tsuchiya and Suzuki, 1962
Halocynthia papillosa	74	28	72	Stiévenart, 1971
Halocynthia aurantium	81	50	50	Smith and Dehnel, 1970

(a) *Phallusia mammillata* Cuvier (Endean, 1961; Stiévenart, 1970). The test is translucent and gelatinous with blood vessels ramifying through it and containing large " bladder cells " as well as a variety of other smaller cells (Fig. 4). Bladder cells or test vesicles are large globular structures containing acid and with a small amount of granular material from which fibres radiate to the periphery of the cell. They appear to be a product of the break-down of morula cells in the test (see also Webb, 1939). Within the hyaline material of the test there is a network of microfibrils 20 mμ to 40 mμ in thickness. Vanadocytes in various stages of disintegration are also discernible and from some of these fine processes with granules extend out into the test. Some of the microfibrils are found to be continuous with the surface of the vanadocyte. The hyaline framework of the test is acid mucopolysaccharide, probably produced by the epithelial cells of the blood vessels. When the test material is hydrolyzed an insoluble residue remains which appears

FIG. 3. Peripheral blood vessels in the test of *Ascidia correlides* (Van Name), showing blind ending ampullae.

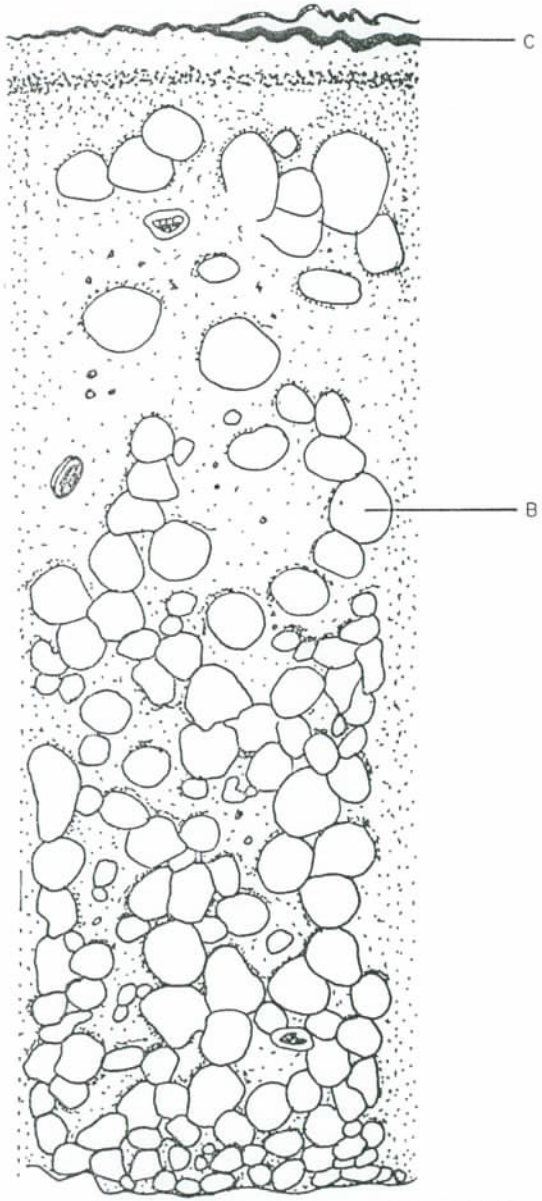


FIG. 4. T.S. of test of *Phallusia mammillata* showing " bladder cells " (B), and external cuticle (C). (Redrawn from Stiévenart, 1970.)

to be cellulose and which Endean considers to arise from the microfibrils; he believes that these microfibrils arise from disintegrating morula cells (vanadocytes). Fibrils of a similar size are also described in *Phallusia* by Meyer *et al.* (1951).

(b) *Ascidiella aspersa* (Müller) (Stiévenart, 1970). The structure of the test resembles that of *Phallusia* except that the bladder cells are

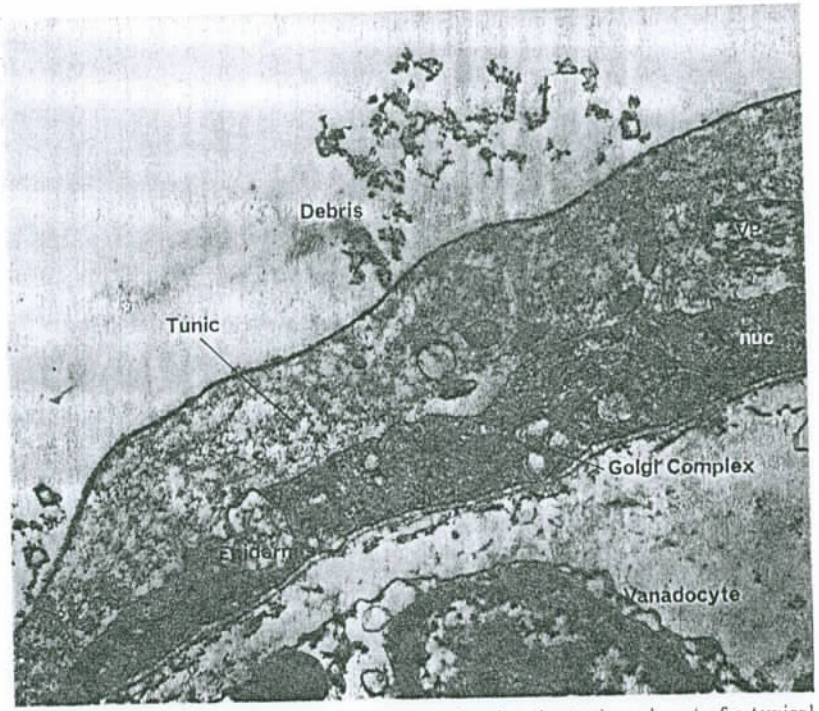


FIG. 5. Low magnification electron micrograph showing the tunic and part of a typical simple squamous epithelial cell in the epidermis of *Perophora viridis*. nuc, nucleus of epidermal cell; vp, process of a vanadocyte in the tunic. × 22 000. (Reproduced by permission, from Deck *et al.*, 1966.)

confined to a narrow region just outside of the ectoderm. The major portion of the test substance contains a fibrillar network with few bladder cells and numerous multivacuolar cells in the process of degenerating. According to Stiévenart the test substance contains acid mucopolysaccharides and neutral polysaccharides but Hall and Saxl (1961) report that the test substance contains microfibrils of carbohydrate associated with a protein resembling collagen and elastin.

(c) *Perophora viridis* Verrill (Deck *et al.*, 1966) (Fig. 5). The test of *Perophora* is much thinner and more translucent than in *Phallusia*. Filaments of about 50 Å diameter lie in a homogeneous ground substance; the structure of these filaments is consistent with their being a cellulose. The matrix of the test contains protein and carbohydrates, and vanadocytes (morula cells) wander through the matrix. Deck and his co-workers believed that the vanadocytes contain glycogen but consider that it does not contribute to the basic carbohydrate of the test. If the glycogen was the precursor of the test matrix it would be expected to occur in the cell in membrane-bounded intra-cellular compartments, and this is not the case. Furthermore, the vanadocytes do not have a well-developed Golgi-complex such as would be found in a cell secreting a glycoprotein material. In the test matrix the vanadocytes appear most frequently as empty shells, having discharged their contents. As in *Pyura* (*vide infra*) labelled glucose appears rapidly in the cells of the mantle wall and during a period of five days passes right through the test. Furthermore the epithelial cells of the mantle, as in *Pyura*, have an abundant endoplasmic reticulum and hypertrophied Golgi zones suggesting this as the site of active secretion.

(d) *Ciona intestinalis* (Bierbauer and Vagas, 1962). The test is made of a fibrillar network containing mucopolysaccharides. Secretion of test material is from the mantle wall. *Ciona* differs from some other solitary ascidians in having no blood vessels in the main part of the test but only in the posterior pedicel (Millar, 1953a) and hence the mantle is the only possible site for secretion of test material. (See also Pérès, 1948b; Dilly, 1969a.)

(e) *Dendrodoa grossularia* (Van Beneden) (Barrington and Thorpe, 1968). The test is thin but tough and devoid of blood vessels. The matrix is composed of both acid mucopolysaccharides and protein some of which is conjugated as mucoprotein. The toughness of the test is attributable to proteinaceous fibres coupled with a quinone tanning system. The test substance appears to be secreted from the mantle epidermis but unlike some other ascidians the morula cells, which are rich in glycoprotein, do not migrate through the mantle and appear to contribute materials directly to the epidermis. According to Barrington and Thorpe the mantle cells probably produce both carbohydrate and protein and wandering " polyphenol " cells in the test are responsible for the quinone-tanning system of proteins. This system may also aid in the process of iodine binding in the tunic (see p. 28). In this context it is noteworthy that in *Ciona* (Barrington and Barron, 1960) iodinated structural proteins are confined to the outer cuticle of the test, whereas in *Dendrodoa* the process of iodination

commences at the level of the mantle although autoradiographs indicate that these structural proteins are concentrated in the cuticle.

(f) *Pyura stolonifera* (Heller) (Endean, 1955b; Wardrop, 1970) (Fig. 6). According to Endean the test is gelatinous, permeated by blood vessels and has microfibrils scattered throughout. These fibrils are up to 60 μ long and 0·3 to 0·4 μ in diameter and mainly run parallel with the long axis of the animal and also parallel with the blood vessels.

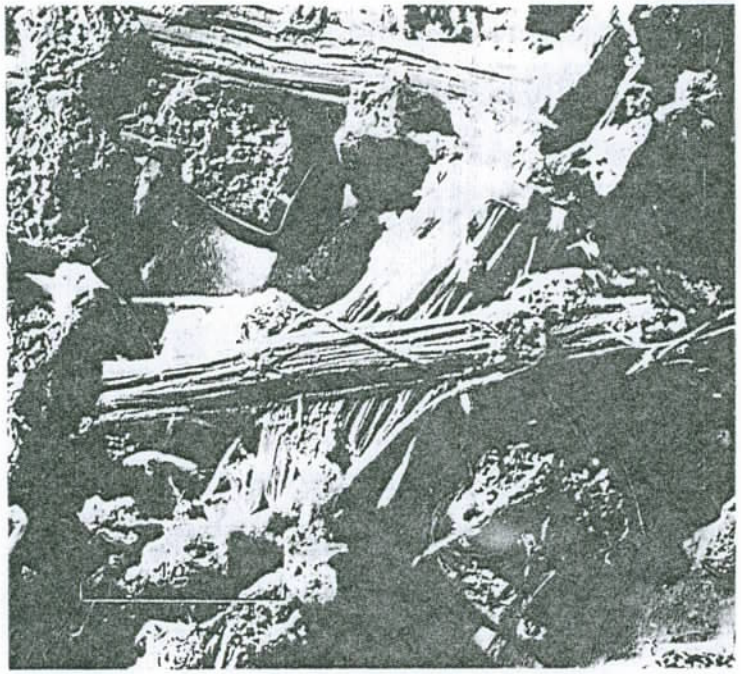


FIG. 6. A frozen etched preparation of the test of *Pyura stolonifera* showing bundles of cellulose microfibrils. (Reproduced by permission, from Wardrop, 1970.)

The fibrils behave like cellulose in being resistant to ordinary solvents, but they do not stain as for cellulose and do not dissolve in cellulose solvents. Endean concludes that they must have a different molecular architecture from cellulose and are probably composed of an insoluble polysaccharide associated with mucopolysaccharide.

Wardrop has re-examined the test of *P. stolonifera* and confirms in electron micrographs the presence of microfibrils but states that they occur as flat ribbons 120 to 130 Å wide and organized in bundles 2000

to 4000 Å wide. Electron micrographs also show that in the disintegration of morula cells the vesicles (vanadophores of Gansler *et al.*, 1963) break free in the test substance and in some cases are found in association with microfibrils thus suggesting that there may be a relationship between fibre production and disintegration of the morula cells. This is supported by Endean's (1955c) observations that in this species the morula cell produces fibres when cytolyzed and that fibre production by cytolysis can be observed in hanging drop preparations of blood. Endean considered that morula cells migrate into the test substance through the walls of the blood vessels or the mantle. He suggested that morula cells either directly secrete fibres or secrete a material into the test which then participates in the formation of fibres. This conclusion is supported by the fact that, in injured test, morula cells migrate to and aggregate at the site of injury and wound healing, an observation also made in *Halocynthia* by Smith (1970a).

Although there is agreement that the morula cells migrate into the test and are involved in fibre production, Wardrop (1970) also shows that the mantle epidermis is actively engaged in carbohydrate metabolism. He found that, in animals immersed in a solution of ^{14}C-labelled glucose, the labelled material accumulated in the epithelia of the mantle and test vessels. Electron-micrographs of these epithelia show that the cells contain extended areas of endoplasmic reticulum and the Golgi bodies have numerous vesicles. Endean (1961) claimed that the morula cells produced carbohydrate in *Phallusia* but this is not borne out for *Pyura* by Wardrop's studies and it seems more likely that the basic carbohydrate for test synthesis is derived from the epithelial cells.

(g) *Halocynthia aurantium* (Pallas) (Smith, 1970a, 1970b; Smith and Dehnel, 1970) (Fig. 7). The test of *Halocynthia* is made up of a fibrous matrix but is thinner than in *Pyura* being only about 1·5 mm thick. It differs also from many other species by having cuticular spines on the surface borne on papillate outgrowths of the test. The fibres are extruded from the tip of the mantle epidermis cells and coalesce to form a laminar structure just distal to the epidermis in a region where morula cells (ferrocytes) are also concentrated. The laminar structure of the test becomes less pronounced toward the periphery where the fibres extend toward the papillae and where dispersed vesicular cells are concentrated. There is therefore a pronounced polarization of blood cells in the test in which morula cells are associated with a zone of test formation and dispersed vesicular cells are associated with the spinous periphery. Both morula cells and dispersed vesicular cells undergo degradative changes in the test and both types

of cell migrate to sites of test injury and aggregate there. The test substance appears to contain a protein polysaccharide complex which has a carbohydrate backbone of glucose, probably extensively substituted and cross linked. Staining reactions of mantle and morula cell suggest that only the mantle contributes carbohydrate and that possibly the morula cells contribute protein or are in some way instrumental in the formation of laminae and fibres in the tunic.

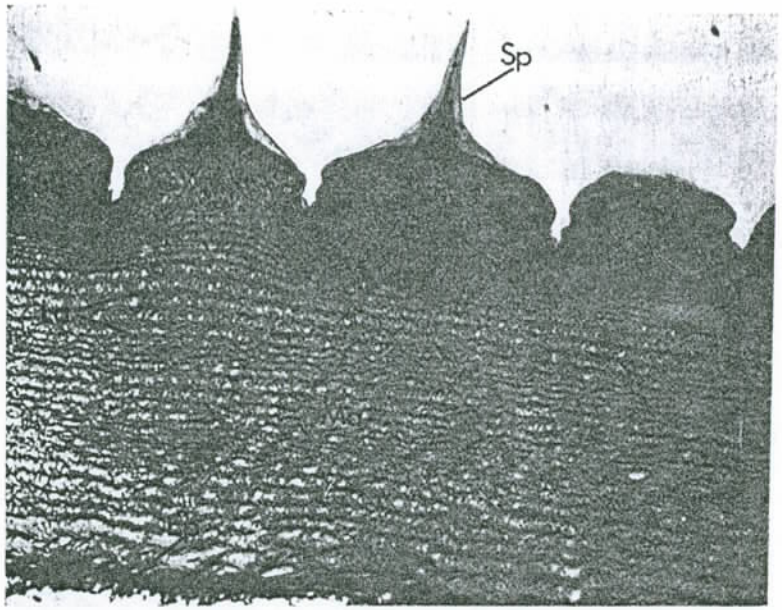


FIG. 7. The test of *Halocynthia aurantium* in transverse section. Ep, epidermis of mantle; La, laminae; Ma, matrix; Pa, papillar outgrowth; Sp, spine. × 120. (Reproduced by permission from Smith, 1970a.)

(h) *Halocynthia papillosa* Gunnerus (Stiévenart, 1971). The organization of the test in this species closely resembles that in *H. aurantium*. The test is thin being only about 1·75 mm in thickness, and has a proteinaceous cuticle bearing small spines. The main body of the test has bundles of fibres arranged parallel with one another about 15 μ apart and joined to one another by small bridges of fibres. All of this is embedded in a matrix which contains granular cells which rupture their contents between the fibres and finally disappear. Stiévenart suggests that this type of formation provides a supple but very strong structure. As in *H. aurantium* the fibres are secreted from the mantle epidermis.

The sub-cuticular portion of the test (Stiévenart's " fundamental substance ") contains both acid-mucopolysaccharides and neutral polysaccharides as well as containing about 20% of the total nitrogen of the test. Some of this nitrogen is in the form of hexosamine which also forms a significant component of the test substance of *H. aurantium* (Smith, 1970b). Stiévenart suggests that " bundles of the fundamental substance are built up with cellulose fibres embedded by amorphous mucopolysaccharides ". However, the picture is probably not as simple as this; neither Stiévenart in *H. papillosa* nor Smith in *H. aurantium* could obtain positive reactions to P.A.S. in the test substance and Smith suggests that if cellulose does exist in the tunic of *Halocynthia* it must be heavily substituted.

A number of other studies have been made to investigate the nature of the microfibrils in the test of various species and there is general agreement that the fibrils are composed of a polysaccharide related to but not identical with cellulose and varying in diameter from 20 to 200 Å according to species (Frey-Wyssling and Frey, 1950; Ranby, 1952; Parker and Leeper, 1969; Mishra and Colvin, 1970; Smith and Dehnel, 1971).

From these recent studies it is possible to draw some general conclusions about the nature and formation of the test. The test is not an inert structure like the molluscan shell but is an active living tissue comparable to the fibrous connective tissue of the vertebrates. It functions in part as a protective covering and in part as a supporting skeleton. In some species it also appears to assist in restoring body shape after muscular contraction (*vide* p. 99). There is usually a hyaline ground substance formed of acid-mucopolysaccharides associated with protein, and embedded in this are microfibrils of a polysaccharide complex closely resembling cellulose. These fibrils are not visible in the light microscope but their identity is apparent from electron micrographs and X-ray diffraction studies (see particularly Mark and Susich, 1929; Meyer *et al.*, 1951; Ranby, 1952; Hall and Saxl, 1961; Deck *et al.*, 1966; Mishra and Colvin, 1969, 1970; Wardrop, 1970).

The degree to which fibre development takes place varies from species to species and may have an important bearing on the mechanical properties of the test. In the phlebobranchiate ascidians in particular (i.e. *Ciona, Ascidiella, Phallusia*) there is a tendency to produce large gelatinous tests in which small fibrils are scattered throughout the matrix. The stolidobranchiate ascidians on the other hand (i.e. *Pyura* and *Halocynthia*) show a tendency to develop fibre bundles in a reduced matrix. These latter tests, particularly *Halocynthia* and *Molgula*, tend

to be thinner and tougher than those of the phlebobranchiates. The differences between phlebobranchiates and stolidobranchiates are further emphasized in studies on gross chemical composition in four species of ascidian (Smith and Dehnel, 1971). In two species of phlebobranchiate the " tunicin " fraction yielded approximately 100% of its dry weight as cellulose equivalents as opposed to 50% equivalents in two species of stolidobranchiates. The amino-acid composition in the two groups is similar and suggests that the protein fraction of the test may be uniform throughout the Ascidiacea. If this is the case the differing physical properties of the ascidian test may be attributable to differences in carbohydrate composition and water content. A comparative study of the architecture and mechanical properties of the test would be of great interest.

The carbohydrate material for test formation appears to be secreted primarily from the epithelium of the mantle wall, the cells of which have an extensive development of endoplasmic reticulum and Golgi bodies. Radio-tracer studies with labelled glucose show that the carbohydrate is accumulating in this region and passing out to the test (Deck *et al.*, 1966; Wardrop, 1970). In Didemnidae the epidermal cells of the terminal bulbs of the stolonic vessels also secrete test material (Millar, 1951) and this may occur in all ascidians which possess such vascular bulbs.

The role of morula cells, whether vanadocytes or ferrocytes, remains uncertain but there is sufficient evidence to show that their primary role is concerned with test formation. Most workers are agreed that morula cells migrate into the test, either directly through the mantle wall or through the epithelial walls of test vessels, and that they discharge their vacuolar contents and finally disintegrate in the test matrix (see especially Endean, 1955c, 1961; Deck *et al.*, 1966; Wardrop, 1970; Smith, 1970a). Endean and Smith have both demonstrated that the morula cells are associated with sites of fibre production. Endean believed that the morula cells actively secreted the fibres but this is probably incorrect. The evidence from tracer studies that carbohydrate is secreted by the mantle and the chemical evidence on the nature of the contents of the morula cell both suggest a different role for these cells. The metal chromogen in the morula cells is a powerful reducing substance maintained in the reduced state by strong sulphuric acid (see p. 75). When the vesicles of the cell are discharged in the test and the chromogen is freed from the acid these reducing properties will become effective. The fact that fibres are produced at the same time suggests that the reducing properties are in some way used to polymerize the carbohydrates in the test to form a fibril of tunicin.

If this interpretation is correct it may lead to an explanation of the acid filled bladder cells or test vesicles found in the test of some ascidians (Webb, 1939; Stiévenart, 1970). These vesicles are not true cells but are merely fluid filled capsules and Endean (1961) has shown how they may form around a disintegrating morula cell. If the purpose of the acid in the morula cell is to hold the chromogen in a reduced state until it is in a position to perform its function of fibre production then, when this function is fulfilled, it is clearly desirable to remove the acid and not to permit it to diffuse through the test substance. This could be fulfilled by encapsulating the acid in the test vesicles and subsequently discharging these through the surface of the test.

III. The Branchial Sac

The branchial sac, or pharynx, must be considered as serving the dual roles of a food collecting apparatus and a site of gaseous exchange with the water. In its simplest form, as found in aplousobranchiate ascidians, it is a sac-like structure the lateral walls of which are pierced by stigmata, each one of which is fringed with cilia; these cilia are responsible for driving the water current through the sac. Along the ventral margin of the sac is an endostyle whose primary function is to secrete mucus which is directed as a sheet passing across the walls of the sac to the dorsal side where it is folded into a mucus cord by the dorsal lamina.* The oral entrance to the sac is guarded by a circle of tentacles which act as a coarse filter for particles entering the sac in the water stream. In phlebobranchiate ascidians the inner wall of the branchial sac is complicated by the presence of internal longitudinal bars borne on pedicel-like outgrowths from the wall of the sac. At the junctions of internal longitudinal bars and transverse vessels small ciliated papillae extend inwards towards the cavity of the sac. Similar internal longitudinal bars, lacking papillae, occur in the stolidobranchiate ascidians but in this group the whole wall of the branchial sac is thrown into a series of folds thus greatly increasing the internal surface area. These differences must be associated with differences in the feeding activity of the animals concerned, but until recently little attention has been given to their functional significance. Nor does anyone seem to have considered the functional significance of differences which occur in the structure of the dorsal lamina.

The feeding mechanism of ascidians has been fully described by

* The word *mucus* is used throughout the text in very general terms to describe the secretion from the endostyle. The precise chemical nature of the secretion is uncertain but it contains large amounts of an iodinated protein in addition to mucus and may be a muco-protein.

Orton (1913), MacGinitie (1939), Werner and Werner (1954), Jørgensen (1966), Millar (1971) and Croxall (1971). It is generally agreed that a continuous mucus sheet is produced by the endostyle and that cilia on the branchial wall pass this dorsally to the dorsal lamina. It is the mucus sheet and not the branchial stigmata which acts to strain off food particles from the water. According to Jørgensen (1949) and Jørgensen and Goldberg (1953) the mucus sheet of *Ciona intestinalis* is capable of retaining particles as small as 1 μ in diameter. In aplousobranchs the mucus sheet must be moved by the cilia of the stigmatal margin, but in phlebobranchs and stolidobranchs the mucus sheet is held away from the stigmatal surface by the longitudinal bars and hence transport of water and transport of the mucus sheet become functionally separated. The mucus sheet is now moved by the cilia of the branchial papillae and longitudinal bars and Hecht (1918a) has suggested that in *Ascidia nigra* waves of muscular contraction in the branchial papillae assist in passing the mucus sheet across the branchial wall. My own observations on *Ascidia nigra* suggest that waves of activity pass across the longitudinal bars and that while the mucus sheet is primarily moved by cilia its passage is aided by undulating movements of the whole system of internal longitudinal bars. A similar observation was made by Orton (1913) on *Ascidia mentula*.

The whole question of food collection and transport of the mucus sheet has recently been studied on a comparative basis by Croxall (1971). In the phlebobranchiate ascidian *Ciona* the longitudinal bars have frontal cilia only (i.e. on the surface facing into the branchial sac) and under conditions of low food density the mucus sheet is transported solely by the ciliary activity of the papillae and bars. In conditions of high food density small but significant papillar movements occur and result in a fast moving sheet of mucus across the branchial wall. In *Corella* (also a phlebobranch) there are no papillae and the longitudinal bars have lateral as well as frontal cilia. In addition the stigmata are spiral, thus increasing the filtration rate, and are three-dimensionally arranged so that they create vortices of water. Unlike *Ciona* the mucus sheet moves at a uniform rate irrespective of particle density; this probably permits maximum efficient use of the vortices and also prevents any tendency toward fragmentation of the mucus sheet in the vortex. In the stolidobranch ascidians *Asterocarpa* and *Cnemidocarpa* there are four branchial folds on either side. The longitudinal bars are not borne on pedicels but are only just raised off the surface of the branchial sac; they are also concentrated towards the tips of the folds and are widely spaced in the troughs between folds. The bars are ciliated frontally and laterally, frontal cilia aid in the passage of mucus, lateral

cilia aid in creating a water current and in holding the mucus sheet against the branchial wall. In the stolidobranchs Croxall finds that there are two separate sheets of mucus crossing the pharynx. One of these sheets is fast moving and passes from crest to crest of the branchial folds aided by waves of activity in these folds which pass from the endostyle towards the dorsal lamina. The other is a slow moving sheet which passes around both folds and troughs. These two systems effectively form a double filtration system of coarse and fine filters; the fast moving sheet from crest to crest is suitable for high densities of particles while the slow moving sheet is more efficient in low particle densities.

TABLE II. FILTRATION RATES OF SIMPLE ASCIDIANS EXPRESSED AS ML/H/G WET WT

Species	*Weight (g)*	*Filtration rate*	*Temperature (°C)*	*Author*
Ciona intestinalis	—	80		Goldberg *et al.* (1951)
C. intestinalis	2·6	230	16–19	Jørgensen (1949, 1955)
Ascidia nigra	100	72	—	Hecht (1916)
A. nigra	10	120	—	Hecht (1916)
A. nigra	4·7	216	27	Goodbody (Unpublished data)
A. nigra	34·5	24	27	Goodbody (Unpublished data)
A. interrupta	5·8	117	27	Goodbody (Unpublished data)
A. interrupta	26	27	27	Goodbody (Unpublished data)
Phallusia mammillata	7·9	104	15	Carlisle (1966)
P. mammillata	128	400	15	Carlisle (1966)
Molgula sp.	—	540	16–19	Jørgensen (1949, 1955)
P. mammillata "Full sized"	—	1	11–14	Hoyle (1953)

Providing that the rate of mucus secretion and of passage of the mucus sheet across the filter can be varied as is suggested above, the efficiency of food collection will depend on the porosity of the filter and the rate of water transport. According to Jørgensen (1949, 1952) particles as small as 1 μ can be retained by mucus filters. The data on filtration rates are very conflicting and must depend in part on the techniques used by different experimenters. The subject has already been reviewed by Jørgensen (1955, 1966) and Millar (1971). Recently Croxall (1971) has provided a wide range for several species under

TABLE III. AVERAGE FILTRATION RATES OF SIMPLE ASCIDIANS UNDER VARYING CONDITIONS, EXPRESSED AS ML/H/G DRY WT (Croxall, 1971)

Size	*Ciona*	*Corella*	*Asterocarpa*	*Styela*	*Microcosmus*	*Pyura*	*Temp. °C*	*Algal conc.*
Small	325	450	502	603	730	675	19	Normal
Large	281	403	448	550	701	624	19	Normal
Medium	300	420	476	575	724	650	19	Normal
Medium	160	234	265	344	517	468	12	Normal
Medium	363	487	541	639	852	815	25	Normal
Medium	202	300	314	376	553	504	19	Low
Medium	402	611	625	774	900	870	19	High

varying conditions of temperature and food supply, but his figures, which are expressed per gramme dry weight, are considerably higher than those for other species where measurements have been expressed in terms of wet weight (Tables II and III). The differences between Croxall's data and those of others are hard to reconcile, but it is clear from all of the data that the phlebobranchiate ascidians filter more slowly than do stolidobranchiates and in general small animals filter faster than do large animals. Croxall has extended this comparative approach to show that the overall feeding efficiency of the stolidobranchs is greater than that of the phlebobranchs (Table IV). Within the phlebobranchs, *Corella*, with spiral stigmata, is slightly more efficient than *Ciona*, and within the stolidobranchs the pyurids are more efficient than the styelids. This is in keeping with the more highly organized nature of the branchial sac in pyurids than in styelids.

TABLE IV. OVERALL FEEDING "EFFICIENCY" RATIOS FOR SIMPLE ASCIDIANS (Croxall, 1971)

	Ratio of filtration rates		*Ratio of digestive efficiency*		*Ratio of food passage time*
	Summer	*Winter*	*Summer*	*Winter*	
Ciona	100	100	100	100	100
Corella	140	149	100	104	105
Astorocarpa	159	155	99	115	137
Styela	192	186	100	107	147
Microcosmus	214	274	115	115	171
Pyura	217	250	115	155	158

Many of the differences in the data on filtration rates of individual species may be due to differences in technique. Hecht (1916) has pointed out that in *Ascidia nigra* it may take as long as eight hours for an animal to recover from handling and to settle down to a uniform and maximum rate of flow. Furthermore ascidians are capable of cutting off the flow of mucus and thereby interrupting the pattern of particle clearance (Jørgensen, 1966) and if this occurs during an experiment it will also affect the results. The very low value for *Phallusia mammillata* recorded by Hoyle (1953) is probably incorrect and due to a pressure head of water acting against the animal in the particular situation (Carlisle, 1966). The pressure created by ciliary activity has been reported as 2 mm of water in *Ascidia nigra* (Hecht, 1916) and *Phallusia mammillata* (Hoyle, 1953). However, Goodbody and Trueman

(1969) using a cannula in the branchial sac of *Ascidia nigra* found a pressure of only 0·3 mm.

In addition to the normal ciliary induced flow of water through the branchial sac, ascidians also exhibit regular contractions of the mantle musculature which result in the discharge of about two-thirds of the volume of water from both branchial and atrial cavities. On relaxation of the animal a fresh supply of water is drawn into these two cavities. Spontaneous squirting of this sort has been studied in *Ascidia nigra* by Hecht (1918a), in *Styela clava* Herdman by Yamaguchi (1931) and in *Phallusia mammillata* by Hoyle (1953), and the subject has been reviewed by Hoyle (*loc. cit.*) and Jørgensen (1966). In all these species the frequency of contraction is about one in every 5 to 8 min and Yamaguchi and Hoyle are in agreement that in the absence of food the rate increases to about once in every 2 min. However, Goodbody and Trueman (1969) in a comparison of two species found that *Ascidia nigra* exhibited a frequency of contractions of about once every 1·5 min, while *A. interrupta* Heller contracted about once in every 7·5 min. In contrast they showed that the pressure pulse induced by contraction in *A. nigra* was about 2 cm of water as opposed to a pressure of 6 cm of water in a similarly sized *A. interrupta*. Taking a human analogy this may be likened to frequent small "coughs" by *A. nigra* and infrequent large "coughs" by *A. interrupta*.

The functional significance of spontaneous squirting is still a matter for debate. Hoyle (1953) considered that it is primarily associated with food intake and is in this respect more important than ciliary activity. He calculated that renewal of the water in the branchial sac by ciliary activity amounted to about 1 ml/min while squirting accounted for a renewal of about 1·5 ml/min. The fact that spontaneous squirting increases in frequency in the absence of food lent further support to Hoyle's argument. However, as pointed out by Jørgensen (1955, 1966) and Carlisle (1966), Hoyle's calculations of renewal by ciliary activity may have been erroneous. Jørgensen also points out that in *Ciona intestinalis* at any rate renewal of water by squirting must be less than 90 ml/h, while renewal by ciliary activity is of the order of 3000 ml/h or at least thirty times as great. Furthermore the water expelled in squirting will probably remove as much particulate matter as is drawn in in the subsequent inhalation (Jørgensen 1952, 1966). Jørgensen (1966) has suggested that one function of squirting might be to renew the water adjacent to the exterior of the siphons and thereby prevent stagnation or any tendency to re-cycle water between the atrial and branchial siphons. While this effect may result from squirting activity it is unlikely to be its primary functional role. The nervous control of

siphon diameter ensures that the exhalant discharge of water is driven a long way from the branchial siphon and in most ascidians the siphons are so orientated as to ensure that mixing will not occur. Furthermore ascidians normally live in places where natural renewal of the water will occur. It seems more likely that squirting is functionally related to the food collecting mechanism rather than to the water supply. One possibility is that it is used to " clean " the branchial wall of accumulated mucus and particulate material not otherwise removed by ciliary activity. However, this will only take place if the atrial siphon is closed and a pressure differential allowed to develop between the two sides of the branchial wall; this appears not to be the case in normal squirting during which water is not exchanged to any degree between atrial and branchial cavities and the mucus sheets are not dislodged (Jørgensen, 1966). Squirting must, however, be important for the maintenance of the atrial cavity and far too little attention has been paid to this aspect of the problem. Faeces and other particulate matter are bound to accumulate in the atrial cavity and while normal reflexes may ensure their discharge, regular spontaneous contractions may materially enhance the sanitary processes of the cavity. Little attention has also been given to the question of pseudo-faeces formation and ejection from the branchial sac and as to whether spontaneous squirting may be associated with such a process as in bivalve molluscs. It seems to be generally assumed that ascidians do not sort their food and that all particulate matter which enters the branchial sac is ultimately transferred to the oesophagus. The oral tentacles act as a coarse filter to exclude particles beyond a certain size, but Croxall (1971) points out that they may also serve to direct food into the centre of the inhalant stream. Once a particle has passed this screen there seems to be no way in which it may subsequently be rejected unless mucus secretion is interrupted and particles are permitted to pass through the stigmata. However, MacGinitie (1939) suggests that ascidians are capable of removing large particles from the food supply and states: " Such large particles as do find their way into the branchial basket are not incorporated in the mucus, but are in some way dropped from it into the branchial basket, and at intervals are forcibly ejected from the oral funnel by a sudden contraction of the body wall of the tunicate. " Later in the same paper he suggests that specially large cilia in the dorsal groove are used to force out unwanted particles from the mucus. No other workers appear to have examined the question of particle sorting and pseudo-faeces production in ascidians and the matter deserves renewed attention especially in relation to spontaneous squirting.

IV. The Endostyle

The endostyle forms a shallow groove extending along the whole length of the ventral side of the branchial sac. The morphology and histology of this structure has been reviewed by a number of authors including Sokólska (1931), Raimbault (1948), Barrington (1957), Olsson (1963) and Orsi and Relini (1966a,b) and details of ultrastructure and histochemistry of the various regions are given by Levi and Porte (1964), Godeaux and Firket (1966, 1968), Ghiani *et al.* (1965), Ghiani and Orsi (1966), Aros and Virágh (1969), Godeaux (1971) and Thorpe *et al.* (1972).

There is considerable uniformity in the structure of the endostyle in different species and in general it is divided into eight regions or zones as illustrated in Fig. 8. An additional small group of cells between zones 4 and 5 has been described by Orsi and Relini (1966a) and Godeaux and Firket (1968) and another small group between zones 7 and 8 in the family Pyuridae by Orsi and Relini (1966a,b) and in *Ciona* by Thiebold (1971).

Zone 1 at the base of the groove is unpaired and is composed of tall cells arranged on either side of a groove marking the median plane of the endostyle. These cells contain large, electron dense granules about 700 Å in diameter and a number of microtubules and microfibrils. At the apex the cells bear long flagella surrounded at their base by numerous finger-like microvilli (Godeaux and Firket, 1968; Ghiani and Orsi, 1966; Aros and Virágh, 1969). According to many authors these cells produce a secretion of mucus (Levi and Porte, 1964; Barrington, 1957; Ghiani and Orsi, 1966; Thorpe *et al.*, 1972), but Olsson (1963) and Godeaux and Firket (1968) both consider that the granular contents of the cell are glycogen and not mucus. The flagella appear to be used to spread secretions from other zones throughout the endostylar groove and to assist in wafting them out towards the walls of the branchial sac.

Zones 2 and 4 are composed of a small number of very large cells arranged like a fan so that the apices adjacent to the endostylar groove are closely crowded while the bases are widely spread adjacent to the underlying blood spaces. The individual cells have a basal nucleus, extensively folded endoplasmic reticulum, numerous secretory vesicles and surface microvilli. The basal membrane of the cell is also deeply folded, especially in zone 2, indicating that considerable metabolic exchange must occur between these cells and the blood spaces. The details of the fine structure are fully illustrated by Aros and Virágh (1969), Levi and Porte (1964) and Godeaux and Firket (1968)

and most workers are agreed that the primary secretion from the cells of both zones into the endostylar groove is protein and not mucus (*cf.* especially Levi and Porte, 1964; Olsson, 1963; Ghiani *et al.*, 1965; Godeaux and Firket, 1968; Thorpe *et al.*, 1972) but Olsson also produced

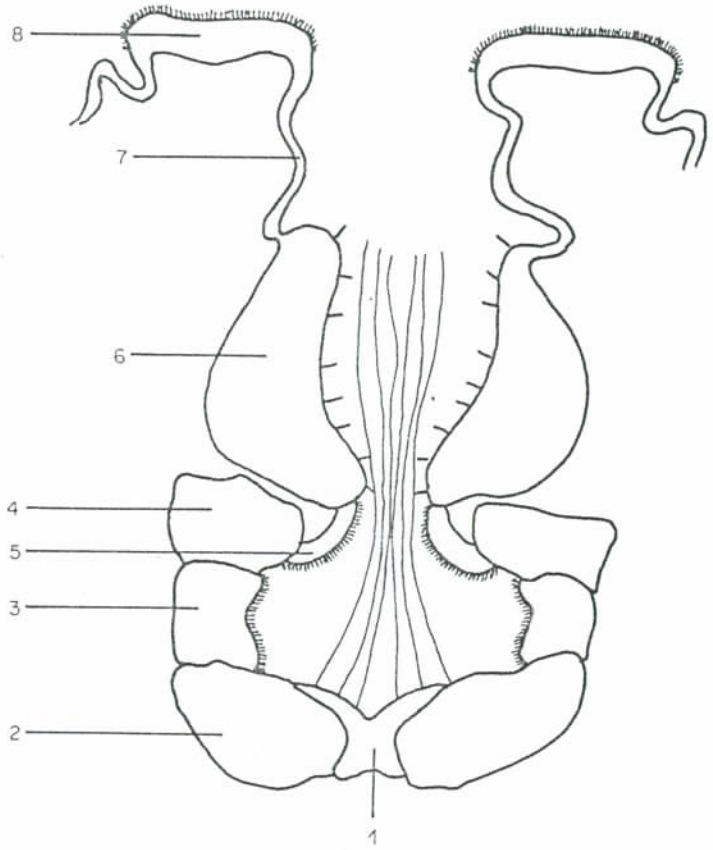


FIG. 8. T.S. of ascidian endostyle to show position of different cell zones. For identification of numbers see text. (Redrawn from Thorpe *et al.*, 1972.)

evidence for some secretion of mucopolysaccharide and Godeaux and Firket (*loc. cit.*) considered that zone 4 is the primary site of mucus secretion in the endostyle of *Molgula manhattensis* (De Kay).

Zone 3 is frequently referred to as the ventral ciliated zone but the work of Ghiani and Orsi (1966) and Aros and Virágh (1969) on *Ciona intestinalis* and Godeaux and Firket (1968) on *Molgula manhattensis*,

makes it clear that this interpretation is incorrect and that in fact it is a highly organized region of flagellated cells. Each cell appears to bear a single flagellum the base of which is surrounded by a collar of microvilli around the margin of the cell; the whole cell thus bears a superficial resemblance to a choanocyte (Milanesi, 1971). Furthermore Ghiani and Orsi have shown that the position of the centriole is different in adjacent cells and periodically repeats the same position every fourth cell. This arrangement results in a rotational movement of the flagella

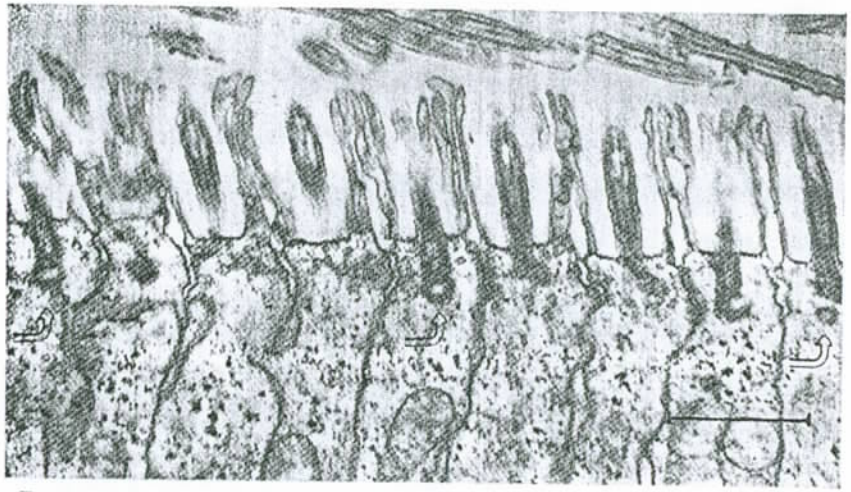


FIG. 9. A frontal section of zone 3 of the endostyle of *Ciona intestinalis*. The position of the centriole at the base of the flagellum occupies different positions in neighbouring cells but repeats its position in every fourth cell as depicted by the arrows. × 20 300 approx. (Reproduced by permission, from Ghiani and Orsi, 1966.)

on the free surface of the epithelium and suggests that the function of these cells and their flagella is to mix the secretions from the two adjacent secretory zones, 2 and 4 (Fig. 9).

Zones 5 and 8 resemble one another but differ from zone 3 in being ciliated and not flagellated (Fig. 10). Each cell bears a bundle of cilia surrounded basally by a ring of microvilli and the ciliary roots run obliquely through the cell, tapering to a common " anchorage " on the lateral cell wall (Olsson, 1963; Levi and Porte, 1964; Ghiani and Orsi, 1966; Godeaux and Firket, 1968). Both zones would appear to be engaged in lateral transport of endostylar secretions by metachronal activity of the cilia. Large deposits of glycogen observed in some of these cells, notably in zone 5, are probably energy stores and not secretory products.

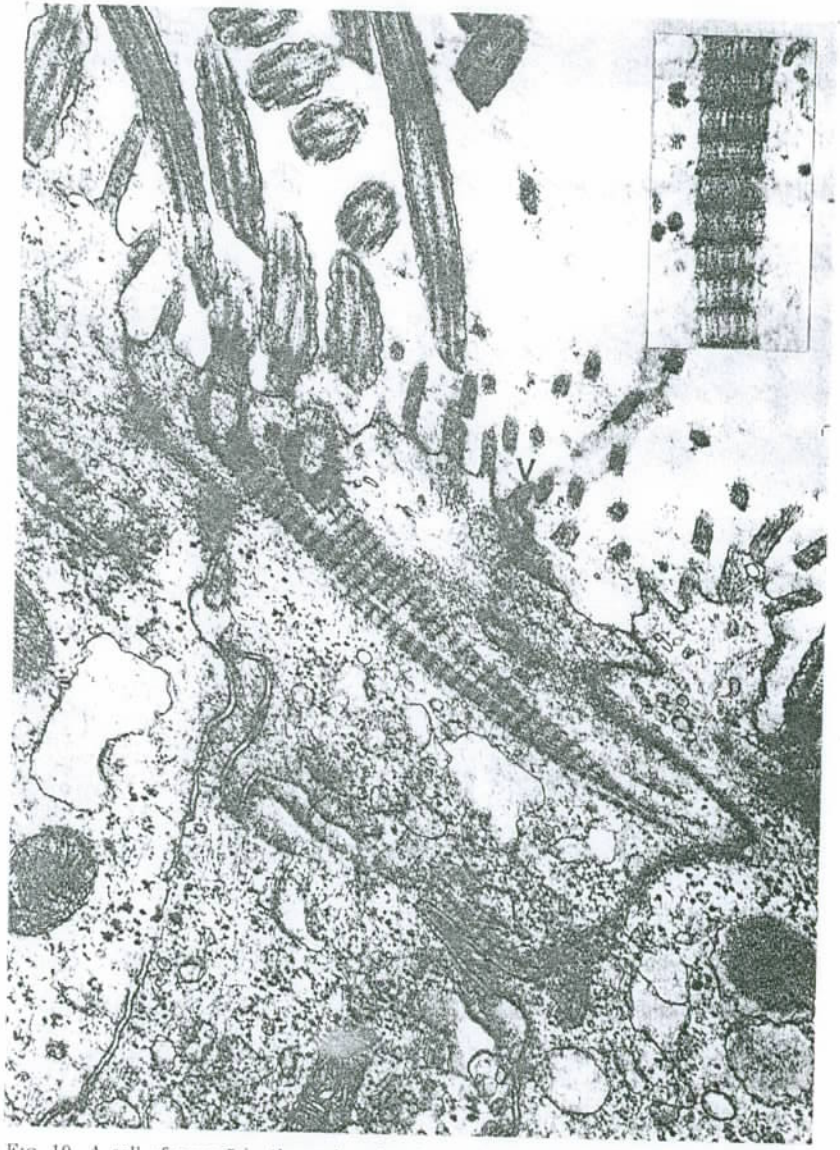


FIG. 10. A cell of zone 5 in the endostyle of *Molgula manhattensis* showing the ciliary roots converging obliquely towards the lateral wall of the cell. × 30 000. (Reproduced by permission, from Godeaux and Firket, 1968.)

Zone 6 is a broad band of secretory cells and does not exhibit the marked fan-like arrangement of zones 2 and 4. A few cilia also occur on these cells and presumably assist in lateral transport across the surface. All authors are agreed that the primary secretion from zone 6 is protein, but Orsi and Relini (1966b) and Thorpe *et al.* (1972) point out that the cells are rich in tyrosine. According to Orsi and Relini it also contains a large amount of tryptophan.

Zone 7 has low columnar cells with an extensive rough endoplasmic reticulum which produces granules of a secretion subsequently "reworked" by the Golgi-body (Gorbunova, 1971; Thorpe *et al.*, 1972). This is the site of iodine binding and secretion of iodinated compounds into the endostyle and is discussed further in the next section.

From all of these details it is possible to re-consider the functional role of the endostyle as the site of production of a complex iodinated protein sheet which is passed out to the pharynx to serve as a filtration membrane. There is only limited evidence of the secretion of muco-polysaccharides and the earlier concept of a purely mucus-based membrane produced by the endostyle would appear to have been incorrect. Nevertheless, the final membrane may be in the form of an iodinated muco-protein. The primary products appear to be produced in the two lower secretory zones, 2 and 4, and these are mixed by the flagella of zone 3 prior to being passed laterally to zone 5. The fan-shaped arrangement of cells in zones 2 and 4 probably ensures that their secretions are concentrated at either margin of zone 3 and thereby adequately mixed. This mixture must then be passed laterally by the cilia of zone 5 to combine with further protein secretions from zone 6 prior to the addition of iodinated compounds from zone 7. The mixture is finally spread out by the combined activity of the flagella of zone 1 and the cilia of zone 8. The precise role of the flagella of zone 1 is not at all clear but they probably serve a dual role of maintaining a steady flow of material out of the endostylar groove and keeping it from "clogging", while at the same time assisting in an even spread of material to either side of the branchial sac.

V. Iodine Binding

The organic binding of iodine in animals is almost entirely due to simple linkages with the amino acid tyrosine to form either 3-mono-iodotyrosine (MIT) or 3,5-di-iodotyrosine (DIT). The iodine involved in this linkage is derived from the oxidation of iodides taken from the animal's environment. The iodotyrosines may be linked with other amino acids in the formation of scleroproteins such as *gorgonin*, *antipathins* and *spongins* and can be identified as such in hydrolysates

of these proteins. Iodotyrosine may also condense to form iodothyronines the best known of which are 3,5,3-tri-iodothyronine (T_3) and thyroxine (T_4) which in vertebrate animals are normally bound by peptide bonds in the protein thyroglobulin but can be released from it by enzymatic action. Tri-iodothyronine and thyroxin are the active hormonal components of the vertebrate thyroid gland (Roche, 1959; Berg *et al.*, 1959; Gorbman, 1969).

In ascidians organically bound iodine has been found to occur in the cuticle of the test, in the endostyle and to a lesser extent in the pharyngeal wall and in the blood. Iodine was first identified in the test of a number of species of ascidians by Cameron (1915) but it is only recently with the introduction of radio-tracer techniques and chromatography that it has been possible to localize and identify these substances. According to Barrington (1957), Barrington and Barron (1960), Barrington and Thorpe (1968), and Roche *et al.* (1962b) most of the iodine in the test is to be found in the proteinaceous cuticle on the outer surface where it is involved in the formation of a scleroprotein. Barrington and Barron (1960) identified only DIT and T_4 in the test of *Ciona intestinalis*, but later, using two-dimensional chromatography, Barrington and Thorpe (1963, 1968) concluded that MIT and DIT could be positively identified in the tests of *Ciona* and *Dendrodoa grossularia*. In the latter species Amaral *et al.* (1972) have identified MIT, DIT and T_4 but not T_3. Roche and his co-workers (Roche *et al.*, 1960, 1962a,b, 1964) found that both T_3 and T_4 are present in hydrolysates of the test of *Ciona intestinalis*, although they conceded (Roche *et al.*, 1964) that these iodothyronines may not be produced *in situ* but may be transported from elsewhere in the body of the ascidian. There is indeed little reason to believe that the iodinated compounds are actually formed in the test. Salvatore (1969) considered most iodine binding occurs in the test and not elsewhere in the body. Roche *et al.* (1963, 1964) found that iodine uptake in *Ciona* was markedly arrested following removal of the test and only resumed after new test formation had commenced. This suggests that uptake of iodine may be a function of the amount of test material present, but on the other hand autoradiographs (Kennedy, 1966; Barrington and Thorpe, 1968) show an active movement of iodine through the test substance and toward the cuticle. Kennedy has also identified iodinated compounds in the blood and it seems more likely that this is the site of iodine uptake rather than that it should occur directly into the cuticle of the test.

The organic binding of iodine in the ascidian endostyle was first demonstrated by Barrington and Franchi (1956) and has been followed up in a series of papers by Barrington and his co-workers (Barrington, 1957; Barrington and Thorpe, 1965a,b; Thorpe and Barrington, 1965; Thorpe *et al.*, 1972) using ^{131}I and ^{125}I as tracers. Iodine is actively taken up and bound in the cells of zone 7 of the endostyle, a simple epithelium lying between the dorsal glandular tract and the ciliated lip (zone 8) of the endostyle (see Fig. 8). According to Thiebold and Illoul (1967) the uptake of iodine by zone 7 is under the control of the neural gland and removal of the gland increases the amount of iodine taken up in a given time. Prolonged immersion in radioactive iodide shows that active secretion of iodinated compounds takes place from these cells and is carried up the wall of the pharynx by the cilia of zone 8 and the longitudinal bars of the pharynx. The cells of zone 8 and of the longitudinal bars also secrete small quantities of iodinated compounds, but nowhere is the intensity of activity so great as it is in zone 7. The relatively high concentrations of iodinated compounds reported from the branchial sac by Suzuki and Kondo (1971) may be due to the incorporation of portions of filtration membrane in their homogenates. Histochemical tests show that the glandular secretions of the endostyle contain only relatively small quantities of acid mucopolysaccharides but are rich in protein, and Barrington and Thorpe have suggested that not only would this account for the remarkable filtering properties of the membrane, but that the iodine secreted by the endostyle is associated with the production of a specialized protein for the membrane. Thiebold (1972) deduced that the proteins secreted by the glandular tracts of the endostyle are iodinated at the apical surface of the cells of zone 7 and that while most of the resulting compound is passed out of the endostyle as the filtration membrane, some of it is reincorporated into vesicles of zone 7 cells. Clearly there are complex biochemical processes being carried out at the level of zone 7 and these terminate in the production of the filtration membrane.

The ascidian endostyle is the evolutionary forerunner of the vertebrate thyroid gland and it is therefore not surprising that it should be engaged in the secretion of iodinated compounds. However, Barrington and Thorpe have found that the principal iodinated compounds in the endostyle are 3-mono-iodotyrosine and 3,5-di-iodotyrosine; there is no evidence for the presence of tri-iodothyronine and only very small amounts of thyroxine are identifiable (*cf.* also Suzuki and Kondo, 1971). This is in marked contrast to the ammocoete larva and to *Branchiostoma* (Amphioxus) both of which are known to produce the hormonal iodothyronines from the endostyle (see Barrington and Thorpe, 1965b, for references). On the other hand, the protochordate *Saccoglossus* only produces mono-iodotyrosine and this appears to be an epidermal secretion (Barrington and Thorpe, 1963).

The overall picture of iodine binding and its significance is at first sight confusing. The two principal sites of bound iodine are the cuticle of the test and the zone 7 cells of the endostyle, and in both cases iodinated tyrosines are the principal compounds involved, but small quantities of an iodinated thyronine (thyroxine) can be detected in the endostyle. On present evidence there is no reason to believe that there is any functional connection between these two processes. It seems likely that iodination in the test is purely associated with the formation of structural proteins for the cuticle. The secretion of iodinated compounds in the endostyle is a specialization of protein metabolism to produce the correct type of filtration membrane passing over the branchial wall (Barrington and Thorpe, 1965b). The presence of iodinated proteins in the epidermal secretions of *Saccoglossus* lends support to the view that the endostylar compounds of ascidians are concerned with the filtration membrane. In *Saccoglossus* and other enteropneusts the epidermal secretions are used as an external food collecting device.

If we accept these ideas as correct it then becomes easier to understand how natural selection may have retained and developed the secretion of iodinated proteins for use in filter feeding and subsequently turned them to advantage as hormones after the change to raptorial feeding in the vertebrates. A discussion of the evolution of the thyroid and its precursors is given by Barrington (1964a,b).

VI. The Alimentary System

A. *Food transport*

Throughout the whole of the alimentary canal food is transported by cilia and there is little evidence of muscular action taking part, except for lateral transport in the branchial sac of certain species where mucus is passed from one branchial papilla to another by muscular action of the papillae themselves (Hecht, 1918a; Berrill, 1950). Roule (1884) has also described very fine bundles of longitudinal muscles in the rectal region and sphincter muscles in the anus of *Ciona intestinalis* which may assist defaecation.

Cilia around the mouth of the oesophagus are responsible for drawing the food cord from the branchial sac into the oesophagus where other cilia impart torsion (Millar, 1953a). The fact that the food cord folds upon itself in the stomach (Plough and Jones, 1939; Berrill, 1950; personal observation) suggests that the cilia of the stomach may be unimportant in food transport. Millar (1953a) describes a ring of ciliated cells at the junction of stomach and oesophagus which he

considers to be specialized for drawing the food cord from the stomach into the intestine where it is passed backward by general ciliation and folded back and forth across the prominent typhlosole (Morton, 1960). The folding of the mucus cord in the stomach suggests that the mucus envelope remains intact and its viscosity is not lowered as reported by Yonge (1935) and Morton (1960). My own observations suggest that any change in viscosity must occur at the junction of stomach and intestine, and is of short duration and that the ring of ciliated cells may have more importance in assisting the passage of a semi-fluid material rather than in pulling the cord. The difference in function is small, but its significance may be important and is returned to later.

B. *Histology of the alimentary canal*

Most studies on the histology of the alimentary canal have been based on *Ciona intestinalis* (Roule, 1884; Yonge, 1925; Van Weel, 1940; Millar, 1953a; Orsi, 1969; Thomas, 1970a,b) and the more complex condition found in stolidobranchs appears only to have been examined by Fouque (1952, 1954, 1959), Degail and Levi (1964), Orsi (1968) and Croxall (1971).

1. *The oesophagus*

Both Roule and Yonge reported the existence of two types of cell in the oesophagus, ciliated cells and mucus secreting cells. Yonge states that the dorsal groove is composed of tall ciliated cells, while the ventral groove is composed of mucus secreting gland cells. In between the grooves on the lateral walls of the oesophagus he found both ciliated cells and mucus secreting cells. Millar also reports on the existence of two types of cell in the oesophagus, mucus secreting ciliated cells in both dorsal and ventral grooves and tall ciliated cells in the remainder of the oesophageal wall. Fouque, Orsi, Thomas and Croxall agree that there is only a single cell-type, the ciliary-mucus cell in which the cilia are confined marginally at the apex of the cell leaving room centrally for the release of mucus.

It is not clear why there should be such a difference of interpretation between different workers using the same species, but Thomas suggests that the cells described by Millar and others as non-secretory may merely be in a transitional phase after discharging their secretion. This seems unlikely in view of the distinct localization of cell types reported by Millar. According to Thomas (1970a) the mucus, which is produced as granules from dilated cisternae of the Golgi-body accumulates at the apex of the cell without any limiting membrane and is finally discharged by rupture of the apical plasma of the cell. Orsi,

Thomas and Croxall all found that oesophageal mucus is preponderantly an acid mucopolysaccharide, but Croxall also identified carbohydrated protein complexes in smaller quantity.

2. *The stomach*

There is a reasonable amount of agreement among different authors concerning the types of cell found in the stomach of *Ciona*, although slightly different interpretations are placed on their function. The subject has been well reviewed by Thomas (1970b) who has added much detail in the form of electron micrographs and histochemical studies. According to Thomas there are between 30 and 40 longitudinal ridges or folds in the stomach and separated from each other by furrows, all of them lined by a simple epithelium. One of these ridges forms a continuation of the ventral oesophageal groove and contains ciliated mucus secreting cells similar to those found in the oesophagus. These are the only mucus secreting cells found in the stomach. The remaining ridges of the stomach possess three types of cell. (1) At the base of the ridge there are undifferentiated cells which probably give rise to other cell types, notably the glandular cells. (2) Gland cells found on the sides of the ridges have very few cilia and produce secretory granules at their apex which are eventually shed into the stomach lumen. (3) Vacuolar cells located at the apex of the ridges contain a succession of vacuoles near the top of the cell each of which contains fragments of membrane and small vesicles. These vacuoles may be shed separately into the lumen of the stomach or sometimes the whole cell is shed. Millar (1953a) also noted the apocrine secretion of vacuolar material from these cells, but Yonge (1925) and Van Weel (1940) believe they were absorptive cells.

In the stomach of members of the family Pyuridae there is a prominent digestive gland discharging into the lumen of the stomach by conspicuous ducts. Fouque (1959) and Degail and Levi (1964) have described the histology of this gland in *Pyura microcosmus* (Savigny) and *Microcosmus claudicans* (Savigny). Mucus secretory cells are reported to occur at the proximal ends of the digestive diverticula and in the lateral walls of the diverticula there are two types of cell. Secretory cells with globules are the principal cell type; they have a basal nucleus and several large vesicles containing flat membranes and granules of secretion. Mitochondria are evenly spread throughout the cell and at the apex there are numerous small vesicles. Interspersed amongst these cells are basophilic secretory cells which do not always extend to reach the lumen of the diverticulum. According to Degail and Levi these cells have a conspicuous endoplasmic reticulum and large Golgi body. Large vacuoles develop near the Golgi body and migrate to the apex of the cell; they contain a soluble substance as well as fibrillar material which stains as for mucus. Croxall considers that these basophilic cells most resemble the vacuolated cells of *Ciona* and that the vacuoles contain carbohydrate–protein complexes, amino groups and acid phosphatase.

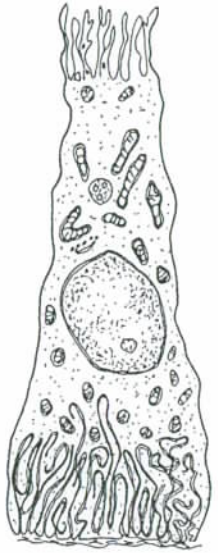


FIG. 11. Schematic drawing of a cell from the terminal region of the digestive gland of a pyurid ascidian showing the infolding of the basal cell membrane. (Redrawn from Degail and Levi, 1964.)

As well as these cells, both Fouque (1959) and Degail and Levi (1964) describe cells at the apex of the digestive diverticulum which are non-secretory. These cells are confined to an apical lobule which projects into neighbouring haemolymphatic spaces and the cells are characterized by having a clear cytoplasm and an infolded basal membrane (Fig. 11). Neighbouring folds of the membrane are so close together that all the cell inclusions including mitochondria are pushed apically. The function of these cells is obscure but the infolding of the basal membrane is similar to that described by Thomas (1970a) for intestinal cells and is characteristic of cells engaged in ionic or osmotic regulation. However, cells engaged in regulatory activities usually have the mitochondria lying between the folds and providing a source of energy.

From these accounts of the gastric epithelium it is possible to identify the following general cell types:

(i) *Mucus secreting cells.* These only occur in conducting areas of the stomach i.e. in the ventral fold of *Ciona* (Thomas, 1970b) and in the ducts of the digestive gland in Pyuridae (Degail and Levi, 1964).

(ii) *Vacuolar secretory cells*, found on the crests of the ridges in *Ciona* (Thomas, 1970b) and in the wall of the diverticulum in Pyuridae (Fouque, 1959; Degail and Levi, 1964). In *Ciona* Thomas found that the vacuoles contain carbohydrate–protein complexes, lipids, free amino groups, tryptophan, non-specific esterase and acid phosphatase activity. Similar secretions have been identified in *Styela* by Orsi (1968) and in *Corella*, *Asterocarpa*, *Cnemidocarpa* and *Styela* by Croxall (1971). Thomas points out that the enzymes, particularly acid phosphatase, are characteristic of cells engaged in intracellular digestion (see de Duve, 1963) and that the fine structure of the vacuole resembles that of a lysosome. Thomas suggests that these cells may produce an unusual secretion even though lysosomal enzymes do not normally have an extracellular role. It is of interest to note, therefore, that these are the cells which Yonge (1925) considered to have an absorptive role in *Ciona* and which Van Weel (1940) reported as taking up particulate iron saccharate. They are probably equivalent to Morton's (1960) Type A cells in *Salpa*.

(iii) *Basophilic cells*, found in the walls of the ridges in *Ciona* and in the walls of the diverticula in Pyuridae. Degail and Levi (1964) believed that these cells secreted mucus, but in *Ciona* Thomas (1970b) reports that they produce a sero-mucus secretion particularly rich in proteins and he notes that their fine structure resembles that of protein secreting cells in other organisms. He concludes that these are enzyme secreting cells. They are probably equivalent to Morton's, (1960) Type B cells described in *Salpa*. Morton considered his Type B cells to be equivalent to those found by Barrington (1936, 1937) in the intestine of the ammocoete and in the digestive diverticulum of Amphioxus (*Branchiostoma*). He further related these to the zymogen cells of the pancreas in higher chordates and therefore concluded that the digestive diverticulum of the tunicate should be regarded as the beginnings of a pancreas and not of a liver.

3. *The mid-intestine*

The histology of the mid-intestine of *Ciona* has been described by a number of authors including Yonge (1925), Van Weel (1940), Millar (1953a), Fouque (1954), Thomas (1970a) and Croxall (1971), but there is a conspicuous lack of precise detail in these accounts. There appear

to be two principal types of cell, absorptive and mucus secreting. Thomas found that the mucus secreting cells are very variable in their morphology and that in general they produce a preponderance of carbohydrate–protein complexes unlike the oesophagus where acid-mucopolysaccharides predominate. Croxall could find no clear distinction between absorptive cells and mucus secreting cells and found that cells intermediate in the degree of ciliation and extent of optical staining frequently occurred. He considered that the " absorptive " cell might represent one phase of activity of the typical mucus secreting cell. Croxall agrees that carbohydrate–protein complexes predominate in the intestinal secretions. Thomas also records that certain mucus cells in the intestine with a holocrine mode of secretion have an infolding of the plasma membrane at the base and sides of the cell. This resembles the infolding of gastric cells noted by Degail and Levi (1964) in Pyuridae, but in the intestinal cells of *Ciona* there are abundant mitochondria between the folds of the membrane suggesting an active regulatory function for these cells. According to Peters (1966) the intestine secretes a peritrophic membrane around the food cord which is rich in chitin.

4. *The rectum*

The rectum consists of low ciliated mucus cells with histochemical reactions similar to oesophageal cells. Some of these cells contain autophagic vacuoles which are voided with the mucus during secretion (Thomas, 1970a).

C. *Digestion*

Digestion in ascidians has been studied by Yonge (1925) in *Ciona*, Berrill (1929) in *Pyura* and *Boltenia* and Van Weel (1940) in *Ciona* and is reviewed by Barrington (1962). Digestive enzymes are secreted into the stomach and digestion is entirely extracellular; unlike many other filter feeding organisms (i.e. bivalves, Amphioxus) there is little evidence for intracellular uptake of solid particles. Van Weel did demonstrate a small uptake of iron saccharate into cells of the stomach epithelium and subsequent passage from these to blood cells, but the extent to which this occurred is insufficient to justify the assumption that it is a normal part of the digestive process.

There is general agreement that ascidians have a powerful amylase, an invertase and a lipase, but only a weak protease. A maltase and a lactase are reported by Berrill for the two Pyurid ascidians, but Yonge denies their existence in *Ciona*. Minor differences of this sort should be further investigated as they may be important in the overall physiological-ecology of ascidians and the manner in which they can utilize the

available resources. These findings, however, are based on a study of a few shallow-water species which feed primarily on finely divided particulate material. A few species of deep-water ascidians such as *Gasterascidia sandersi* Monniot and Monniot (1968) are possibly active predators and it would be of particular interest to know the way in which their digestive physiology may have been adapted and whether proteases are more important than amylases.

The earlier work reported on above was limited by the techniques then available. Recently Koch and Marsh (1972) have examined in detail the hydrolysis of carbohydrates by the digestive gland of *Pyura stolonifera* (Tables V and VI). A number of polysaccharides are hydrolysed, but a cellulase is specifically absent although the soluble derivative of cellulose, carboxymethyl cellulose, is broken down. Yokoe and

TABLE V. THE ACTION OF DIALYSED DIGESTIVE GLAND EXTRACT FROM *Pyura stolonifera* ON VARIOUS POLYSACCHARIDES (From Koch and Marsh, 1972)

Substrate	Enzyme activity (μmoles reducing sugar/12 h/g tissue)
Starch	6·1
Carboxymethylcellulose	3·3
Alginate (guluronic acid-rich)	2·2
Alginate (mannuronic acid-rich)	1·6
Hyaluronic acid	1·1
Chondroitin sulphate	1·6
Pectin	1·8

Yasumasu (1964) failed to find cellulose in four species of ascidian while Elyakova (1972) also found that ascidians can hydrolyse carboxymethyl cellulose. True celluloses are usually confined to micro-organisms and therefore it is to be expected that they would be absent from ascidian tissues. In addition to the enzymes noted above Sova *et al.* (1970) have shown that laminarin (a β-glucan) is hydrolysed by extracts of *Halocynthia* digestive gland and Elyakova (1972) reports that a chitinase is present in species of the same genus.

Alarcon and Cid (1963) report on the presence of an amine oxidase in the digestive diverticulum of *Pyura*, using benzylamine as a substrate, and stated that it was stimulated *in vivo* by high concentrations of chlorhydrate of adrenalin. Aminoxidase is an enzyme system that catalyses the oxidation of many amines, and possible substrates in the

living organism are discussed by Blaschko (1952). It is widely distributed in animal tissues and amongst invertebrates amine oxidase has been found in high concentration in the hepatopancreas of *Octopus* and *Sepia* (Blaschko, 1941; Blaschko and Hawkins, 1952), in echinoderms, but not in annelids and coelenterates (Blaschko *et al.*, 1937).

Berrill (1929) reported that ascidian amylase had an optimum pH for activity near to 7·5 and that the proteases were active between 6·0 and 10·0. Van Weel (1940) found that all the enzymes worked at a pH in excess of 7·0 and that the protease and lipase worked best between 8·8 and 9·6. Koch and Marsh (1972) found that glucuronidase activity in *Pyura* has an optimum pH of 4·5.

TABLE VI. GLYCOSIDASE ACTIVITY OF CRUDE EXTRACT OF THE DIGESTIVE GLAND OF *Pyura stolonifera* (From Koch and Marsh, 1972)

Enzyme	Aglycon	Enzyme activity (μmoles aglycon/h/g tissue)
α-D-Glucosidase	*p*-Nitrophenol	8·6
β-D-Glucosidase	*p*-Nitrophenol	10·4
α-D-Galactosidase	*p*-Nitrophenol	21·3
β-D-Galactosidase	*o*-Nitrophenol	9·6
α-D-Mannosidase	*p*-Nitrophenol	0·61
β-D-Mannosidase	Phenol	14·0
α-D-Glucuronidase	*p*-Nitrophenol	9·3
β-D-Glucuronidase	Phenolphthalein	4·9
β-D-N acetylglucosaminidase	*p*-Nitrophenol	230

Yonge (1935) recorded a pH of 5·4 in the stomach of *Phallusia* and associated this low pH with the iso-electric point of the mucus (pH 5·6) in the food cord. When the pH of the medium is the same as that of the iso-electric point the mucus will be at its least viscous and therefore enable the enzymes to penetrate into the food cord and mix with it. In contrast to this, Yonge found that in the hind gut the pH was 7·1 and thereby caused the mucus to become more viscous and enable the formation of faecal pellets to take place.

Berrill also carried out experiments to show that at different environmental temperatures the time taken for food to pass through the alimentary canal was roughly equal to the time taken for 75% of enzyme activity to be completed and that in consequence the animals are physiologically adapted to make maximum use of their enzymes,

discarding them in faeces when they have passed the peak of their activity.

Almost nothing is known about the detailed process of absorption in the ascidian alimentary canal and subsequent metabolism of food. All authors agree on the existence of absorptive cells throughout the intestine. Schneider (1897), Yonge (1925) and Van Weel (1940) claimed that the vacuolated cells in the stomach are also absorptive and would take up iron and olive oil, but later workers (Millar, 1953a; Thomas, 1970b) believe they release their vacuoles from the apex, although the precise function of these vacuolated cells is still in doubt (cf. p. 32). In this context it is of interest that Davidson *et al.* (1971) report the presence of goblet cells in the gut of *Ciona intestinalis* which give staining reactions similar to the insulin producing " β-cells " of mammalian pancreas. They do not specify the precise location of the cells in the gut, but bio-assays of the stomach and adjacent parts of the intestine yielded 5·7 mU of insulin per gram wet weight of tissue.

The existing picture of digestion in ascidians then suggests that food wrapped in " mucus " enters the stomach where a change of pH to the iso-electric point of the mucus softens it and permits penetration of the enzymes to the food; it is assumed that digestion commences in the stomach and continues throughout the length of the alimentary canal alongside absorption of the products of digestion in cells of the intestine. There are two difficulties in the way of accepting such a simple hypothesis. Recent work on the endostyle suggests that the material in which food is wrapped is not a simple mucus, but is primarily formed of an iodinated protein (see p. 27). Examination of the food cord in living transparent animals suggests that the food cord is not softened in the stomach but remains firm enough for it to fold back and forth without disruption. Actual softening of the food cord appears to take place at the pyloric junction of stomach and intestine. In those species in which there are folds in the wall of the intestine the grooves or furrows lining the inside converge to a ciliated ring at this point, and this coincides with the point at which the duct of the pyloric gland enters the alimentary canal. In Pyuridae with digestive diverticulae of the stomach, the ducts from these enzyme-secreting diverticulae also discharge toward the pyloric end of the stomach. Millar (1953a) described the ciliated ring and concluded that it was used to pull the food cord through from the stomach to the intestine, but an alternative viewpoint is that it is used to mix the food and digestive enzymes at a point where the food cord is temporarily softened. Is the function of the pyloric gland to bring about this change by the secretion of appropriate substances?

D. *The pyloric gland*

The pyloric gland is a system of tubules, terminating in small swellings or ampullae, which ramify over and are closely applied to the alimentary canal lying between it and the connective tissue sheath which surrounds it. The tubules unite to form a single pyloric duct which usually opens by a small aperture into the alimentary canal at the junction of the stomach and intestine. In *Ciona* Millar (1953a) described it opening on the left-hand side of the anterior ciliated ring of the intestine. In certain species of the family Didemnidae the ramifying tubules are absent and there is a large pyloric vesicle in the digestive loop; a single duct enters this from the region of the intestinal wall and another duct goes from the vesicle to the pyloric region of the stomach.

The most detailed account of the morphology and histology of the gland is given by Fouque (1954) who suggests that it may have its origin in a structure like the siphon of the Echinoidea. From this it may have evolved into its present complex form as shown in Fig. 12. In the most highly developed state as in *Styela plicata* (Lesueur) the gland may be divided into three morphological areas. Over the posterior end of the intestine and rectum it forms ramifying tubules and poorly developed ampullae, and a similar type of organization is also to be found around the oesophagus. In the region of the mid-intestine the ampullae are more numerous and conspicuous and form what Fouque refers to as the zone of contiguous ampullae. Throughout the gland the tubules and ampullae are lined by a single layered cuboidal epithelium. There is considerable confusion in the literature as to whether these cells may have cilia projecting into the lumen of the canal. Colton (1910) describes the cells as bearing long flagella, but Azéma (1937) believed they had small bundles of cilia. Millar (1953a) states that in *Ciona* the epithelium bears " a thin clothing of long cilia ". Fouque (1948) describes cilia throughout the tubule in *Diplosoma* but in a later paper (Fouque, 1954) he denied the existence of cilia in the tubules and believed that the presence of cytoplasmic strands in the lumen gave a false impression of the presence of long cilia. There are, however, cilia in the pyloric duct which beat towards the alimentary canal.

Within the pyloric gland and in particular in the region of contiguous ampullae there is a cytonuclear cycle of events which has been described similarly for *Microcosmus sulcatus* (Coquebert) by Azéma (1937) and for *Styela plicata* by Fouque (1954). The similarity of events described for the two species suggests that this is a normal cycle of events. In the course of the cycle the cytoplasm becomes strongly basophile and vacuolated and the nucleus contracts and expels chromatin which

comes to form a ring around the degenerating nucleus. The cytoplasm then starts to break up often forming refringent spherules which fall into the lumen of the tubule and may aggregate to form concretions. The remnant of the nucleus is also expelled and finally all that remains

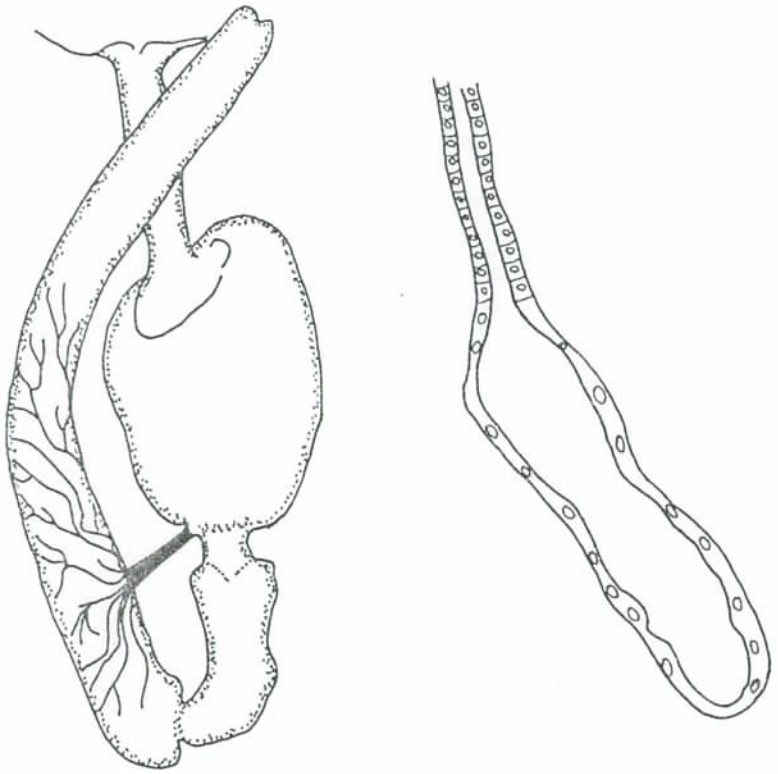


FIG. 12. The pyloric gland. (a) Distribution of the gland on the alimentary canal of *Amaroucium*. (b) Longitudinal section through a terminal ampulla. (Redrawn from Berrill, 1950.)

of the epithelium is a cytoplasmic network. Frequently only the basal membrane remains of the original lining of the tubule. There is thus a drastic form of holocrine secretion into the lumen of the tubule, but these events do not occur simultaneously throughout all the lining epithelium and regeneration can occur from neighbouring intact areas.

This cytonuclear cycle is most pronounced in that portion of the gland adjacent to the mid-gut and Fouque (1954) describes how the epithelium of the intestine is modified in this area so that the cells become flatter and more basophile than elsewhere in the intestine and have characteristics similar to those of the intact cells of the pyloric ampullae. In addition Fouque describes free cells in this area which he has termed " parapyloric cells "; these cells are believed to have their origin usually from the cells of the ampulla, but occasionally, e.g. in *Botryllus*, they may arise from the intestinal epithelium, while in *Diplosoma* they are absent but the pyloric vesicle is stated to be surrounded by " haemolymph " cells. The role of the parapyloric cells is further discussed below.

In the more distal parts of the gland the cytonuclear cycle is less pronounced and the ampullae frequently contain concretions which Fouque believed to be formed through the breakdown of haemolymph cells which have migrated into the ampulla. The concretions are often bi-refringent and may be partly purine in nature. Azéma believed that in Ascidiidae the cytonuclear cycle did not occur and he described hyaline concretions in the ampullae. Concretions have been described in the pyloric gland by several authors including Chandelon (1875), Isert (1903), Millar (1949, 1953a) but are not mentioned by Colton (1910). Isert believed they arose from masses of matter formed by the accumulation of secreted substances, while Millar considered that they may crystallize out from the liquid contents of the tube.

The physiological role of the pyloric gland is still a matter of speculation but most authors have ascribed to it either an excretory or a digestive function or both, but throughout the literature there is insufficient evidence of adequate experimental work to enable any definitive statements to be made. The older literature is reviewed by Azéma (1937), Fouque (1954) and Millar (1953a) and the reader is referred to them for further detail.

The evidence for an excretory role is based partly on the presence of solid concretions in the ampullae and comparisons have been made between the storage excretion of the nephrocytes and renal vesicles of many species and the possible purine nature of the concretions. However, the deposition of purine granules appears to be a widespread phenomenon in ascidians and the quantities deposited (if any) in the pyloric gland would be too small to be of any overall significance in the animal's metabolism. If purines are deposited in the gland this must be a secondary function and possibly even accidental.

The pyloric gland will also pick up vital dyes (Fouque, 1954; Godeaux, 1954) and thorium dioxide (Brown and Davies, 1971) from

the haemolymph and concentrate them in ampullae or tubules. In this respect the gland may be considered to have an excretory function but it does not imply that this is its primary role.

The presence of a holocrine secretion in the gland and the undoubted fact that in many, if not all, species there are cilia in the pyloric duct which beat towards the gastro-intestinal junction, suggests that a part of the function of the gland is to discharge some substance into the alimentary canal. From the point of view of digestive physiology this region of the canal is the most active and critical of all, the region where enzymes and food are being mixed and prior to the main site of absorption. It seems inconceivable that any true excretory organ would discharge at such a place and some other function is more likely. It is a region in which one might expect digestive enzymes to be discharged but all the available evidence at present suggests that the stomach is the sole site of production of digestive enzymes. If the pyloric gland was producing notable quantities of digestive enzymes they should be identifiable in homogenates of the intestine, but they have not been identified there (Yonge, 1925; Berrill, 1929; Van Weel, 1940). Fouque (1954) dismisses the idea that the gland may secrete digestive enzymes on the grounds that the duct is not always open but this may be an abnormal phenomenon. Berrill (1950) on the other hand put forward a novel suggestion that the gland was used to recover enzymes from the intestine. However, in an earlier paper (Berrill, 1929) he suggested that the timing of passage of food through the alimentary canal was adjusted to make maximum use of the enzymes during slow passage through the intestine. If this is true then any system for enzyme recovery should be concentrated in the posterior part of the intestine, but the work of Fouque (1954) suggests that the most active region of the gland is around the mid-intestine. Furthermore, any system of recovery would require a mechanism for re-absorbing large protein molecules.

According to Fouque the intestinal epithelium in the neighbourhood of the ampullae is modified and there appears to be an intimate association between the two. Fouque considered that the ampullae are engaged in a " re-working " process of substances which are absorbed by the intestinal cells, passed on to the cells of the ampulla which further treats them and subsequently passes the re-worked material back to the parapyloric cells which play the role of trophocytes. In view of the holocrine nature of the ampullary epithelium and the fact that vital dyes such as indigocarmine, neutral red, methylene blue and toluidine blue are picked up from the haemolymph and concentrated in the ampullae and tubules (Fouque, 1954; Godeaux, 1954) it seems unlikely that metabolites are treated in this way by the gland. Active

transport appears to be from haemolymph to gland and not vice versa. This still leaves the role of the parapyloric cells unaccounted for but they may be concerned with direct uptake from the intestine rather than from the ampulla.

There are two other hypotheses which should be explored and which do not appear to have been considered by previous authors. The gland may be concerned either with ionic regulation of the blood or with the control of pH in the alimentary canal. Ascidians are known to regulate sulphate but the mechanisms for doing so are unknown. Thomas (1970b) has drawn attention to certain cells in the stomach which he believed might perform this function, but neither these nor the cells of the pyloric gland appear to be a suitable site for such regulation; sulphate ions withdrawn from the blood by these cells would be released in such a position that they would be carried back over the intestinal absorptive surface.

Attention has already been drawn to the fact that the enzyme secreting channels of the stomach converge towards the gastro-intestinal junction where the pyloric duct enters the alimentary canal and where there is a ring of cilia. The food cord is still wrapped in mucus or muco-protein and it would be reasonable to believe that the pyloric secretions are involved in some way in softening the food cord to permit mixing of the enzymes. This might be achieved by control of pH at this particular point.

VII. Respiration

In view of their sessile mode of life and low level of activity it is to be expected that respiratory exchange in ascidians will be small and can be accommodated without the intervention of specialized respiratory carriers in the blood. Ascidian blood contains a complex organometal compound usually including either vanadium or iron but this is concerned with test formation and not with oxygen transport (p. 81).

The only available data on oxygen uptake by ascidians are those of Jørgensen (1952) and Goodbody and Young (unpublished). Jørgensen gives values of 850 μl of oxygen per hour taken up by five specimens of *Ciona intestinalis* and comparable values for *Molgula manhattensis* but these are not related to any specific body weight. Goodbody and Young found that *Ascidia nigra* at 28°C consumed 806 μl O_2/h/g total dry weight or 1630 μl O_2/h/g body weight excluding the test. Because the test is largely inert it is difficult to know how metabolic activities in ascidians should be related to body weight and it is advisable to express them in these two ways (Goodbody, 1957). The level of oxygen

uptake by *A. nigra* is very similar to that of other sessile animals metabolizing at similar temperatures (Table VII).

TABLE VII. OXYGEN UPTAKE (μL/H/G DRY WT) IN *Ascidia* COMPARED WITH THREE BIVALVES AT SIMILAR ENVIRONMENTAL TEMPERATURES (°C)

Animal	*Temp.*	*Oxygen Uptake*	*Author*
Ascidia nigra (excluding test weight)	28	1630	Goodbody and Young (unpublished)
Ascidia nigra (including test weight)	28	806	Goodbody and Young
Crassostrea virginica (Gm.)	24·5	645–1504	Galtsoff and Whipple (1930)
Mytilus edulis L.	25·2	686	Read (1962)
Brachidontes demissus Lamarck	28·2	1059	Read (1962)

The low rate of gaseous exchange in ascidians makes the existence of specialized respiratory organs unnecessary; nevertheless, the primary site of gaseous exchange is by no means certain. It is usually assumed that the large surface area of the branchial sac provides the major area of respiratory exchange but, while this is a reasonable assumption, it is also likely that the inner surface of the mantle wall may provide a secondary site of exchange.

VIII. THE CIRCULATORY SYSTEM

The ascidian circulatory system consists of a tubular heart enclosed in a pericardium and connecting at either end to a system of blood vessels and channels which ramify throughout the body and test. The principal features of this system are illustrated in Fig. 2 (p. 5). At one end of the heart, the hypobranchial end, a large sinus is given off to run under the endostyle and a second sinus crosses through the body wall to supply the test. The subendostylar sinus supplies branches to the body wall and to the branchial sac. From the branchial sac blood collects into a dorsal branchial vessel which also receives blood from the body and itself delivers blood to the alimentary canal. A series of blood spaces and channels circulate the blood around the viscera before collecting again into a visceral vessel which rejoins the heart at the visceral end where a second test sinus also joins. None of the blood spaces is a true blood vessel as an endothelial lining is lacking, but the principal channels, such as the subendostylar sinus have a connective

tissue lining on the lumenal wall. For a fuller description of the ascidian blood system see Millar (1953a).

The heart and circulatory system have no valves and the blood is pumped around the body by a series of peristaltic contractions passing from one end of the heart to another at a frequency of about 25 to 50 beats per minute according to the species and size of the animal concerned. The direction of the peristaltic wave changes at fairly regular intervals so that at one time the wave is *advisceral* (i.e. passes from the hypobranchial end toward the visceral end) while at other times it is *abvisceral* (passing from the visceral end to the hypobranchial). This reversal of heart beat is found universally throughout the Tunicata but is not unique to this group of animals. It is well known in certain groups of insects (Jones, 1965) and has been recorded in several other groups of animals (Azariah, 1965; Beklemishev, 1969; Mislin, 1969).

The ascidian heart poses a number of interesting questions namely:

(1) How is the heart beat controlled? Is it neurogenic (under nervous control) or is it myogenic (under the intrinsic control of its own musculature)?
(2) How is diastolic filling of the heart effected in the absence of valves or extrinsic musculature?
(3) What controls the reversal of peristalsis and what is its functional significance?

The literature on the physiology of the ascidian heart is extensive. Most of the early work has been reviewed by Skramlik (1938), Krijgsman (1956), Krijgsman and Krijgsman (1957) and Millar (1953a).

A. *Structure of the heart*

The tubular heart is enclosed along its whole length within a fluid filled pericardium. In development the heart is formed as a longitudinal invagination of the pericardium the lips of which approximate together and close off a tubular fold. The heart remains attached to the pericardium along the length of the original suture, this dorsal attachment being referred to as the *raphe*. The walls of the pericardium are formed of a flattened basement epithelium in which the membranes of adjacent cells are folded in such a way as to interdigitate closely with one another (Kalk, 1970) (Fig. 13). The interlocking of cells coupled with a thickened basement membrane probably provides rigidity to the wall and enables it to exert pressure upon the fluid. A few strands of smooth muscle surround the pericardial wall (Millar, 1953a) and presumably provide a mechanism for altering tension in the fluid.

The heart itself consists of a single layer of spindle-shaped myoendothelial cells oriented at an angle of about 60° to the long axis of the heart. The size of the cells varies from species to species but in *Ciona intestinalis* they are about 90 μ long, 10 μ high, 2 μ wide at the base or lumenal and 5 μ wide at apex (Millar, 1953a; Kriebel, 1968b; Anderson,

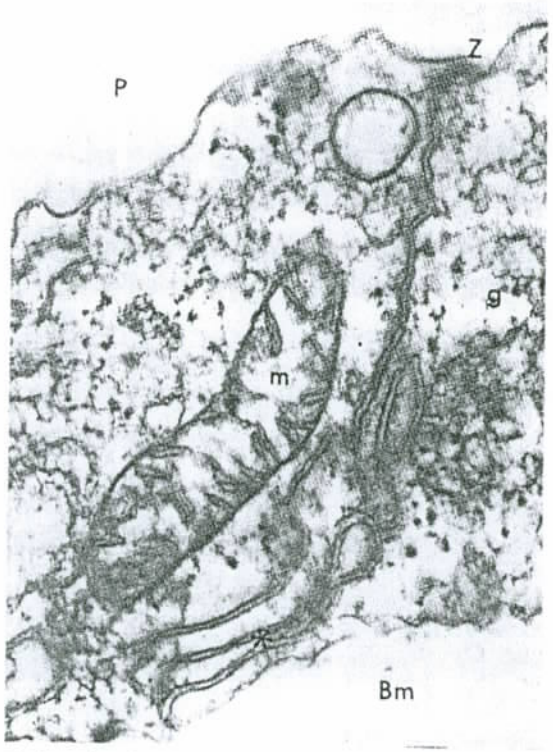


FIG. 13. A vertical section through the interdigitating plasma membranes (*) of adjacent pericardial cells of *Ascidia* where a *zonula occludens* (Z) occurs on the border of the pericardial cavity (P); Bm, basement membrane; g, glycogen granules; m, mitochondrion. × 20 000. (Reproduced, with permission, from Kalk, 1970.)

1968; Kalk, 1970). Phase contrast and electron microscopy shows several distinctive features in the cells (Kawaguti and Ikemoto, 1958; Ichikawa, 1966; Kriebel, 1968b; Kalk, 1970; Lorber and Rayns, 1972). Each cell has a single bundle of myofibrils near the base and adjacent cells are linked by apical nexuses (*zonulae occludentes*) and occasional spot nexuses (*maculae occludentes*) (Fig. 14). According to Kriebel, who

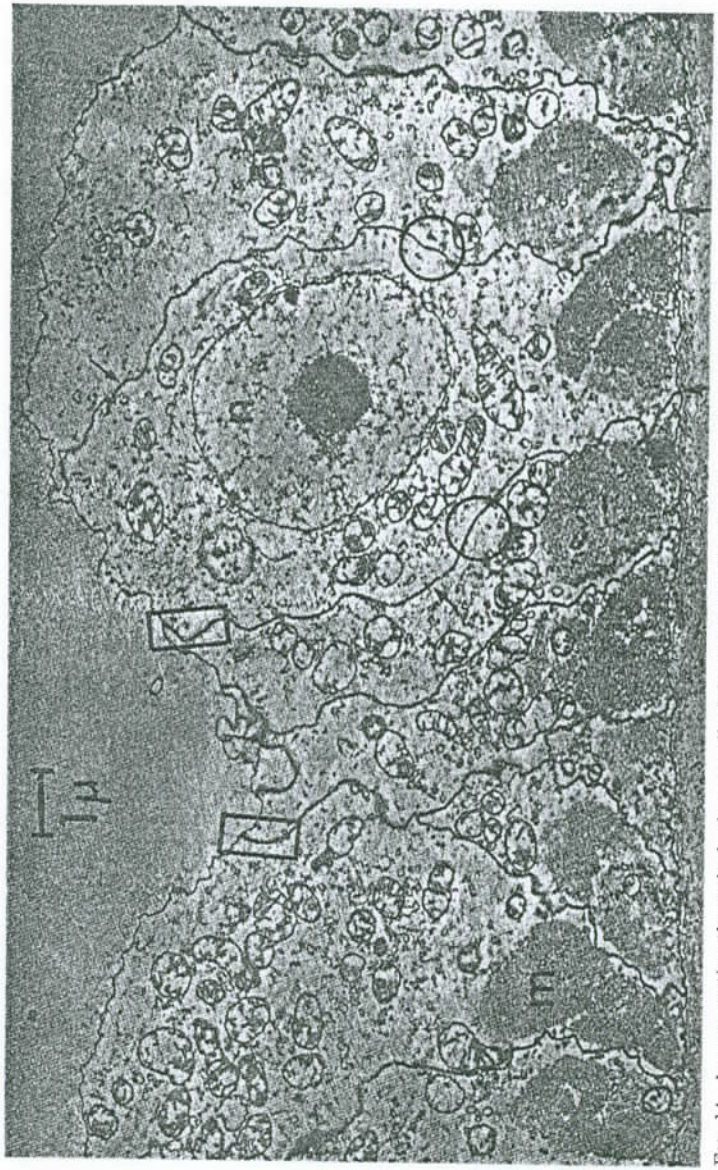


FIG. 14. A cross section through the heart wall of *Ciona intestinalis* showing several adjacent cells. Apical nexuses between cells are enclosed in boxes; spot nexuses are encircled. Note the absence of nexuses near the lumenal surface (arrows); m, myofibril; n, nucleus. × 9100. (Reproduced, with permission, from Kriebel, 1968b.)

has measured the trans-epithelial resistance in *Ciona intestinalis*, the intercellular space between adjacent cells must be about 150 Å and the maximum gap at the apical nexus is only 0·12 Å. Following Waltman (1966) who studied a similar situation in the Ampullae of Lorenzini, Kriebel suggests, in the light of these data, that the apical nexus forms a barrier to the transepithelial passage of ions. (For a general account of the nexus in cells see Farquhar and Palade (1963) and Dewey and Barr (1964).) In addition to the nexus Kalk has described peg and socket cytoplasmic junctions between adjacent myoendothelial cells.

Kawaguti and Ikemoto (1958), Schulze (1964) and Kalk (1970) have all described cellular processes or cytoplasmic lobes projecting from the apical cell surface into the lumen of the heart and according to Kalk each lobe contains 1–3 multivesiculate vacuoles which she considers may be extruded into the heart lumen during contraction. Schulze and Kalk describe a fibrous matrix lining the heart and into which the cytoplasmic lobes protrude. Further detail of the ultrastructure of the myoendothelial cell in *Corella willmeriana* Herdman is given by Oliphant and Cloney (1972).

Apart from the myoendothelial cells described above there is a line of undifferentiated cells, about 2–5 cells wide, running the length of the heart on the side opposite to the raphe and a ring of similar cells connects the heart at either end to the main blood sinuses. Millar (1953a) considers that these undifferentiated cells give rise to new muscle cells and he describes the processes whereby older muscle cells degenerate and are cast off into the pericardial cavity to form the pericardial body.

The nervous innervation of the heart and pericardium is discussed later (p. 92) and it is concluded that the heart itself is devoid of innervation, but there does appear to be a fine innervation of the pericardium which may be a sensory motor reflex controlling pericardial pressure.

B. *Contraction of the heart*

In an active undisturbed ascidian a series of peristaltic contractions will pass from one end of the heart to another pushing the blood forwards as it does so. After a period of time the wave of contraction will slow up and stop and either immediately or after a pause the heart commences beating again but in the opposite direction. The series of beats in any one direction is known as a *pulsation series* and the period during which a pulsation lasts and until reversal occurs is known as a *reversal period* (Kriebel, 1968a). There is no regularity about reversal and successive reversal periods may be of different lengths, but the

frequency of contractions is fairly uniform in any given animal. Several workers have noted, however, that the frequency of advisceral beating is usually slightly greater than for abvisceral beating (Hecht, 1918c; Skramlik, 1926a, 1930; Anderson, 1968; Kriebel, 1968a) (Fig. 15).

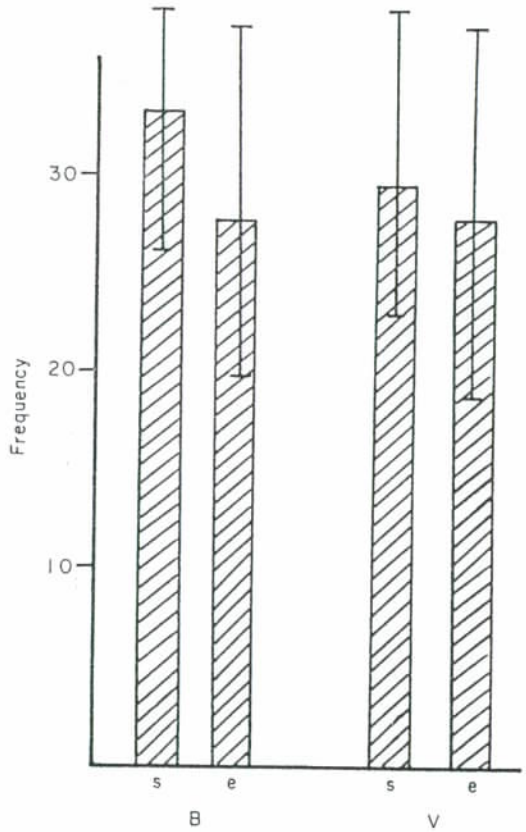


FIG. 15. The average frequency of sixteen isolated hearts at the start and end of branchial (B) and visceral (V) pulsation-series. The range is shown by the centre line in each bar. s, start of series; e, end of series. (Redrawn from Kriebel, 1968a.)

No matter which way the heart beat is travelling the wave of contraction normally starts at one end of the heart and travels to the other but occasionally under abnormal conditions a wave may commence at the centre of the heart and pass simultaneously to either end (Hecht, 1918c; Skramlik, 1926a; Anderson, 1968). Brocas *et al.* (1966)

claimed that there is a relay centre in the middle dividing the heart functionally into two parts. I have also observed an apparent relay in the heart of *Corella willmeriana* but it is most likely, as suggested by Hecht (1918c), that it is due to a shifting of the position of the raphe and this gives a false impression of the existence of such a centre. Reversal of the heart may take place immediately without any pause, there may be a pause of varying duration between opposing waves, or the two opposing waves may momentarily collide at the centre of the heart before the new wave comes to dominate the other.

The peristaltic wave of contraction forces blood out of the heart but in a valveless system it is not easy to see how diastolic filling of the heart is achieved and what prevents backflow through the system. In the absence of elastic fibres or other attachments there must be other mechanisms for refilling the heart. If the pericardium is punctured the heart immediately swells indicating that it must normally be held under pressure by the pericardial fluid. Skramlik (1929) was the first to point out that pressure in the pericardial fluid was necessary for proper functioning of the heart and this was later confirmed by Benazzi (1935). Skramlik determined that the optimum pressure in the pericardium was 2 cm water but Kriebel (1968a) found that the optimum is 5 cm water. Kriebel also pointed out that this pressure is responsible for the production of a contraction valve by forcing the wall of the heart into the venous ostium. As pressure rises in the circulatory system the " venous " pressure comes to exceed the pericardial pressure and forces open the " valve " and refills the heart.

C. *Pacemaker activity*

The rhythmic alteration of heart beat suggests the existence of two active centres or pacemakers one at either end of the heart. Although this was first postulated by Ling and Loeb in 1900 (see Bancroft and Esterly, 1903) and was accepted by Hecht (1918c) experimental proof came much later. The evidence may be summarized as follows:

(a) Heating a resting end induces spontaneous activity, that end becoming dominant. Heating an active end increases the frequency but heating other parts of the heart usually only alters the amplitude of beat (Skramlik, 1926a,b; Krijgsman and Krijgsman, 1957; Kriebel, 1968a).

(b) Hearts ligatured in the middle continue to beat from both ends towards the centre, and similarly if the heart is cut in two (Schultze, 1901; Hunter, 1903; Krijgsman, 1956; Kriebel, 1968a).

(c) By the use of suction electrodes attached to either end of the heart, and by application of suitable stimuli it is possible to drive the heart and artificially alter the beat (Kriebel, 1967; Anderson, 1968).

(d) Mechanical stimulation of a quiescent end will activate spontaneous contractions and stretching of an active end increases the frequency of beat (Anderson, 1968).

Although localized pacemakers can be identified in this way at either end of the heart it is clear that spontaneous contractions can take place at any point in the heart and in isolated strips of muscle (Schultze, 1901; Hunter, 1903; Krijgsman and Krijgsman, 1957; Mislin and Krause, 1964; Anderson, 1968; Kriebel, 1968c). Kriebel and Anderson have both shown that small pieces of myoendothelium lacking any portion of the raphe or undifferentiated line will beat spontaneously and synchronously, and in some cases will exhibit reversal of beat. It seems clear therefore that rather than having defined pacemaking centres at either end, the heart is composed of a series of multiple pacemakers and that any portion of it may have potential for driving the system. Under normal circumstances the threshold of the two end regions must be different from that in the rest of the heart so that they remain in control and the critical question is to determine what causes them to maintain this situation and how do they exercise control over the whole system.

Such control could be exercised through nervous pathways, by neurohumoral transmission or by conduction through the myoendothelium itself. All evidence points to the fact that the heart itself is lacking in any nervous innervation. Hunter (1903) claimed to have found nerve cells at either end of the heart but subsequent workers, using more refined techniques, have been unable to confirm this and Millar (1953a) suggested that Hunter really saw connective tissue elements at the junction of the heart and blood sinuses. Alexandrowicz (1913) and Jones (1971) both reported fine nerve fibres extending onto the heart wall, but the very careful work of Bone and Whitear (1958), Kriebel (1967), Anderson (1968) and Kalk (1970) amongst others has failed to confirm these findings. Furthermore the illustration in Jones (1971) suggests that the fibres in question were in the pericardium and not in the heart.

Hunter (1903) destroyed the ganglion of *Molgula manhattensis* by cauterization and reported that this caused irregularity of beat. Ebara (1971) stimulated the ganglion of *Perophora orientalis* Arnback and found that frequency of beat first declined but later increased again, and that this effect was transmitted to the heart of a second zooid attached by a stolon. He claimed that the effect is potentiated by eserine and concluded that the heart is under neural and neurohumoral control.

Ebara stimulated the ganglion by means of electrodes placed one inside the branchial sac and the other outside and considerable mechanical stress must have occurred. Similarly in Hunter's work mechanical stress occurred and in both cases this is likely to have had very considerable effects on the heart beat. In the case of *Perophora* the effect on the second zooid is a natural part of the co-ordination found between neighbouring zooids (Ebara, 1961).

D. *Cardio-regulatory drugs*

The evidence concerning the effects of cardio-regulatory drugs on the ascidian heart is very conflicting, but this may in part be due to differences in the techniques used (Table VIII). Acetylcholine normally acts to depress cardiac activity in animals. Bacq (1934a, 1935a), Krijgsman and Krijgsman (1959), Scudder *et al.* (1963) and Sugi and Matsunami (1966) found no response to acetylcholine while Waterman (1942, 1943) and Ebara (1953a,b) both found different types of cardiac enhancement. Adrenalin is a cardiac accelerator and Scudder *et al.* and Sugi and Matsunami claim to have found such an effect in ascidians. Sugi and Matsunami also found that serotonin caused an increase of beat frequency when applied to the visceral pacemaker but either reduced the frequency or had no effect when applied to the hypobranchial pacemaker.

Kriebel (1968d) has criticized all earlier work on the grounds that whole hearts or even whole animals were being exposed and that the drugs could not penetrate through the myoendothelial wall because of the tight junctions between cells. Kriebel tested the effects of adrenalin and acetylcholine on hearts which had been slit open to allow access of the drugs and found that both drugs caused a temporary cessation of heart beat when used in pharmacological concentration. The effect of acetylcholine was inhibited by atropine. Kriebel interprets this to mean that cardio-acceleratory nerves are absent but that " the tunicate heart may receive cardio-inhibitory innervation and this innervation is cholinergic ". Although the existence of cholinergic somatic nerves in ascidians has been claimed by Florey (1963, 1967) the evidence does not seem to warrant Kriebel's conclusion. No nervous innervation of the heart has been demonstrated and all evidence suggests that it is under intrinsic and not extrinsic control (p. 54). Furthermore both Bacq (1935b) and Florey (1951) have reported very low concentrations of cholinesterase in ascidians and Durante (1956) states that it disappears at metamorphosis; under these circumstances it would seem unlikely that acetylcholine could be used as an important cardio-regulator. The existence of a pharmacological effect by a drug on the

TABLE VIII. THE EFFECTS OF ACETYLCHOLINE, ADRENALIN AND ESERINE ON ASCIDIAN HEARTS

Species	*Acetylcholin*	*Adrenalin*	*Eserine*	*Technique*	*Author*
Ciona intestinalis	No response	No response	No response	Whole heart	Bacq (1934a, 1935a)
C. intestinalis	Mild acceleration at 10^{-6} g/ml. In high conc. depresses activity	—	No effect and does not potentiate effect of Ach.	Intact ligatured hearts	Krijgsman and Krijgsman (1959)
C. intestinalis	No effect	Increased frequency (10^{-4} g/ml)	—	Whole hearts; recording from pericardium	Scudder *et al.* (1963)
C. intestinalis	Stops heart temporarily at conc. 10^{-8} g/ml. Blocked by atropine	Stops heart temporarily at 10^{-5} g/ml. Increased frequency at 10^{-4} g/ml	—	Slit hearts	Kriebel (1968d)
C. intestinalis	—	Decreased frequency	—	Whole heart	Keefner and Akers (1971)
Perophora viridis	Enhances abvisceral dominance	Enhances abvisceral dominance	Enhances abvisceral dominance	Whole heart	Waterman (1942, 1943)
Polycitor mutabilis Oka	Accelerates heart beat	—	Potentiates effect of acetylcholine	Whole animal	Ebara (1953a)

heart does not have to imply that that drug is part of the normal physiological equipment of the heart.

In summary then, the existing evidence is totally against the presence of a neurogenic control mechanism for the ascidian heart but the evidence in favour of hormonal control is inadequate and contradictory. It is possible that some form of intrinsic regulation may be exercised by secretions from the cytoplasmic lobes of the myoendothelium (Kalk, 1970).

E. *Conduction*

In the absence of neurogenic or neurohumoral control it seems probable that heart beat is under myogenic control. Myogenic control requires that there should be an adequate means of electrical conductance between cells and that they must be capable of co-ordination.

The resting potential of ascidian myoendothelial cells has been given as from −48 to −75 mV (Miller and McCann, 1963; Kriebel, 1967; Anderson, 1968) and there is very little overshoot during the action potential. The electrical characteristics of these cells remain the same irrespective of the direction in which the wave of mechanical contraction is moving (Ebara, 1957; Kriebel, 1967) and this suggests that there is only a single physiological cell type which can drive the heart mechanically in either direction. Nevertheless, Anderson (1968) has shown that all cells in the heart are capable of a certain amount of independent activity; the action potential in a cell may for instance show small pre- and post-potentials, small potential changes may occur independent of any mechanical or electrical stimulation or a cell may show no potential change at all during a contraction of the heart.

Mechanical conduction in heart tissue will take place through strips which are completely isolated from the raphe and the undifferentiated line and Kriebel (1967) has shown that electrical conduction can pass through isolated strips of heart tissue and will cross the undifferentiated line but not the raphe. Furthermore (Kriebel, 1968b) the heart can continue to function and pump blood when the raphe has been crushed or sectioned. The raphe appears to be no more than a suspensory structure with little physiological significance.

Kriebel (1967) has shown that under normal circumstances the wave front of activity passes along the heart perpendicular to the long axis of the heart cells. The average speed of conduction along the heart is 13 mm/sec and, since each cell is only 4 μ across, the wave front must cross the cell in 0·3 msec. Kriebel brings forward evidence to show that this is too fast to be mediated by non-electrical means and he concludes that conduction in the heart must be by local current flow

from cell to cell. In a later paper Kriebel (1968b) has shown that the cell membranes in the heart have a resistivity of 210 ohms/cm² but the nexuses (see p. 46) have a cell resistivity of only 0·2 ohm/cm². This suggests that the nexuses, as well as forming a transepithelial ionic barrier, are also low resistance pathways for electrical conduction. In order to show that such conduction is possible Kriebel replaced the intercellular fluid by a sucrose barrier and demonstrated that an electrical impulse could still pass along the heart. The only pathway left to it under these circumstances is through the nexuses.

On the basis of all this evidence it is reasonable to conclude that propagation of the wave of contraction in the ascidian heart is by electrical conduction from cell to cell.

Under normal conditions the heart is controlled by the pacemakers located at either end of the heart, but in *Ciona intestinalis* during longitudinal contraction a third pacemaker may become active and drive the blood towards either end. This is the C centre of Skramlik (1938). The C centre can be activated experimentally by applying pressure to this region of the heart. The cells of the remainder of the heart all have pacemaker capabilities but do not at any time take control of the heart. The question arises then as to how the two end pacemakers maintain their dominance over the rest of the heart and thereby control heart beat. Most of our understanding of this is due to the work of Kriebel (1968c, 1970) and Anderson (1968).

In order to generate a wave of contraction in a passive heart it is necessary to apply several stimuli to the end of the heart and in order to manipulate heart beat reversal it is necessary to apply a train of impulses of a higher frequency at the other end. The heart cells have a " low safety margin for conduction " (Kriebel) and a low degree of excitability which permits them to engage in independent activity. For co-ordinated activity and the generation of a contraction wave a number of cells must depolarize simultaneously; the input of a train of impulses must in fact synchronize the beat of a large number of cells and thereby produce a contraction. According to Kriebel conduction velocity is faster in the middle of the heart than at either end (Fig. 16) and he attributes this to differences in the level of excitability of cells; those near the ends being less excitable than those in the centre. While this does make it more difficult for an impulse to spread from the ends it also means that only well co-ordinated contractions involving a number of cells can make their way out from these terminal pacemakers. Sugi *et al.* (1965) maintain that the threshold for provoking contraction is highest in the centre of the heart in *Ciona*. This would imply that the cells in the centre are less excitable than at the ends. Occasionally

extrasystoles or interpolated contractions may arise in the vicinity of a terminal pacemaker (Quincke and Stein, 1932; Mislin, 1964; Kriebel, 1967; Anderson, 1968) and it is possible to create these artificially by stimulating an end pacemaker in between ordinary contractions. The existence of such extra systoles suggests that there may be more than

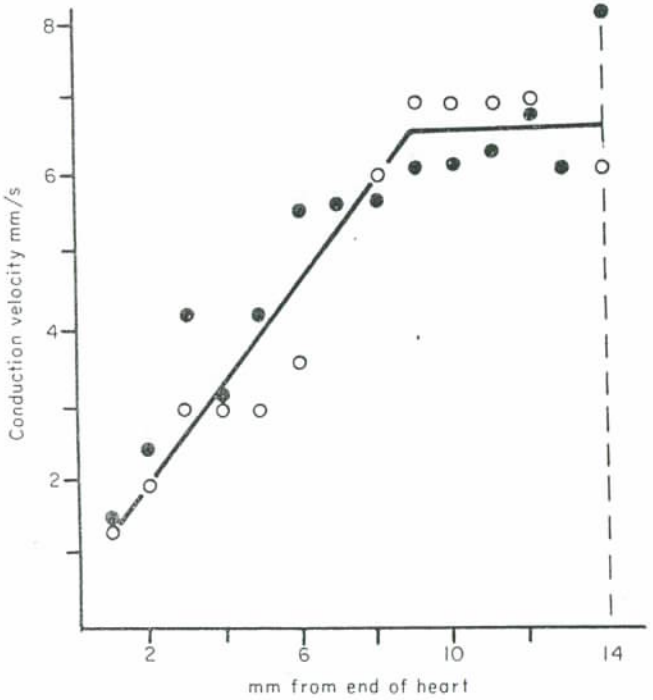


FIG. 16. The speed of conduction in 1 mm long segments of the heart of *Ciona intestinalis*. Open circles represent velocities in visceral arm of heart, solid circles velocities in branchial arm. The dashed line at 14 mm represents the position of the bend of the heart. (Redrawn from Kriebel, 1970.)

one centre of activity at the end of a heart and that it is possible for these to beat out of phase and possibly at different frequencies. Kriebel has in fact demonstrated that at least two centres occur at each end, one on either side of the raphe. Arhythmic contractions of the sort described can be obliterated by " driving " the heart with appropriate electrical stimuli or by increasing the cardiac pressure. The latter is equivalent to stretching the myoendothelium; Anderson reports increased frequency of heart beat when the heart is stretched, while Kriebel (1970) reports on increases in conduction velocity.

While the evidence is still incomplete it does seem likely that the dominance exerted on the heart by the two terminal pacemakers is linked to differences in excitability and activity thresholds between cells of the pacemaker area and those of the rest of the heart. It is also apparent that a stretch stimulus may alter the frequency of a pacemaker and the speed of conduction from it.

F. *Heart beat reversal*

In the absence of nervous innervation of the end pacemaker the control of heart beat reversal must be intrinsic to the heart itself. A number of theories have been put forward to explain this phenomenon, the most important of which are based on the following:

(a) Changing partial pressure of carbon dioxide in the heart (Brocas *et al.*, 1966).

(b) The development of extrasystoles or interpolated contractions (Mislin, 1964, 1965; Mislin and Krause, 1964).

(c) Back pressure in the arterial system (Lahille, 1890; Haywood and Moon, 1950; Kriebel, 1968a).

(d) Pacemaker fatigue (Krijgsman, 1956; Krijgsman and Krijgsman, 1957).

(e) Changes in the frequency level of pacemakers (Anderson, 1968).

Brocas *et al.* (1966) considered that the hypobranchial pacemaker is dominant to the visceral and that the frequency of contraction in the visceral pacemaker varies with the partial pressure of carbon dioxide. In high levels of carbon dioxide the visceral pacemaker increases in frequency and at the same time the hypobranchial pacemaker is simultaneously depressed by an undefined relay mechanism and not by the influence of carbon dioxide. When the carbon dioxide level drops the visceral pacemaker declines in frequency. While such an effect could be due to changing pH and not directly to carbon dioxide there is no clear evidence to show how such metabolic changes might be taking place in the heart so as to vary their influence on the pacemaker. Nevertheless, in the light of Anderson's (1968) findings that there is a rhythmic variation in the frequency of the visceral pacemaker (see below), this work of Brocas *et al.* merits careful consideration.*

* Anderson (1968) appears to have misinterpreted the conclusions of Brocas *et al.* She states (p. 383): " Their results indicated that the activity of the hypobranchial end of the heart is slowed by increasing the p CO_2 while the visceral end is not greatly affected." Brocas *et al.* (1966, p. 92) state: " C'est donc le niveau d'activité du centre viscéral qui détermine le sens de la circulation, ce niveau d'activité étant fonction de la pression partielle du gaz carbonique. "

Mislin (1964, 1965) and Mislin and Krause (1964) reported the existence of extrasystoles or interpolated contractions occurring at the moment of reversal and considered that these may have disturbed pacemaker frequency so as to permit reversal. This has not been confirmed by other workers and probably the extrasystoles recorded were the result of " collisions " during the moment of reversal when one pacemaker is gaining dominance over another (Anderson, 1968).

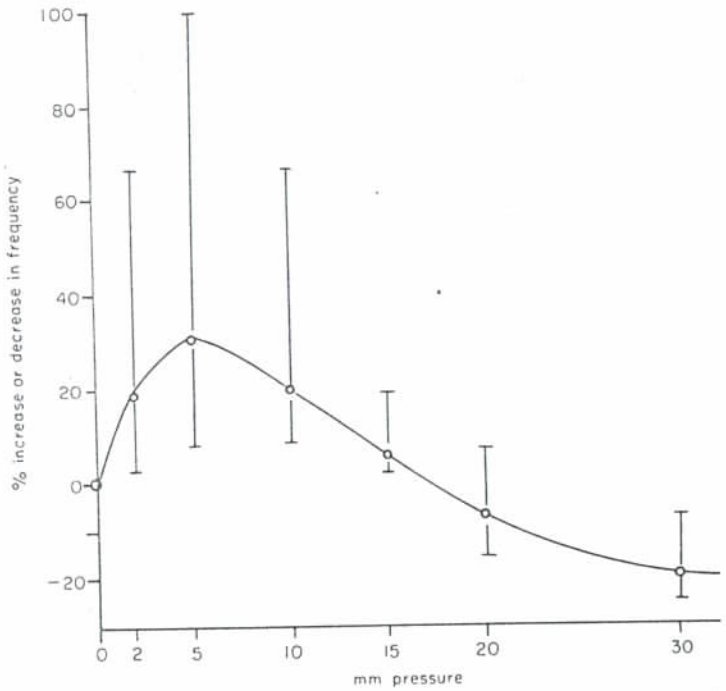


FIG. 17. The effect of varying intra-cardiac pressure on pacemaker frequency in the heart of *Ciona intestinalis*. (Redrawn from Kriebel, 1968a.)

The back pressure theory suggests that pressure builds up in the peripheral circulation as the heart continues to beat in one direction and that increasing effort is required by the heart muscle to overcome this resistance. Eventually the pressure is relieved by reversal. Haywood and Moon (1950) examined this theory by the use of mathematical models and concluded that the evidence was in favour of back pressure causing cardiac arrest, but they did not attempt to account for the commencement of beat in the opposite direction. Their work has been

criticized by Krijgsman on the grounds of over-simplification and that " they adjusted their (arbitrary) constants to fit the observations . . . ".

Kriebel (1968a) has recently revived interest in the back pressure theory and puts forward several pieces of evidence in its favour:

(a) During a reversal pause blood may be seen to flow back into the heart indicating a pressure build-up in the arterial end.

(b) Cinematographic analysis of beating hearts shows that during the first few beats after reversal there is a fall-off in the stroke volume of blood suggesting a fall in venous pressure.

(c) A lowering of intra-cardiac pressure by removal of blood causes a change in the pulsation period (interval between reversals).

(d) Raising the intra-cardiac pressure by occluding the artery causes a reversal.

(e) In experiments with cannulated hearts it can be shown that pacemakers are very sensitive to changes in pressure (Fig. 17) and pacemaker frequency may be altered by adjusting the blood volume or intra-cardiac pressure.

Critics of the back pressure theory (see Millar, 1952, 1953b; Krijgsman, 1956; Krijgsman and Krijgsman, 1957; Anderson, 1968) point out that isolated hearts opened to ensure equal pressure inside and outside will still exhibit reversals. Furthermore, if hearts are ligatured in the middle *in situ* the two end pacemakers will beat toward the centre of the heart for several hours and in spite of the pressure build-up do not exhibit reversals.

Although back pressure cannot be entirely responsible for heart beat reversal it may be a contributory factor by inducing mechanical stimulation of changing frequency in pacemakers (Anderson, 1968).

Pacemaker fatigue has been suggested as the cause of reversal by Krijgsman (1956) and Krijgsman and Krijgsman (1957). Their conclusions are based on the fact that isolated hearts continue to reverse and that in half-heart preparations there are periods of activity alternating with periods of rest. Since there is no opposing pacemaker in a half heart it is assumed that the periods of rest are the result of fatigue of the active pacemaker.

In natural conditions such periods of rest following fatigue would be very brief indeed as during dominance of any one pacemaker the other end of the heart is being driven and continues to contract. Krijgsman and Krijgsman concluded that pacemaker activity and contractility are separate functions and that fatigue is not in the contractile element but in the active pacemaker component. They suggested therefore that the pacemakers respond to some secretion or

metabolite, behaving as sensory receptors. It is then postulated that in an active pacemaker the threshold of response to this " metabolite " slowly increases until the pacemaker no longer responds to it and becomes inactive. At this point the threshold of response in the other pacemaker has fallen and it can now respond to the stimulus. Pacemaker fatigue may then be explained, not in terms of muscle fatigue, but in terms of increasing resistance to sensory stimulation.

If we accept the postulate that all heart cells have pacemaker properties (see p. 51) we can only accept the hypothesis of pacemaker fatigue if it can be shown that the terminal pacemakers respond differently to other cells. That this is probably the case is shown by the differences in electrical activity already demonstrated.

Positive identification of a " metabolite " which might control pacemaker activity awaits confirmation but it is significant that in *Ascidia* Kalk (1970) has demonstrated the existence of multivacuolar vesicles in cytoplasmic lobes projecting into the heart lumen (see p. 48) and Kalk believed that these vesicles are discharged into the blood as it passes through the heart. She calculated that there are about 100 of these vesicles per cell and suggested that during contraction the active end of the heart will be slowly depleted of the substance secreted by the vacuoles while the other end of the heart will be subjected to a continued flow of the material secreted by the cells preceding it in the heart. She further suggested that a concentration gradient will develop along the length of the heart with the highest concentration at the opposite end to the active pacemaker. This ensures that this end is the first part of the heart to be subjected to a high enough internal concentration of the substance to start " firing " before the rest of the heart. Kalk states that " In this way pacemaking will be taken over by alternate ends of the heart after a period of quiescence. " She suggested that acetylcholine may be the substance involved but in view of evidence presented earlier on the effect of acetylcholine on the ascidian heart (p. 52) this is unlikely.

Anderson (1968) has suggested that fatigue as such need not be involved to account for heart beat reversal but that it is due to changes in the levels of frequency at either end of the heart. As long as one portion of the heart has a higher frequency than the rest it will remain dominant and she has shown (Fig. 18) that while frequency remains fairly constant at the hypobranchial end it fluctuates at the visceral end between periods of high and low frequency. She has also shown that the electrical characteristics of the visceral end are different and that the threshold voltage for one-to-one driving of the heart at different frequencies shows a linear response in the case of the hypobranchial

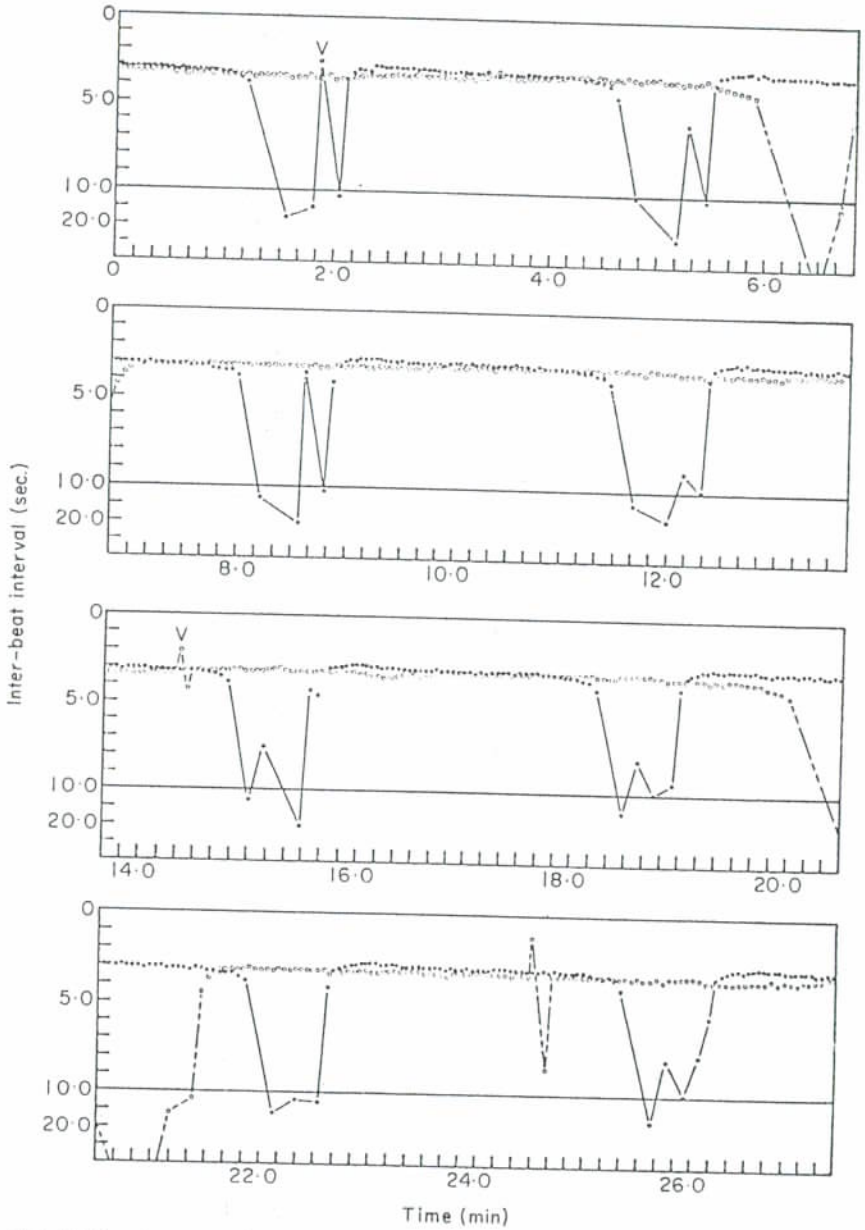


FIG. 18. Spontaneous activities recorded simultaneously from the ends of a heart of *Ciona* ligatured in the middle. Interbeat intervals (ordinate) were plotted against time (abscissa). The visceral end (closed circles) was characterized by regularly varying levels of high and low frequency; in this preparation the hypobranchial end (open circles) varied more or less regularly between levels of high and low frequency but with longer periods of high frequency than those of the visceral end. (Reproduced, with permission, from Anderson, 1968.)

pacemaker and a non-linear response in the visceral one. Anderson concluded that heart-beat reversal can be entirely explained by the intrinsic properties of the heart cells all of which have pacemaking properties. She considered that central areas of the heart do not normally become dominant because their electrical activity can be dissipated in two directions along the heart, while at the ends of the heart all of the electrical activity is concentrated in one direction and promotes dominance.

It is clear from the foregoing paragraphs that there is still insufficient evidence available to enable us to draw any firm conclusions concerning the mechanisms which control the reversal of heart beat in ascidians. It is possible that a full explanation of the phenomenon requires a synthesis of the various mechanisms involved. In view of the behaviour of isolated hearts and portions of hearts it is inconceivable that back pressure alone can explain reversal. At the same time the work of Kriebel leaves us in no doubt that changes in pressure can strongly influence the behaviour of the heart and that it functions best under a pressure of 5 cm water. However, the fact that cardiac behaviour is influenced by pressure does not necessarily mean that pressure changes cause reversal. The discovery by Anderson (1968) that there is an inherent rhythm of high and low frequencies in the visceral pacemaker but not in the hypobranchial suggests that it is the varying responses of this end of the heart which controls cardiac reversal. Brocas *et al.* (1966) suggest that carbon dioxide tension or pH change may affect the activity of this end of the heart. Alternatively, the visceral rhythm may be controlled by the cardiac secretions noted by Kalk and such control might be exercised by a varying threshold of response as suggested by Krijgsman (1956). One might therefore visualize the heart being controlled in this way by stimulation of the visceral centre but being modified by changes in pressure or mechanical stress.

G. *Conclusion*

The ascidian heart is a simple tube constructed from a single layer of myoendothelial cells and completely enclosed by a tough pericardium which maintains a pressure of between 2 and 5 cm water around the heart. Blood is forced through the heart by peristaltic waves of contraction passing from end to end of the heart and in the absence of structural valves a functional valve forms at the venous end due to pressure from the pericardial fluid. It is possible that the pericardial body which floats freely in the pericardial fluid may also play some role in forming functional valves. At periodic intervals, usually of two to three minutes duration, the heart reverses the direction of contraction.

There is no innervation of the heart and conduction and co-ordination are entirely myogenic. All cells of the heart have potential for acting as pacemakers and small clusters of cells can behave as independent units with a single rhythm. A similar type of activity is found in isolated heart cells and groups of cells from the early chick embryo (Hecht, 1965) and it may be a general property of unspecialized heart cells. In the active ascidian heart co-ordination is achieved through two pacemaker centres, one at either end (terminal pacemakers), and conduction through the heart is by means of apical junctions between the cells. The terminal pacemakers probably maintain their dominance by having slightly different thresholds for activity than other heart cells. Myogenic hearts are known from other animals and in the chick embryo the heart is totally myogenic in the early stages of development before neural connections are made (Hecht, 1965). In the hagfish (*Myxine*) the systemic heart is myogenic and never has a nervous innervation, and heart cells having the characteristics of pacemakers are found throughout all chambers of the heart (Jensen, 1964). In molluscs the heart is essentially myogenic but is influenced extrinsically by accelerator and inhibitor nerves (Hill and Welsh, 1966). Krijgsman and Divaris (1955) state that the molluscan heart is of a diffuse myogenic type and, like the ascidian, isolated hearts and fragments of heart will continue to contract endogenously.

The physiological mechanisms which control reversal of heart beat are still not fully understood but it seems likely that the visceral terminal pacemaker is subject to frequency variations by secretions from the myoendothelial cells themselves. The direction of beat may then depend on whether the visceral pacemaker is in a phase of low frequency or high frequency. The precise moment of reversal may, however, be modified by mechanical stimuli resulting from back pressure in the arterial system. A similar mechanism for the maintenance of contraction, but not reversal, in the molluscan heart has been suggested by Krijgsman and Divaris (1955).

Recent advances in our knowledge of the ascidian heart enable us to have a much clearer understanding than before of the physiological mechanisms controlling the heart's activity. However, we still do not know what is the functional necessity for reversing the circulation of blood and this problem does not appear to have been examined at all. The phenomenon of reversal is found without exception in all members of the Tunicata—the pelagic forms as well as the sessile ascidians—and it therefore seems likely that it is of functional importance and not just a physiological accident. Its general occurrence in the group suggests that it is associated with some special feature of the tunicate circulation.

The main circulation through the body does not exhibit any unusual features and there is a through circulation in which the blood completes a single circuit in about one minute in *Ciona intestinalis* (Skramlik, 1929). The unusual feature of the system is the additional circulation provided for the test and it is probably to this circulation that we should look for a solution to the problem of heart reversal. In many ascidians the circulation in the test is of great importance as growth and maintenance of the structure is carried out by blood cells. The nature of the circulation varies from genus to genus and in *Ciona* is restricted to the posterior peduncle. In many other genera (e.g. *Ascidia* and *Corella*) there are several main channels running through the test and arising from these are peripheral channels which run toward the surface where they end in ampullary swellings. The main channels are divided by a mesenchymatous septum and the two halves connect to opposite ends of the heart, but many of the peripheral vessels have no septum and "two way" circulation in them is impossible. During normal activity of the heart the blood in such peripheral vessels oscillates to and fro with each pulsation of the heart but at the moment of heart beat reversal when blood pressure momentarily drops blood tends to drain out of these vessels into the main channels and thus effect a renewal of blood. This suggests that some form of elastic recoil must take place in the ampullae and peripheral vessels to help force blood into the main circulation. These observations only suggest the sort of functional problem which may be associated with heart reversal and considerable biophysical research needs to be done to determine the forces involved and whether any connection between the two exists.

IX. Ascidian Blood

Ascidian blood is characterized by three unusual features.

(a) The plasma, while isotonic with sea water, has low concentrations of sulphate and of carbon dioxide.

(b) There are at least eight different types of blood cell.

(c) Certain blood cells contain organo-metal compounds incorporating either vanadium or iron, and these cells frequently, if not always, contain high concentrations of sulphuric acid.

Earlier reviews on the blood are to be found in George (1939), Webb (1939, 1956), Pérès (1943) and Eliassen (1954).

A. *The plasma*

Henze (1911, 1912) was the first worker to analyse the plasma of an ascidian (*Phallusia mammillata*) and his findings have been confirmed by

Webb (1939) and Robertson (1954) for *P. mammillata*, and Endean (1955a) for *Pyura stolonifera*. Robertson has also made determinations on a pelagic tunicate, *Salpa maxima* Forskål. Other and conflicting determinations have been made by Bialaszewicz (1933) for *Phallusia mammillata* and *Ciona intestinalis* and by Kobayashi (1935) for *Chelyosoma siboja* Oka. All of these results are summarized in Table IX. Although there are considerable differences in the ratios found by various authors, especially those of Bialaszewicz, there is a clear indication that the plasma is normally near to neutral in pH, is isosmotic with sea water and contains a very low concentration of sulphate ions. Ionic balance is achieved by elevation of chloride concentration. The blood also has a low carbon dioxide capacity; Robertson (1954) provided data to show that in *Phallusia mammillata* a normal gradient of carbon dioxide exists between the blood and sea water but that the alkali reserve of the blood is less than in the surrounding water.

The significance of the low sulphate concentration remains uncertain. Robertson (1954) has pointed out that such concentrations could be maintained against inward diffusion from the sea if the sulphate was removed from the plasma by blood corpuscles which are then eliminated from the body. The maintenance of high concentrations of sulphuric acid by certain blood cells suggests a mechanism for withdrawal from the plasma. The ultimate fate of the cells is concerned with the deposition of test substance and during this process cytolysis occurs and the sulphuric acid must be lost to the surrounding medium. It is tempting therefore to suppose that a dynamic situation might exist in which sulphate ions are removed from the plasma by blood cells and released into the test. If this was correct we should expect to find large quantities of sulphur or sulphates in the test but, in *Halocynthia aurantium*, Smith and Dehnel (1970) find negligible quantities of sulphur. In many ascidians, but not in *Halocynthia*, the test contains large vesicular cells, or bladder cells, with strong acid properties (Webb, 1939; Endean, 1961) and it is conceivable that these accumulate the excess sulphuric acid eventually discharging it to the surface of the test.

There is thus a potential pathway for the elimination of sulphate from the body through the test but according to Bielig *et al.* (1961b) the uptake of ionic sulphate from the plasma is very slow. If sulphate ions are not being actively removed by morula cells and discharged through the test there must be some other mechanism for maintaining a low plasma concentration. One such mechanism might be through a Donnan equilibrium, but Robertson (1954) has shown that the concentration of plasma proteins is low (about 0·3 mg/ml) and Bielig and

TABLE IX. THE CONCENTRATION OF VARIOUS IONS IN THE PLASMA OF TUNICATES EXPRESSED AS A PERCENTAGE OF THEIR CONCENTRATION IN SEA WATER

	Na	K	Ca	Mg	Cl	SO_4	pH	Author
Phallusia mammillata	—	109	99	99	104	51	Neutral	Henze, 1912
P. mammillata	—	—	—	—	102·9	61·6	5 to 7	Webb, 1939
P. mammillata	99·4	100·2	93·3	98·8	103·6	52·5	6·6	Robertson, 1954
P. mammillata	—	114·58	111·7	96·6	109·9	99	—	Bialaszewicz, 1933
Ciona intestinalis	—	104·2	101·9	87·3	105·7	73	—	Bialaszewicz, 1933
Pyura stolonifera	96·3	97·5	102	101·5	102·6	52	6·7	Endean, 1955a
Chelyosoma siboja[a]	97	128	98	83	99	77	7·2	Kobayashi, 1938
Halocynthia aurantium	—	—	—	—	—	—	7·2	Smith, 1970a
Salpa maxima	100·3	112·9	95·9	94·9	102·4	64·9	7·5	Robertson, 1954

[a] The original data for *Chelyosoma siboja* given by Kobayashi (1935) must have included material from ruptured blood cells and gave a sulphate value of 175%. The data included here are from fluid termed perivisceral by Kobayashi (1938) but which was probably from the pericardium. For further discussion see Robertson (1954).

his co-workers (1961b,c) consider that this is insufficient to permit such a mechanism; furthermore, labelled sulphate absorbed by *Ciona* is retained in a completely dialysable form and cannot be protein bound. Using labelled sodium sulphate, Bielig *et al.* (1961b,c) found that after 48 hours immersion the ratio of activity in sea water to plasma was 100 to 52; uptake of sulphate is temperature dependent and has a Q_{10} of 1·8. The rate of uptake is enhanced by the addition of vanadate and there is a shift in the ratio of sulphate in sea water and plasma resulting in a higher plasma concentration. Ascidians are totally lacking in any form of conventional excretory organ and in the light of the above it seems likely that sulphate regulation is maintained by active transport across the branchial epithelium (Bielig *et al.*, 1961b). It is of interest that the situation is paralleled in the mesogloea of the scyphomedusan, *Aurelia aurita* L., where similar large reductions of sulphate have been recorded in the absence of any excretory organ (Robertson, 1949).

B. *Blood cells*

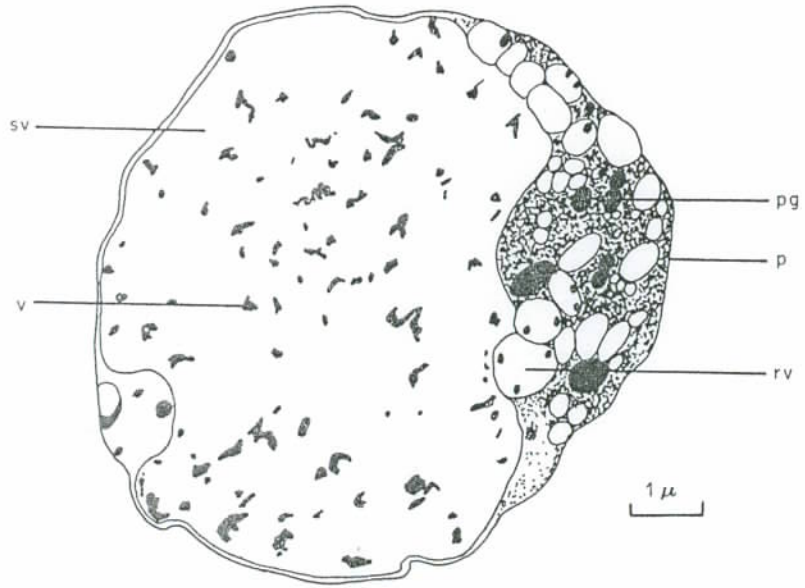
All ascidian blood cells arise from haemoblasts of mesenchymatous origin located in the vicinity of connective tissue particularly around the alimentary canal and branchial sac (Cowden, 1968; Millar, 1953a; Pérès, 1943, 1945). The haemoblasts are able to give rise to either connective tissue cells or lymphocytes from which all other blood cell types are derived.

Before attempting to discuss the functions of the blood it is necessary to clarify the nature of the cells of which it is composed. This is made difficult by confusion in description and terminology presented by various authors. The first comprehensive description of the cellular elements is probably that of Fulton (1920), but George's (1939) review provides a more accurate account from which to base later studies. Since the publication of George's review, descriptions of ascidian blood have been published by a number of workers including Pérès (1943), Endean (1955a, 1960), Millar (1953a), Sabbadin (1953, 1955), Freeman (1964), Overton (1966), Vallee (1967a), Smith (1970a). The terminology used by these authors is not always the same but from a study of their papers and of the blood itself it is possible to identify eight types of cell as described below.

(*a*) *Lymphocyte.* This is an undifferentiated cell about 5 μ in diameter and resembling the mammalian lymphocyte. It has a large nucleus but no nucleolus and apparently gives rise to all other blood cell types. The lymphocyte also plays an important role in the development of young buds during asexual reproduction of such species as *Perophora*

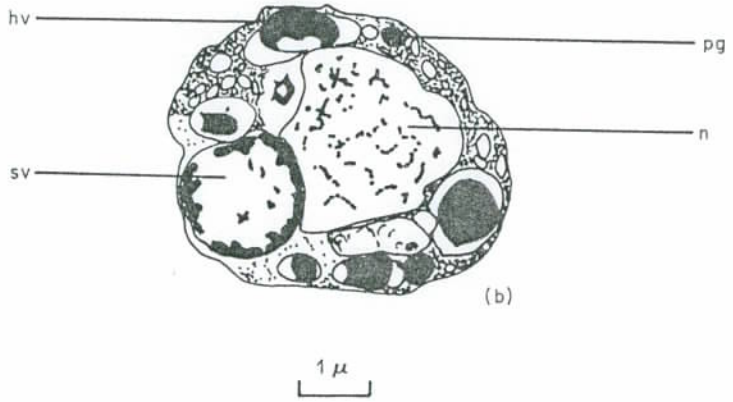
viridis (Freeman, 1964). The name lymphocyte may erroneously suggest a scavenging function for this cell which is incorrect. In comparative haematology the lymphocyte is regarded as " the primitive multipotential type of cell " (Andrew, 1965; see also Liebmann, 1946, Pérès, 1942) and there can be no justification for changing the name in ascidian literature to " stem cell " as has been done by Smith (1970a). Vallee (1967a) referred to these cells as spherical cells.

(b) *Granular amoebocyte.* There is a lot of confusion in the literature concerning the nature of the amoebocytes, probably because many of them are in transitional phases to other cell types. The granular amoebocyte seems to vary from 6 μ to 10 μ in diameter and to be capable of considerable phagocytic activity. Although little positive evidence is available, this would seem to be the primary cell concerned in metabolic transport. According to Overton (1966) granular amoebocytes contain spherical bodies embedded in a mass of dense particles 200 to 300 Å in diameter and having the appearance of glycogen. These cells have also been referred to in the literature as macrophages or phagocytes.



(a)

FIG. 19.



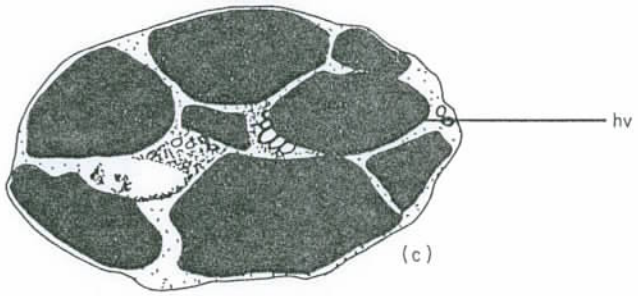


FIG. 19. Vacuolated cells in the blood of *Ascidia pygmaea*. (a) A signet-ring cell showing a large storage vacuole containing granules of vanadium complex and a cytoplasmic polar cap. (b) An early compartment cell showing the distribution of haemovanadin globules in peripheral vacuoles. (c) An early stage of a vanadocyte morula cell with large globules of haemovanadin and a compressed intervacuolar cytoplasm. hv, haemovanadin; n, nucleus; p, polar cap; pg, protein granules; rv, vacuoles forming in cytoplasmic cap by ropheocytosis; sv, storage vacuole; v, vanadium complex. (Redrawn from photographs in Kalk, 1963b.)

(c) *Hyaline amoebocyte.* These vary from 6 μ to 12 μ, have a less granular cytoplasm than the granular amoebocyte and are variously described as having few or no vacuoles. They are also referred to as macrophages or amoebocytes with granules. Differences in the published descriptions may be due to differences in metabolic state and probably include cells in transition to one of the vacuolar types. According to Fulton (1920) and Ohuye (1936) both types of amoebocyte

are phagocytic, but Smith (1970a) considered that only the hyaline amoebocyte will phagocytose carbon particles. Anderson (1971) found that phagocytosed material was subsequently released through the branchial epithelium.

(d) *Signet-ring cells* (Fig. 19*a*). There are three types of vesicular or vacuolated cell in the blood named respectively " signet-ring ", " compartment " and " morula ". Signet-ring cells are about 8 μ to 10 μ in diameter and contain a single large vacuole which presses the cytoplasm and nucleus to the periphery of the cell. The vacuole contains granules in Brownian movement, and the periphery is lined by electron dense material (Overton, 1966; Kalk, 1970). Signet-ring cells appear to be derived from hyaline amoebocytes (Kalk, 1963b) and according to Andrew (1961) have fine protoplasmic processes extending from them and giving them specific adhesive properties. In the transition from hyaline amoebocyte to signet-ring cell there is an intermediate vacuolated amoebocyte.

(e) *Compartment cell* (Fig. 19*b*). These are about 8 μ in diameter with several vacuoles filling most of the cell and providing the compartmented appearance. Each vacuole contains an accumulation of granular material which in some species has a greenish appearance. According to Kalk (1963b) the vacuoles of the compartment cell are a new development arising in the cytoplasm of the signet-ring cell and extracting material from the large vacuole of that cell. This would explain the apparent anomaly in Endean's (1960) description of these cells. He states " Each (cell) contains a large vacuole which varies in size from cell to cell . . . A number of subspherical compartments, peripheral in position, obscure the vacuole except at one pole of the cell. " Clearly these are transitional stages toward the completed compartmental cell.

(f) *Morula cells* (*Green cell type*) (Fig. 19c). These cells which are 8 to 10 μ in diameter are tightly packed with granule filled globules or vacuoles measuring 2 to 3 μ in diameter and called *vanadophores* by Gansler *et al.* (1963). When released from the blood stream and after standing a short while they take on a berry-like appearance, perhaps due to distortion of the globules, and it is this appearance which has caused them to be called morula cells. Due also to the fact that in some cases they are known to contain vanadium (Henze, 1913; Webb, 1939, 1956) and in others to contain iron (Endean, 1953) they have been respectively termed vanadocytes and ferrocytes. There is a need to rationalize this terminology, particularly in view of the discovery of niobium and titanium in some species of ascidian. Although the mulberry-like appearance only appears after freeing of the cell from the

animal, it seems best to continue to refer to these cells as " morula " cells irrespective of whether they contain vanadium, iron or any other metal. The morula cells are also unique in containing in many species large quantities of sulphuric acid. This and the significance of metal accumulation are discussed below.

(g) *Orange cells* (Fig. 20). Most ascidians have a small number of blood cells containing a bright orange pigment. According to George (1939)

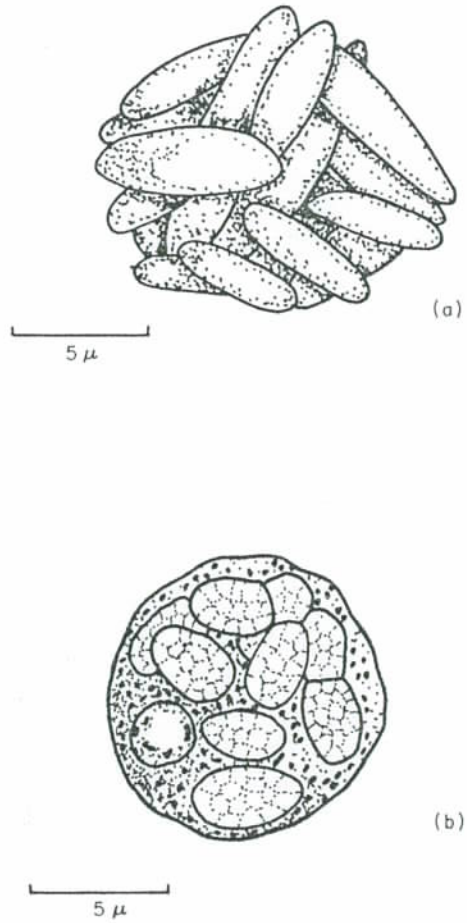


FIG. 20. (a) An orange pigment cell in *Phallusia mammillata*. (b) Developing pigment bodies in an orange pigment cell. (Redrawn from Endean, 1960.)

this pigment is not suspended in fluid-filled vacuoles, but is in granular form invested by dense cytoplasm. Azéma (1929a,b) and Lederer (1934) concluded that the pigment is a carotenoid, but according to Webb (1939) this is only true of certain clear orange morula cells in a few species. In *Ascidia mentula* Webb found that in true orange cells the pigment exists in the form of flat elliptical plates showing strong birefringence; he also found it to be soluble in water and acetone, but not in other organic solvents, and that it is stable to most oxidizing agents. Webb concluded that the pigment in *A. mentula* and several other species could not be a carotenoid. Endean (1960) found orange cells

TABLE X. TOTAL CELL COUNTS PER MM^3 AND PERCENTAGE OF DIFFERENT CELL TYPES IN ASCIDIAN BLOOD

Species	*Cell number per mm^3*	*Morula cells and their precursors*	*Amoebocytes*	*Lymphocytes*	*Author*
Ascidia nigra	53·4 × 10^3	93·5	4·9	0·4	Vallee, 1967a
Phallusia mammillata	68·0 × 10^3	92	7·0	Rare	Endean, 1960
Pyura stolonifera	37·0 × 10^3	90	3·0	5·0	Endean, 1955a
Halocynthia aurantium	17·24 × 10^3	33·7	59	5·0	Smith, 1970a

with similar properties in *Phallusia mammillata* and concluded that some of the contents were melanin. Melanin has also been reported in the orange cells of *Ciona intestinalis* by Pérès (1943). The resistance of the orange pigment to many reagents was also noted by Cuénot (1891, quoted by Webb, 1939) and George (1939), but its precise nature remains uncertain. Some vanadium compounds are orange or red in colour (Sidgwick, 1950) and it is possible that the orange pigment is a by-product of vanadium metabolism in the animal.

(h) *Nephrocytes.* These are large cells up to 20 μ in diameter and contain granules or larger concretions of a purine nature, bathed in fluid-filled vacuoles. (For further details see George, 1939.) They are further discussed on p. 82.

This confusing array of blood cell types can be rationalized into four groups comprising (1) lymphocytes; (2) morula cells and their precursors, the signet-ring and compartment cells; (3) amoebocytes; (4)

storage cells, i.e. orange cells and nephrocytes. The proportion of different cell types in the blood appears to differ widely between species and may be a reflection of different types of activity either in different species or in animals at different seasons of the year (Table X).

It is clear that much of the confusion in the literature must arise from the existence of cells in transition from one cell type to another, particularly in the formation of morula cells. The sequence of events involves changes through hyaline amoebocyte, vacuolated amoebocyte, signet-ring cell, compartment cell and finally the mature morula cell. This is a dynamic process involving considerable metabolic activity and synthesis within the cell and the changes cannot be accommodated adequately in simple morphological descriptions. It is further discussed below.

C. *Heavy metals in ascidian blood*

Sea water contains between 0·3 and 3·0 μg of vanadium per litre (Harvey, 1955) but values as high as 3700 p.p.m. organic dry weight have been found in ascidians (Ciereszko *et al.*, 1963) so that the degree of concentration of the metal in the body is very great. In the blood cells of some ascidians the concentration of vanadium may be in excess of one million times as high as that in sea water (Rummell *et al.*, 1966).

Henze (1911) was the first to show that ascidians are capable of accumulating vanadium in their bodies as a complex organic molecule and this observation has been confirmed by many authors since that time. The earlier literature has been reviewed by Webb (1939, 1956) and Table XI provides a summary of the occurrence of vanadium in the Ascidiacea. It is apparent that most species of ascidians are able to concentrate the metal in small amounts but only in a few families of phlebobranchiate ascidians, notably Ascidiidae and Perophoridae, does the metal occur in large quantity. In the Pyuridae iron appears to be concentrated instead of vanadium (Endean, 1953, 1955a, Smith, 1970a) and niobium, tantalum and titanium have all been reported in other species (Table XII). It is generally agreed that the iron and vanadium are concentrated in the blood and particularly within the morula cells (see especially Webb, 1939, 1956; Baltscheffsky and Baltscheffsky, 1953; Endean, 1955a, 1960; Bielig *et al.*, 1961a; Kalk, 1963b; Koval'skii and Rezaeva, 1963; Smith, 1970a; Rummel *et al.*, 1966), but there does not seem to be any concrete evidence that niobium, tantalum or titanium are concentrated in blood cells. Assuming that the mechanisms of uptake are the same for all metals (see below) it is likely that they will become concentrated in the blood. It is of interest that in the pelagic tunicate *Salpa*, where a number of metals are also concentrated,

TABLE XI. OCCURRENCE OF VANADIUM IN ASCIDIANS

Species	*p.p.m organic dry weight*	*p.p.m dry weight*
Morchellium argus (Milne Edwards)	—	0·0–3·7
Sidnyum elegans (Giard)	—	0·0
Aplidium pallidum (Verrill)	—	4·3
Amaroucium pellucidum (Leidy)	320–410	—
Clavelina picta (Verrill)	150	—
C. lepadiformis (Müller)	—	7·0
Eudistoma olivaceum (Van Name)	200	—
Didemnum candidum Savigny	—	2·6
D. maculosum (Milne-Edwards)	—	30·8
Ciona intestinalis (Linnaeus)	400†	166–1300
Diazona violacea Savigny	120–240	—
Rhopalea neapolitana Phillipi	1700–2000	—
Ecteinascidia turbinata Herdman	600–1300	0·0–8·3
E. conklini Berrill	1800	—
Perophora viridis Verrill	470–1150	0·0
Ascidiella scabra (Müller)	1120†	—
A. aspersa (Müller)	690–790 1450†	—
Ascidia mentula Müller	470–1400	982
A. correloides (Van Name)	1130	—
A. nigra (Savigny)	2700–3700	—
Phallusia mammillata (Cuvier)	1700*†	—
Botryllus schlosseri (Pallas)	—	0·0–7·8
B. leachi (Savigny)	—	0·0
Distomus variolosus Gaertner	—	6·4
Dendrodoa grossularia (Van Beneden)	48†	10·2
Pyura microcosmus (Savigny)	—	7·1
Styela partita (Stimpson)	—	0·0
Microcosmus sulcatus (Coquebert)	—	0·0
M. glacialis (Sars)	—	0·0
Molgula manhattensis (De Kay)	—	31·8

Only data for which numerical values are available have been used. Data are expressed as p.p.m dry weight and p.p.m organic dry weight. Data for dry weights are taken from Bertrand (1950). Data for organic dry weights are taken from Webb (1956) and Ciereszko *et al.* (1963). Items marked † are from Webb, the remainder from Ciereszko *et al.* Items marked * do not include the weight of the test. Additional data on the occurrence of vanadium are given in Kobayashi (1949), Webb (1956) and Bertrand (1950).

vanadium is present only as 7 p.p.m. of the ash weight, which compares with 460 p.p.m. ash weight in *Ascidia obliqua* Alder (Vinogradov, 1934) but copper amounts to 500 p.p.m. in *Salpa* (Nicholls *et al.*, 1959).

TABLE XII. DISTRIBUTION OF METALS OTHER THAN VANADIUM IN THE TISSUES OF ASCIDIANS

	Niobium	*Titanium*	*Chromium*	*Manganese*	*Iron*	*Tantalum*
Ciona intestinalis		1·7[a]		120[a] 95[b]	250[a] 3000[f]†	
Molgula manhattensis	0–75[b]					
Pyura stolonifera					** 3400 to 5100[c]	
Eudistoma ritteri Van Name		1361 to 1512[d]	72 to 144[d]			
Styela plicata	150 to 300[e]					100 to 410[e]

Figures are in parts per million dry weight of body except as follows: ** p.p.m of blood; † p.p.m of ash dry weight.

[a] = Noddack and Noddack (1939); [b] = Carlisle (1968); [c] = Endean (1955a); [d] = Levine (1962); [e] = Kokubu and Hidaka (1965); [f] = Koval'skii *et al.* (1962).

1. *The chemical nature of haemovanadin*

Henze (1911) recognized the existence of vanadium as an organic compound in the blood cells where it occurs as a pale green solution. On haemolysis in distilled water a red-brown acid solution is released which rapidly oxidizes in air to give a blue-green precipitate which is the stable form of the compound. These three phases have been recognized by later workers as the true green haemovanadin of the cell, Henze's red haemovanadin and blue oxidized haemovanadin. Califano and Boeri (1950) showed that haemovanadin remains stable only at pH's below 2·4 and that in less acid media it rapidly oxidizes. Boeri (1952) and Rezaeva (1963) have both calculated that the pH inside the vanadocyte is less than 2·4 and it would seem possible that the sulphuric acid first recognized in the vanadocyte by Henze (1911) and later confirmed by Webb (1939) exists primarily to maintain the haemovanadin in a reduced form. Vanadium can exist in a number of different

states of valency from 2 to 5 (Sidgwick, 1950) and while it appears to occur in sea water in a pentavalent form it is agreed by most recent workers that in the blood cell and in Henze's red haemovanadin the vanadium exists in a trivalent form (Lyding, 1953; Boeri and Ehrenburg, 1954; Bielig and Bayer, 1954; Rezaeva, 1964; Koval'skii and Rezaeva, 1965; Bielig *et al.*, 1966) but that the blue oxidized haemovanadin is tetravalent. These authors are also agreed that the haemovanadin acts as a powerful reducing agent.

The precise nature of the chemical compound is still uncertain but it has been established that within the cell it occurs as a dialysable organic molecule loosely linked to a non-dialysable protein (Califano and Caselli, 1949; Bielig *et al.*, 1954; Bielig and Bayer, 1954; Baltscheffsky and Mendia, 1958; Koval'skii and Rezaeva, 1964). Bielig *et al.* (1966) have suggested that the vanadium occurs as an equilibrium between different sulfato-vanadium (III) ions, protein and hydrogensulphate ions. A fuller discussion of the chemical composition and possible interactions is not relevant here but the reader is referred especially to the papers of Bielig *et al.* (1954 and 1966).

2. *The uptake of vanadium by ascidians*

We have already seen that the concentration of vanadium in the blood cells of ascidians may be 10^5 or 10^6 times as great as it is in sea water so that it is of particular interest to know how the animals manage to collect and accumulate the metal. It is unlikely that it could be obtained through the food supply as this would entail concentration by other organisms such as phytoplankton or accumulation in detritus and there is no evidence for this. Goldberg *et al.* (1951), Baltscheffsky and Baltscheffsky (1953), Bielig *et al.* (1961a), Kalk (1963a,b) and Rummel *et al.* (1966) have all produced evidence to confirm that the primary site of uptake is through the single celled epithelium of the branchial wall. Rummel *et al.* (1966) have summarized much of the work on the uptake of labelled vanadium and show that this takes place by a process of active absorption across the branchial wall (see Fig. 21). When animals are exposed to ^{48}vanadium for six hours the branchial sac yields more than half of the vanadium taken up, the gut only about 10%, thus confirming that the metal is obtained directly from sea water and not from the food. Vanadium absorbed through the branchial sac is initially transferred to the blood plasma and after six hours exposure only 8% of labelled vanadium in the blood is found in cells and 92% is in the plasma. However, if the animal is then kept in unlabelled sea water for a further five days, about 95% of the labelled vanadium is now found in blood cells (Fig. 22). The

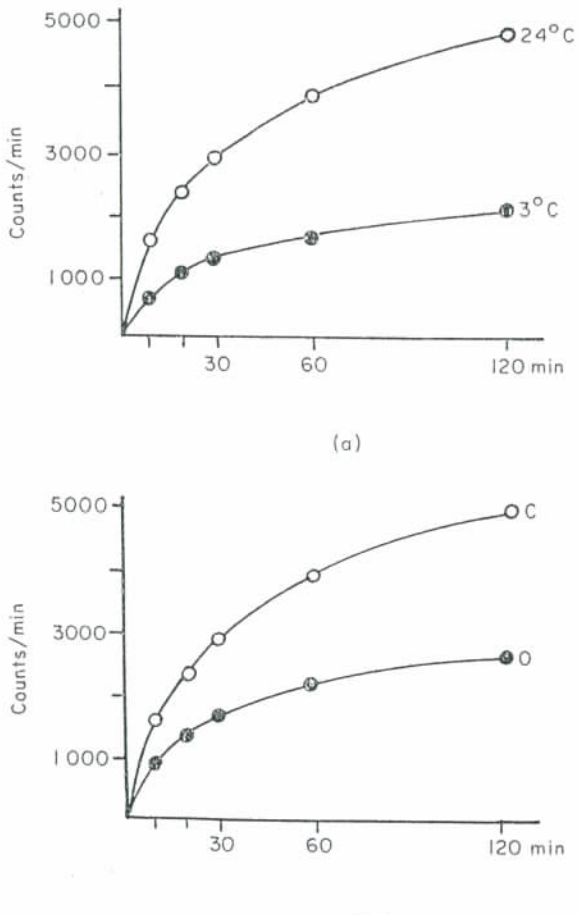


FIG. 21. The uptake of radioactively-labelled vanadium in the branchial sac of *Ciona*, (a) in relation to temperature, and (b) in the presence of ouabain (O); C, control at 24°C. (Redrawn from Rummel *et al.*, 1966.)

process of uptake from sea water appears to be similar to the uptake of phosphate but the latter accumulates in the plasma and not in the blood.

Kalk (1963a,b) considers that pentavalent vanadium in sea water is linked to either a sulphate or a protein group in the mucus passing over the branchial wall and that by a process of pinocytosis droplets of

mucus pass through the branchial epithelium to the blood vessels where they are picked up by amoebocytes and accumulate in vacuoles. Kalk goes on to suggest that the mucus is then digested in the cell and the vanadium accumulates on the vacuolar membrane where an insoluble complex of vanadium, protein and sulphate forms coupled with a change of valency from pentavalent to trivalent vanadium. It is

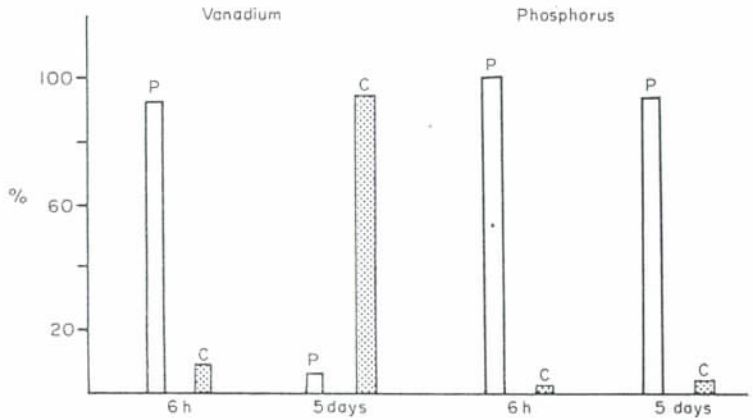


Fig. 22. The distribution of labelled vanadium and phosphorus in the blood of *Ciona* six hours and five days after exposure to sea water containing ^{48}V vanadate or ^{32}P phosphate. P, plasma; C, blood cells. (Redrawn from Rummel *et al.*, 1966.)

at this stage that the signet-ring cell begins to form and the insoluble complex is deposited in the large vacuole. By a process of ropheocytosis (Policard and Bessis, 1958) the granules are reabsorbed into the cytoplasm of the cell where the compartments form and according to Kalk haemovanadin is now being synthesized and acid produced resulting in a solution of haemovanadin in the compartments. Kalk suggests that the haemovanadin molecule is made in three stages as follows.

(a) Protein granules are made by ribosomes in small vacuoles at the vesicular ends of Golgi bodies.

(b) Vanadium is transferred from the compact granules to the small vacuoles by dispersion of the grains and solution of their

matrix and by apposition of vacuoles containing vanadium-complex and protein.

Pentavalent vanadium in sea water
↓
Uptake on branchial wall
↓
Absorption into blood
↓
Uptake from plasma to amoebocytes
↓
Accumulation of vanadium in vacuolar amoebocytes and reduction to trivalent form
↓
Insoluble granules of vanadium-protein-sulphate complex in signet-ring cell
↓
Synthesis of haemovanadin in compartment cell and production of acid
↓
Mature morula cell (vanadocyte)

Fig. 23. An outline scheme for the uptake of vanadium in ascidians. (Adapted from Kalk, 1963b.)

(c) During the distribution of the vanadium-protein-complex granules in vacuoles around the periphery the contents become acid and soluble.

Kalk finally concludes that the absorption of water and sugars into the compartments converts the cell into a morula cell or vanadocyte where the sugars are polymerized to form a polysaccharide. Kalk's work is based almost exclusively on electron microscopy, using the electron dense properties of vanadium as a natural tracer, and of necessity some of her conclusions must be considered as speculative (Fig. 23).

While the overall picture of vanadium absorption and accumulation is becoming clear through the work of these various authors there is considerable divergence of opinion on certain matters. Kalk's suggestion of uptake by pinocytosis for instance is in conflict with that of Bielig and others who indicate that it must be an energy consuming biochemical process, and indeed Rummel *et al.* (1966) provide evidence to suggest that the vanadium passes first into the plasma and is only

slowly absorbed from there to the cells. Bayer (1955) has put forward a model to suggest the type of chemical pathways which may be involved in vanadium accumulation but until a great deal more is known about the actual pathways we cannot equate his model with the processes described by Kalk and Rummel *et al.**

There is some doubt also as to whether morula cells contain carbohydrate. Endean (1955a) reported the presence of polysaccharides in the ferrocytes of *Pyura stolonifera*, and Endean (1960) and Kalk (1963a) both believed that vanadocytes synthesize polysaccharides from simple sugars in *Phallusia mammillata* and *Ascidia pygmaea* Michaelsen respectively. Smith (1970b), on the other hand, believed that polysaccharides are absent from the ferrocytes of *Halocynthia aurantium*. The evidence from studies on test formation already given (p. 15) suggests that carbohydrate is formed directly from mantle epithelium and not from morula cells.

3. *The iron compound in Pyuridae*

Although it is twenty years since Endean (1953) first discovered that iron and not vanadium was present in the blood cells of *Pyura stolonifera*, surprisingly little work has been done to follow up this interesting discovery. The only confirmatory evidence that this may be a characteristic of the stolidobranch ascidians comes from Smith (1970a) who worked with *Halocynthia aurantium* in which iron is also present. According to Endean the iron compound is organically bound in the ferrous state and is non-dialysable. It has marked reducing properties but is not such a powerful reducing agent as the vanadium compound in Ascidiidae, a fact also evident from Endean's (1955a) and Smith's (1970a) results, in which it is shown that ferrocytes give only a greyish colour with osmium tetroxide and not black as in the vanadocytes of *Ascidia*. In other respects the iron compound is also similar to haemovanadin. Cytolysis of the ferrocyte gives a brown acid solution comparable to Henze's red solution of vanadium and this oxidizes in air to give an orange-brown precipitate. The ferrocyte also contains sulphuric acid but probably not in such strong concentration as in vanadocytes and Endean (1955a) considers that it is present to maintain the iron compound in a reduced condition. The evidence therefore points to the fact that in all essential respects the iron compound of the Pyuridae is

* This model is also followed by Bielig *et al.* (1954) and by Koval'skii and Rezaeva (1965) and seems to be incorrectly attributed as the work of these latter authors by Carlisle (1968).

similar to the vanadium compound of the Ascidiidae and probably serves a similar function.

D. *Agglutination*

According to Hecht (1918c), Fulton (1920) and Vallee (1967a, 1967b, 1968) the blood of ascidians does not clot but in drawn blood the cells aggregate and if left standing for some time separate again. Vallee (1968) has shown that the vanadocytes have free SH groups which bind to one another in agglutination but can alternatively bind to a free SH group in cysteine which prevents agglutination. Anderson (1971) also found that vanadocytes aggregated around foreign bodies implanted in the tissues and were capable of secreting a protective layer of test material over such bodies. In addition Fuke and Sugai (1972) report that the body fluid of two species of ascidian (*Styela plicata* and *Halocynthia hilgendorfi* (Oka)) contains haemagglutinins which cause aggregation of mammalian erythrocytes but does not aggregate blood cells of other ascidian species. Unlike other haemagglutinins the substance in ascidian fluids is a polysaccharide and not a protein.

E. *Blood function*

It is possible now to consider the role and function of various types of cell in ascidian blood. The *lymphocyte* seems to be a transient multipotent cell type which can give rise to all other blood cells as required. *Vacuolar amoebocytes*, *signet-ring* cells and *compartment* cells are developmental phases in the formation of *morula* cells which are concerned with test formation. Morula cells contain organic compounds of heavy metals notably vanadium or iron, but there is no evidence to suggest that these play a role in oxygen transport (Webb, 1939). The metabolic level of ascidians is low and Goodbody and Young (unpublished data) have shown that oxygen consumption in *Ascidia nigra* ranges from 806 to 1630 μl/h/g ; a specific oxygen carrier in the blood seems unnecessary. The precise role of the morula cell in test formation is still unclear, but is most likely concerned with the deposition of microfibrils of cellulose-like polysaccharides within the ground substance secreted by epithelial cells of the mantle (p. 15). The metal chromogen of the cell is a powerful reducing agent and since the morula cell disintegrates in the test substance it is logical to assume that its prime role is to function in a reducing capacity. In view of the necessity to produce the fibrils *in situ* it may be that the reaction is in some way concerned with

the polymerization of simple carbohydrates to more complex polysaccharides.

The precise role of the two types of *amoebocyte* is difficult to define and there is little in the literature to enable firm conclusions to be reached. Both the hyaline and the granular amoebocyte are reported to be phagocytic and the former has considerable powers of amoeboid movement. Azéma (1937) has drawn attention to the fact that the distinction between these two amoebocytes is not always clear and that in fact they may be different stages of a single cell type. The strongly amoeboid properties of the hyaline type may be associated with phagocytic activity, while the less mobile mature granular cell may be a contrasting metabolic phase. In any event it seems likely that both types are concerned in some way with the basic nutrition and metabolism of the animal; modern techniques in biochemistry should be able to throw more light on this problem. Kalk (1963b) believed that the hyaline amoebocyte phagocytizes beads of vanadium-laden mucus in the pharynx and gives rise to vacuolar amoebocytes and signet-ring cells.

Both the *nephrocyte* and the *orange pigment cell* appear to be depositories for metabolic waste products. The absence of certain uricolytic enzymes results in incomplete metabolism of purines and related compounds (p. 85) and these are stored initially in *nephrocytes* which either accumulate in the body or are transported out across the epithelium. Although little is known about the orange cells their almost universal occurrence in ascidians suggests that they play a significant role in metabolism. The pigment is not, as first suggested, a carotene but may be a derivative of melanin or perhaps of vanadium. However, orange cells occur in many species in which vanadium does not occur.

Ascidian blood may thus be considered to have three functions: nutrition, storage excretion and test formation.

X. Nitrogen Excretion

Ascidians do not possess any form of regulatory organ such as a kidney or a nephridial system, but in a great many species there are structures in which certain types of metabolic waste may be accumulated in solid form (Fig. 24). In the simplest case, and occurring in most ascidians, granular concretions are deposited in special blood cells termed nephrocytes. The concretions are usually considered to be purine in nature and the nephrocytes either remain in circulation or are deposited in the tissues perhaps eventually being passed to the

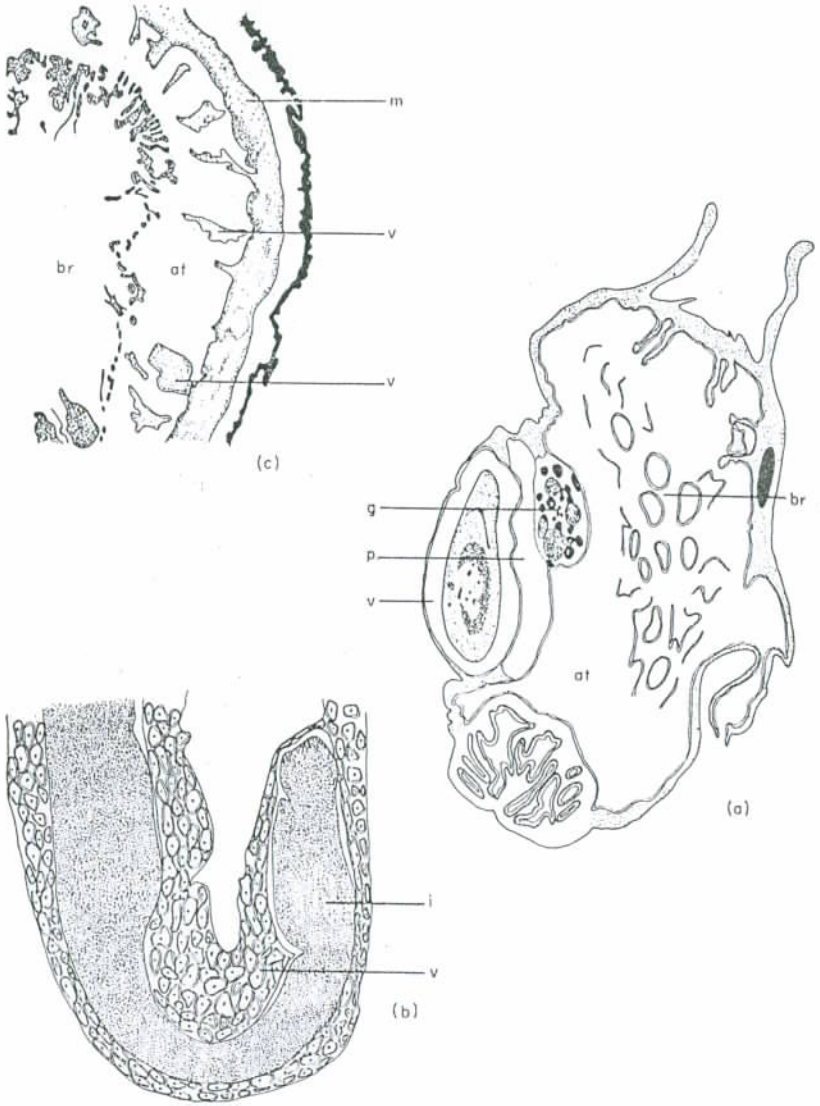


Fig. 24. Storage excretory organs in ascidians. (a) The single vesicle of *Molgula citrina* Alder and Hancock adjacent to the pericardium; sagittal section. (b) Numerous vesicles surrounding the alimentary canal in *Ascidiella aspersa*. (c) Parietal vesicles on the inner surface of the mantle in *Dendrodoa grossularia*; transverse section. at, atrial cavity; br, branchial sac; g, gonad; i, intestine; m, mantle wall; p, pericardium; v, vesicle. (Redrawn from photographs in Goodbody, 1954.)

exterior. In the Molgulidae there is in addition a large vesicle adjacent to the heart which contains a single concretion, partly composed of uric acid, and in the Ascidiidae there are large numbers of small concretions usually but not always composed of uric acid or urates. These vesicles are often referred to as renal vesicles but as they can in no way be compared to a kidney and are derived from the epicardium which is a coelomic structure (Berrill, 1955) they are better referred to as coelomic vesicles. In Pyuridae and Styelidae vesicular evaginations of the mantle wall, termed endocarps, hang into the mantle cavity and accumulate concretions or nephrocytes. In some Styelidae and Pyuridae large deposits of uric acid may also accumulate beneath the endostyle and in other tissues. For a fuller description of these structures and their contents see Azéma (1937), Das (1948a), Goodbody (1954, 1965).

The existence of solid concretions of purines or uric acid led many earlier workers to conclude that ascidians were wholly uricotelic in their nitrogen metabolism and Burian (1924) even went so far as to suggest that they resembled snails, insects, reptiles and birds in this respect. Most aquatic animals take advantage of the water to get rid of their nitrogenous wastes as soluble ammonia and it would be surprising if ascidians did not do the same. Goodbody (1957) has shown that in three species, *Ciona intestinalis*, *Ascidiella aspersa* and *Molgula manhattensis*, ammonia is excreted in sufficient quantities to suggest that their protein metabolism is essentially ammonotelic, and Sabbadin and Tondonati (1967) have confirmed on a qualitative basis that ammonia is also excreted by *Botryllus schlosseri* (Pallas) and *Botryllus leachi* (Savigny). These four species represent different types of ascidian in terms of uric acid or purine storage. *Ciona intestinalis* has an open coelom (epicardium) and no concretions and according to Lambert, quoted by Nolfi (1970), uric acid has not been demonstrated in its tissues; *Ascidiella aspersa* has numerous coelomic vesicles but the concretions are primarily formed of calcite (Goodbody, 1965); *Botryllus schlosseri* and *B. leachi* have no visible coelom or vesicles but have a very large quantity of nephrocytes, the concretions in which are almost solely formed of uric acid (Tondonati and Sabbadin, 1966; Sabbadin and Tondonati, 1967). *Molgula manhattensis* has a single coelomic vesicle containing a large concretion which is composed of about 48% uric acid (Goodbody, 1965; Nolfi, 1970) but may vary from 3% to 75% (Nolfi, *loc. cit.*).

The evidence favours the conclusion that ammonia is the principal end-product of nitrogen metabolism but that most, if not all, ascidians accumulate uric acid and possibly other purines such as xanthine, guanine and adenine. The significance of such accumulations is less

easy to understand. Goodbody (1965) suggested that it was entirely due to a deficiency in the uricolytic enzyme system and showed that uricase, allantoinase, allantoicase and urease were all absent from homogenates of a large number of species of ascidian. A fuller understanding of the problem requires a knowledge of the biosynthetic pathways in the ascidian by which uric acid is formed. Nolfi (1970) has shown that *de novo* synthesis of uric acid in *Molgula* from smaller molecules does not occur but that it must be entirely produced from pre-formed purines. He has also shown that uric acid makes up about 3% of the total nitrogen excreted by *Molgula* and thus confirms Goodbody's (1965) suggestion that the uric acid is probably derived from nucleic acid metabolism and not from protein metabolism.

There remains the question as to what functional purpose is served by the accumulation of uric acid in ascidians. Nolfi (*loc. cit.*) has pointed out that there are four possible biological functions which could be served by purine storage, viz. (1) storage excretion; (2) pigmentation; (3) a source of reserve of carbon or nitrogen, and (4) a stable purine source for nucleic acid synthesis.

It is unlikely that pigmentation is the primary reason for uric acid storage as in most species it is stored deep in the tissues away from surface areas of pigmentation, and most authors have assumed that it is solely a means of removing solid wastes. However, there are a number of pieces of evidence which suggest that the uric acid may be mobile and involved in some active metabolic process in the animal. Hertwig (1871) found that the concretions of *Phallusia mammillata* were soluble in acids with an intense evolution of gas and he suggested that they were composed primarily of carbonates. Goodbody (1965) working with the same species found almost no carbonate and 62·5% of uric acid in the concretion. Goodbody also found that the concretions of *Ascidiella aspersa* were largely composed of calcium carbonate in the form of calcite and Nørrevang (1966) found dense granules of glycogen and acid mucopolysaccharides in the renal vesicles of the same species but noted that seasonal differences in the state of the vesicle occur. Nolfi (*loc. cit.*) found that uric acid content of the vesicle in *Molgula manhattensis* varies from 3 to 75% of the dry weight in the concretion. Nolfi quotes Buchner (1953) as having reported the presence of fungi in the renal vesicle of *M. manhattensis* which he considered engulfed and digested the concretions in an amoebocytic fashion. Nolfi suggests that if some of the metabolic intermediates "leaked" back to the ascidian host this could be a mutualistic relationship. It is difficult to see how such a relationship could function and how the fungus could initially invade the closed coelomic vesicle. My own histological

preparations of *Molgula* suggest that the concretion has a connective framework in which the uric acid granules are embedded, and this framework is likely to be a polysaccharide. Although the evidence available is limited there is a suggestion throughout that the composition of renal concretions may not remain stable and hence that they may be engaged in some active metabolic process. One such function suggested by Nolfi is that they provide a stable purine source for nucleic acid synthesis. However, this would involve *in vivo* synthesis of hypoxanthine from uric acid and as Nolfi points out there is no evidence to suggest that this is possible.

In summary, ascidians appear to be wholly ammonotelic in their protein metabolism but are uricotelic in respect of nucleic acid metabolism and in this respect differ from most other aquatic invertebrates which are wholly ammonotelic. The stored uric acid is derived by degradation from purines and not by *de novo* synthesis and the absence of uricolytic enzymes prevents further degradation to allantoin or urea. The functional significance of uric acid storage remains obscure but it is of such general occurrence in ascidians that further research is highly desirable.

XI. The Nervous System

A detailed review of the physiology of the nervous system in the Tunicata has recently been published (Bullock and Horridge, 1965) and little new information has appeared since that time. In the present review an account is provided of our present knowledge of structure and function in the ascidian nervous system with emphasis on those features which are of special interest or significance in the physiology of the group.

A. *Structure of the nervous system*

Ascidian nerves do not show up well with normal histological techniques such as silver impregnation or methylene blue staining and in consequence of this and the fineness of many of the fibres there is still some uncertainty about the details of the peripheral system. The best accounts of the anatomy of the system are those of Hilton (1913) who described the central ganglion of *Ascidia nigra*, Fedele (1923a, 1923b, 1927, 1937a) who described the peripheral nervous system in *Ciona intestinalis* and Millar (1953a) who provides a general account of nervous organization in *Ciona intestinalis*. Other review articles are to be found in Seeliger and Hartmeyer (1893-1911), Huus (1937), Brien (1948) and Bullock and Horridge (1965). In addition Markman (1958) has reported on a methylene blue and phase contrast study of young

specimens of *Ciona intestinalis* and Aros and Konok (1969) have redescribed the central ganglion of *C. intestinalis*. The following account of the nervous anatomy is based upon the findings of these authors amongst whom there is general agreement.

There is only one ganglion in the adult ascidian and this is situated in the dorsal mantle wall mid-way between the two siphons and adjacent to the neural gland (Fig. 25). The ganglion may measure as much as 3 mm in length in an adult *Ciona*. It is surrounded by a connective tissue sheath and has main nerve fibres arising at either end. Millar and Hilton both describe also some very fine fibres arising at the periphery of the ganglion and apparently innervating the musculature in the surrounding mantle tissues.

The internal structure of the ganglion is typical of that found in most invertebrates with an outer cellular cortex and a fibrous medulla. Within the centre there are two sizes of neuron, large ones which according to Millar are 9 μ × 18 μ and small ones measuring between 5·25 μ and 6 μ in diameter. Millar and Aros and Konok considered that all of these cells are unipolar but Hilton believed that as well as the unipolar cells there are a few multipolar ones. Aros and Konok also recorded the presence of glial cells in the cortex and several authors (see below) have described " neurosecretory " granule-laden cells in the cortex. Hilton and Millar both described the presence of minute fibrillae in the neurons but it is not certain whether this was a misinterpretation of the nature of the granular " neurosecretory " cells.

The central medullary region of the ganglion is composed of a dense aggregation of nerve fibres mostly oriented along the length of the ganglion. The axons arising from the cortical neurons enter the medulla and turn along its length although a few may cross it diagonally so as to emerge in nerves on the opposite side, and some appear only to be fibres of association neurons within the ganglion. Millar also mentions the presence of a few small multipolar cells in the medulla while Aros and Konok indicate the presence of glial cells in this region. Within the neuropile Dilly (1969b) has described polarized synaptic junctions in which vesicles of two different sizes occur adjacent to the pre-synaptic membrane ; granules occur in some of the large vesicles. The significance of these two types of vesicle is not known but Dilly suggests that they may release different transmitter substances which perform separate functions or that one is potentiated by the other. Alternatively there may be an intimate association between normal synaptic transmission and neurosecretion ; granule bearing vesicles assumed to have a neurosecretory function occur in the peripheral neurons of the ganglion (see p. 106) but until a great deal more is known about these and the

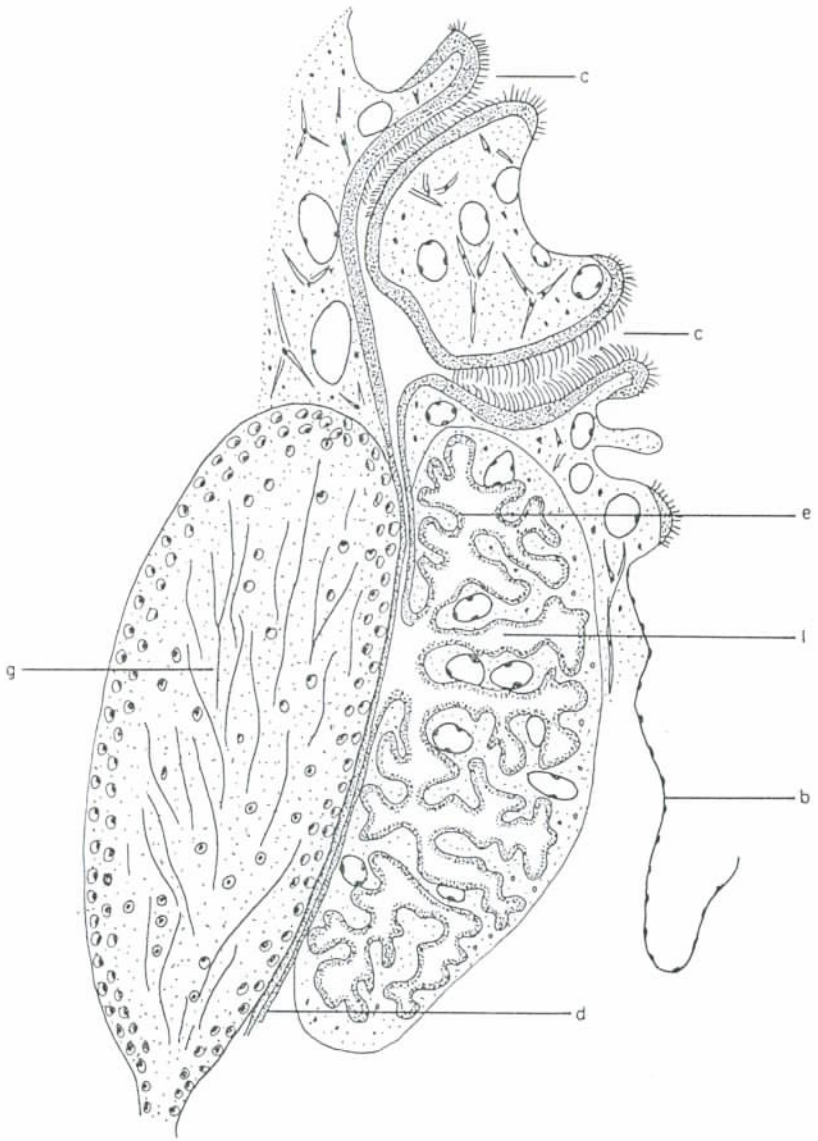


FIG. 25. The neural gland and ciliated funnel in relation to the nerve ganglion; longitudinal section. b, branchial epithelium; c, ciliated funnel; d, dorsal strand; e, glandular epithelium; g, ganglion; l, lumen of the gland. (Redrawn from Brien, 1948.)

synaptic vesicles it is impossible to do more than speculate on their function.

Apart from the fine nerve fibres arising peripherally and reported by Hilton and Millar there are five main nerves arising from the ganglion. At the anterior end there are two nerves each of which gives rise to four branches as follows: (1) To the dorsal side of the branchial siphon, (2) to the ventral side of the branchial siphon, (3) and (4) to the musculature of the mantle. Posteriorly there are two similar nerves which innervate the atrial siphon as well as the mantle musculature and arising medially between these two nerves there is an unpaired visceral nerve which enters the roof of the branchial sac and extends posteriorly toward the main visceral mass. Millar states that while the branchial siphon is wholly innervated from the anterior region of the ganglion and the atrial siphon from the posterior end, the mantle musculature is innervated from both ends although the nerves of the anterior end contribute more to the ventral side than do the posterior nerves which contribute more to dorsal musculature.

B. *The visceral innervation*

From the generalized account of the ascidian nervous system it would appear that the sensory motor innervation is entirely associated with the paired anterior and posterior nerves while the visceral innervation is confined to the single unpaired posterior visceral nerve. This is probably true for the sensory motor system but there is considerable doubt and uncertainty about the nature of the visceral innervation. Some of the visceral functions which may be expected to be under nervous control are as follows:

(a) *Ciliary activity in the branchial sac.* The beating of the cilia is not continuous but intermittent. This is difficult to demonstrate on a large solitary ascidian but can be observed readily in small zooids from colonial forms or in young ascidians with only a few rows of stigmata. Observation of these reveals that at intervals all of the cilia stop beating momentarily and simultaneously. This co-ordinated activity and inhibition of the cilia is under nervous control (Mackie *et al.*, 1974).

(b) *The activity of the heart and pericardium.* The peristaltic activity of the heart is reversible and the heart beats for a certain period in one direction then stops and recommences beating in the opposite direction (see p. 48). Although this type of activity could be under neurogenic control it has now been established that this is not the case, that it is entirely myogenic and there is no direct innervation of the heart (see p. 54). Nevertheless there is an innervation of the pericardium which is further discussed below.

(c) *Gastro-intestinal activity*. There is no muscular coating to the intestinal tract except for a small sphincter muscle around the anus, and there is little evidence to suggest that any other part of the canal has a special innervation.

(d) *Gonadial activity*. The gonads might be innervated by neuro-secretory fibres and the sphincter muscle of the gonoduct may also be subject to visceral innervation.

(e) *Secreto-motor activity*. Various regions of the alimentary system, and in particular the endostyle, are engaged in mucus secretion some of which activity might be under viscero-motor control.

Fedele (1923a, 1927) reported the presence of a visceral plexus and visceral nerve cells in *Ciona* and in the pelagic tunicate *Salpa*, but neither Millar (1953a) nor Bone (1959) were able to find any nerve cells on the gut of *Ciona* although Bone did find a few cells on the alimentary canal of *Salpa*. In *Ciona* Bone found some multipolar stellate cells on the gonoduct which had connections to the dorsal nerve but there is nothing to enable us to determine the function of these cells. Markman (1958) was unable to locate any nerve cells outside of the dorsal ganglion in young *Ciona*, and found no innervation at all in the intestine.

Innervation of the branchial sac and endostyle has been reported by several workers. Hunter (1898) illustrates nerve fibres from the anterior region of the ganglion innervating the endostyle and the branchial sac but he believed that they were sensory, not motor. Markman (1958) also reports the presence of sensory cells in the endostyle of *Ciona intestinalis* but shows the endostyle innervated from the visceral nerve. In pelagic tunicates Bone (1959) describes a pair of anterior visceral nerves passing either side of the neural gland, passing the peripharyngeal band and innervating the endostyle. He speculated that they may perform a secreto-motor function. Fedele (1923a) considered that the intermittent beating of the branchial cilia is not directly controlled by the visceral nerve and that special visceral nerve cells either on the gut or perhaps in the pharyngeal wall itself must control this activity. Bone (1959) was unable to substantiate this and Markman (1958) concluded that all the gill slits are innervated from the paired posterior nerves and not from the visceral system.

In two later papers Fedele (1927, 1938) described the visceral nervous system in *Ciona* and suggested that there may be a system of autonomous nerve nets connected to the ganglion through the dorsal strand. Surrounding the dorsal strand there seems to be a sheath of nerve fibres and scattered nerve cells which Fedele considered to be of " no less importance than the ganglion " and which he believed provided

the innervation for a nerve network in both the branchial sac and the viscera (cf. p. 124). The system of fibres around the dorsal strand is particularly well developed in the vicinity of the branchial sac and Fedele believed that this is associated with the nervous control of ciliary activity. Similarly Fedele believed that the visceral elements around the dorsal strand supply a fibrillar network surrounding the alimentary canal and which controls ciliary activity in the gut. Fedele noted that a feature of all these fibrillar networks is their lack of polarity and this is further substantiated by the work of Mackie *et al.* (1974) on the branchial sac of *Corella*.

Mackie and his colleagues have studied branchial innervation and ciliary control in *Corella willmeriana*. A network of fine nerve fibres runs through the branchial sac sending branches to some of the ciliated cells lining the stigmata; neuro-ciliary junctions have been identified in this epithelium by electron microscopy. The nerve network is connected to the ganglion by way of the visceral nerve but electrophysiological studies provide evidence of alternative conduction routes into the branchial sac along the endostyle and around the anterior margin; these alternative routes have not been identified histologically. The branchial nerve network is through conducting and unpolarized and it is presumed to be responsible for spreading excitation to the ciliated cells of the stigmata. The spread of response can be identified by ciliary arrests and accompanying electrical potentials. Ciliary arrests usually occur in a rhythmical series along with muscular contractions in the mantle and siphons. Isolated parts show inherent rhythmicity but in the intact animal a single rhythm prevails. There are evidently numerous, scattered peripheral pacemaker sites which are normally coupled and whose output controls ciliary and muscular effectors alike.

Ciliary arrests are accompanied by large electrical signals recordable externally from the branchial surface with suction electrodes; intracellular recordings with micro-electrodes show 50 millivolt depolarizations lasting over one second. Depolarizations probably spread from cell to cell in the ciliated epithelium since most of the cells are not directly innervated. 50 Ångström close junctions, associated with intercellular electrical communication in a number of other tissues are shown to be present between the stigmatal ciliated cells. Observations on ciliary arrest behaviour in artificial sea waters containing differing ionic balances suggest that the response depends on the sudden influx of calcium ions as in the comparable case of ciliary reversal in *Paramecium*. Resumption of normal beating would occur when the resting potential is restored by outward pumping of calcium ions.

Co-ordination between muscular contraction and ciliary arrest is

probably protective in function. It ensures that at the moment of contraction the cilia are relaxed and the stigmata are " wide open " thus permitting a free flow of water and pressure equalization between the atrial cavity and the branchial sac without any risk of damaging the ciliary mechanism.

Although Alexandrowicz (1913) reported finding nerve fibres, from bundles on the pericardial raphe, innervating the heart muscle in *Ciona intestinalis* it now seems certain that this is incorrect and the ascidian heart muscle is totally devoid of nervous innervation (see Bacq, 1935a; Krijgsman, 1956; Bone and Whitear, 1958; Kalk, 1970). According to Bone and Whitear who studied living hearts with phase microscopy there is a plexus of nerve fibres in the wall of the pericardium as well as a fine plexus on the surrounding epicardium. This plexus arises from bundles of fibres which pass through the connective tissue layer between epicardium and pericardium and thereafter branch into single fibres across the pericardial wall. Nerve bundles, similar to those seen by Alexandrowicz, were sometimes seen on the raphe above the heart but no fibres go from these to the heart. Around the pericardial wall there is a band of smooth muscle, but Bone and Whitear were unable to locate any nervous innervation of it and they concluded that the nerve plexus in the wall must be sensory. Skramlik (1929) found that a pressure of 2 cm of water in the pericardium is necessary for proper functioning of the heart and Bone and Whitear suggested that possibly the nerve plexus is part of a sensory-motor reflex involving the pericardial muscle which might control this pressure. They further suggested that since no ganglion cells are visible in this region the system is probably connected to the central ganglion. Markman (1958) stated that the pericardium has a double innervation, one end innervated from the visceral nerve and the other from a branch of one of the anterior nerves. Markman also illustrates elongate structures in the raphe of the pericardium which he considered may be sensory. Hunter (1898) described similar structures which he also believed to be sensory.

The gonad appears to be innervated directly from the visceral nerve but is also intimately associated with the dorsal strand arising from the neural gland (p. 123).

In summary, the existing evidence for a visceral innervation in ascidians is still incomplete and fragmentary and until further critical microscopical and electro-physiological techniques can be brought to bear upon this system our understanding must remain limited. The main visceral nerve emanating from the posterior part of the ganglion is supplemented by a system of fibres and cells forming a meshwork around the dorsal strand. It is proposed here that this meshwork around

the dorsal strand should be named the " *visceral fibre complex* " to distinguish it from the visceral nerve. The dorsal strand is not an intimate part of this visceral system but may nourish it or may continue to provide new nerve cells as the animal grows (p. 125). The visceral innervation to the branchial sac and to the alimentary canal is derived from the visceral fibre complex and in both cases forms a non-polarized network which controls ciliary activity. Other visceral nervous elements also enter the branchial sac particularly through the endostyle, and the alimentary canal may also have a second innervation. The endostyle is also innervated by anterior nerves arising from the ganglion but these are probably secreto-motor in function. The heart muscle has no innervation and is entirely myogenic but the pericardium has a fine nerve plexus, possibly part of a sensory-motor reflex.

C. *The sensory system*

The sessile mode of life adopted by ascidians makes only relatively small demands upon the sensory system. There are no problems of locomotion and exploratory activity and almost all of the sensory system and its motor responses are concerned with protection or defence. Once metamorphosis is complete the animal has no further choice in selection of the environment and food supply available to it and becomes adapted toward either accepting or rejecting what is presented. The only motor responses available to the animal are to expand the mantle wall and siphons and thereby accept the environment, or to contract the mantle and close the siphons thereby rejecting the environment. For a few ascidians such as *Ciona* the whole body can contract longitudinally thus providing a limited " escape " reaction, but for the majority the rigid limitations of the test restrict their activity primarily to mantle contraction and only limited shortening of the body. Within the framework of these limitations it is to be expected that mechanoreceptors and chemoreceptors should predominate and that photoreceptors might also be present. Sensory cells and afferent fibres have been described by a number of authors but until more precise electrophysiological work can be carried out the exact functions of some of these must remain in doubt.

Hunter (1898) described cells in the endostyle and pericardium which he believed were sensory. These are figured as simple bipolar cells scattered throughout the endostyle and in the raphe of the pericardium. Lorleberg (1907) believed that there were no sense cells in ascidians but that free nerve endings existed scattered throughout the body wall. Das (1948b) also claimed the existence of such free nerve endings throughout the body wall although elsewhere (Das, 1936) he

described multipolar nerve cells throughout the test. In the same paper he suggested that the epithelial cells surrounding the vascular ampullae of the test are tactile receptors innervated by these fibres. However, considering the nature of the test and its formation, it is unlikely that nerve cells or receptor cells are present in it. Das used methylene blue staining to observe these structures but this is an exceedingly difficult technique with which to demonstrate nervous elements in ascidians and is likely to stain other structures as well. Hecht (1918b), who made a careful study of *Ascidia nigra*, was uncertain about the presence of receptors in the test and considered that responses to stimulation of the test are due to mechanical transmission through the test to receptors in the siphonal region, a viewpoint also supported by Fedele (1923a). However, the experiments of Hoyle (1952) who stimulated the test electrically suggest that there may be some sensory innervation in the test, but the innervation need not include the cell body. Fedele (1923a) and Millar (1953a) have described pear-shaped epithelial sense cells surrounded by a number of supporting cells; each sense cell has a vacuole and a distal process which penetrates through the test substance and a nerve fibre leads away from the narrow end of the cell. Bone (1959) has described similar cells in pelagic tunicates and suggests that they are epithelial sensory cells characteristic of tunicates in general. Fedele and Millar both found these cells in *Ciona intestinalis* to be concentrated on the inside and outside of the siphonal margins and elsewhere to a lesser extent. Cells of this sort could account for all of the simple responses of ascidians outlined below, but whether different groups of cells have different physiological characteristics and responses must await the refinement of techniques for studying them.

The epithelial sense cells described above are probably the primary receptor cells of ascidians but two other types of specialized structures have been reported. These are *cupula organs* and *ocelli*. Cupula organs are described by Fedele (1923a) and Millar (1953a) in *Ciona intestinalis* as dome-shaped evaginations arising from the mantle wall just inside the atrial siphon (Fig. 26c). The dome consists of several ordinary sense cells surrounded by supporting cells and the whole thing covered by test material which is drawn out at the apex into a long process or flag. This is the only part of the inner mantle wall where the power of test secretion has been retained. Similar structures are known from pelagic tunicates (Fedele, 1923c; Bone, 1959) but their function remains obscure. The mantle cavity is concerned with two principal types of response. It must respond to the presence of faeces so as to eject them through the atrial siphon; this is a simple reflex action which hardly requires such specialized structures as the cupula organs. The mantle

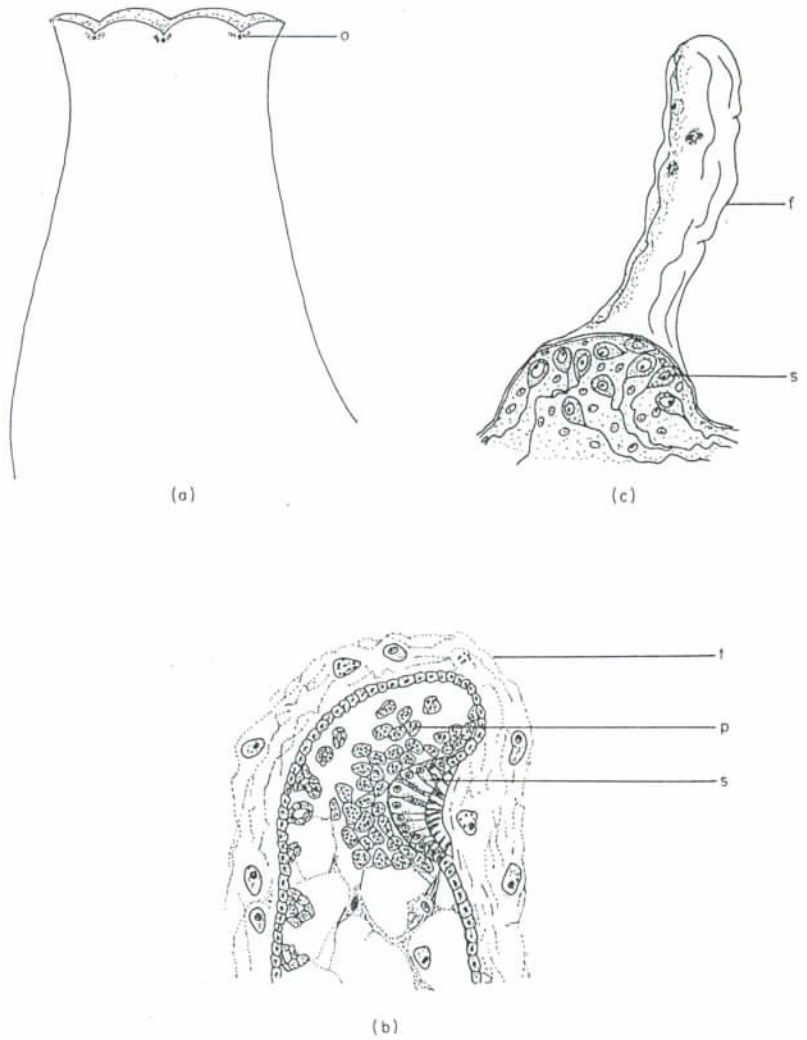


FIG. 26. Sense organs in *Ciona intestinalis*. (a) Position of ocelli between siphonal lobes; (b) an ocellus in longitudinal section; (c) cupula organ. f, flag of test substance; o, ocellus; p, pigment cells; s, sensory cells; t, test. ((a) and (c) redrawn from Millar, 1953; (b) redrawn from Huus, 1937.)

cavity is also concerned with maintenance of water pressure and the speed with which water flows out through the atrial siphon. It is possible that the cupula organs are concerned with this activity (Fedele, 1923a).

The ocelli are pigmented spots found in many, if not all, ascidians in the notches between the lobes of the siphons (Fig. 26). They are very conspicuous in *Ciona intestinalis* and in the very young stages of species of *Ascidia* but in the latter may later be masked by the development of other pigment. Descriptions of these structures have been given by several authors including Seeliger and Hartmeyer (1893–1911), Haffner (1933), Huus (1937), Brien (1948) and Millar (1953a). Each ocellus consists of a small depression lined by sensory cells and the whole backed by an accumulation of pigment granules. Haffner describes minute hair processes arising from the apex of each cell but Millar was unable to detect them. Seeliger also illustrates such processes but arising from the cell boundaries. Neither Millar nor Scudder and Karczmar (1966) were able to detect any nervous innervation of the ocelli but Markman (1958) reported fibres extending from them back to the ganglion. The ocelli are further discussed in the next section.

D. *Photoreception*

The responses of ascidians to light were studied by Hecht (1918b, 1918d, 1926) in *Ascidia nigra* and *Ciona intestinalis* and no further study of significance seems to have appeared since that time. In *Ciona* Hecht showed quite conclusively that the " ocelli " are not light receptors and therefore must have some other function. The name " ocellus " leads to confusion about their function and it would be appropriate to find a new terminology, but perhaps this should wait until their true role is made certain. *Ciona* does, however, show two very distinctive types of response to light, one producing a positive phototropism, the other producing a sharp retraction of the body or " escape " reaction.

Hecht (1918d) showed that the posterior visceral region of *Ciona* is totally insensitive to light, the anterior tip is only sensitive in the region of the ganglion, and the whole branchial region is highly sensitive. If the branchial region is illuminated on one side the mantle musculature of that side will contract so that the animal bends towards the illuminated side. Millar (1953a) suggested that the ocelli may play a role in this phototropic response but in doing so seems to have overlooked Hecht's very convincing studies. Hecht was further able to show that the phototropic response is independent of the cerebral ganglion and unaffected by its removal, and he suggested that purely local pathways, perhaps through a nerve net, must be involved. There are no specialized

light receptors in the branchial region and sensitivity must rest in unspecialized dermal light receptors (Steven, 1963). A phototropic response of this sort has not been reported from any other species, but this is not surprising as the majority of species have pigmented tests which must prevent the light penetrating to the body wall.

Ciona also responds to sudden changes in illumination by a sharp retraction of the body. *Ascidia nigra*, however, only responds in this way if the source of illumination is directed into the branchial siphon. Hecht was able to show that the sensitive region in both cases is in the vicinity of the cerebral ganglion, and this was substantiated for *Ascidia mentula* by Day (1919) (Table XIII). The whole process is the product of a typical photochemical reaction in which a period of sensitization, during which the system must be illuminated, is followed by a latent period quite independent of illumination. The duration of the period

TABLE XIII. THE REACTION OF *Ciona intestinalis* TO ILLUMINATION OF VARIOUS PARTS OF THE BODY

(From Day, 1919)

Site of stimulation	*Oral siphon*	*Atrial siphon*	*Ganglion*	*Body*
No. of tests	70	41	65	35
No. of +ve responses	6	3	58	1

A positive response is indicated by contraction of the siphons.

of sensitization is inversely related to the intensity of illumination. Le Borgne-David (1972) implanted micro-electrodes into the nerve ganglion of *Ciona* and demonstrated the existence of a similar latent period between a light stimulus and the initiation of increased nervous activity in the ganglion. It is of some interest that it was from studies on *Ciona* and related studies on the mollusc *Mya arenaria* L. that Hecht first formulated the general principles relating to the photochemical basis of light sensitivity.

It is not immediately obvious what is the functional significance of these two types of photic response in ascidians. What advantage is to be gained by *Ciona* in orienting itself towards the prevailing light? It would seem of greater advantage to the organism to orient itself in relation to prevailing water movements and yet it remains true that the majority of ascidian species when growing on exposed surfaces tend to grow up towards the surface or to orient the siphons toward the surface which is the direction from which the maximum light intensity is

received. Perhaps the answer is to be found in the retraction response to sudden changes in the intensity of illumination. This response is not very sensitive but it is necessary to focus light on the ganglion on the interior of the siphon before it can be elicited. Possibly under natural conditions it will only function to produce a shadow reflex if the maximum possible illumination is falling on the sensitive regions. Alternatively, ascidians respond through these mechanisms to long term changes in solar or lunar illumination, and therefore orient towards the brighter centre of illumination.

Because of their structural similarity to the simple light receptors of many other invertebrates the ocelli have often been assumed to be photoreceptors as well, but in fact there is no evidence at all to support this assumption. Das (1948a) mentions them as photoreceptors but Hecht (1918b), Millar (1953a) and Millott (1957) all categorically deny a response to light by these structures, although Millar concedes that they might play a role in the phototropic response exhibited by the siphons of *Ciona intestinalis*. Fedele (1923a) also concluded, on purely morphological grounds, that they could not be photoreceptors. If the ocelli are not photoreceptors and if they are innervated as suggested by Markman then they must serve some other function. It is unlikely that they are " vestigial structures " as suggested by Huus (1937). They might, however, be a specialized form of mechanoreceptor engaged in monitoring siphonal activity. Situated in the notch between siphonal lobes they are ideally situated to be subjected to mechanical deformation as the siphons close and open.

E. *The somatic motor system*

Before considering the nature of ascidian response mechanisms it is necessary to examine the nature of the musculature and how it controls the animal. It is probable that *Ciona* represents the most primitive type of ascidian organization (Berrill, 1955; Millar, 1966) and the muscles in that genus may also represent the basic type of system. In *Ciona* the viscera lie posterior to the branchial sac and there are two well-defined opposing sets of body muscles. Six major longitudinal bands run from the siphons to the posterior end of the body and act as body retractors and siphon retractors. These are opposed by a set of much finer and more numerous circular muscles girdling the body throughout its length and modified anteriorly to form sphincter muscles around the siphons. The test in *Ciona* is soft and almost jelly-like in its consistency and can play little if any role in the mechanics of movement. A similar type of musculature persists in most aplousobranch genera, all of which form colonies of zooids, and where the mechanics of movement has not been investigated. In the majority of phlebobranchs other than *Ciona* the alimentary canal has been reflected forward to one side of the pharynx accompanied by a reduction of mantle musculature on that side. This reduction reaches its highest degree in the genus *Ascidia* where mantle musculature is almost entirely absent on the left-hand side. As a consequence of this there has usually been a modification of the musculature on the mantle of the right side and while longitudinal and circular components are still apparent there is a tendency to develop a network of crossing fibres. At the same time the test has become thicker and more rigid than in *Ciona* and, while developing elasticity, has imposed considerable limitations on body movement. *Ciona* is capable of longitudinal retraction and body shortening to a degree quite impossible in the heavier framework of *Ascidia*. Goodbody and Trueman (1969) have likened the movement of the mantle in *Ascidia* to a diaphragm which contracts the mantle cavity but there is no opposing muscle system to enable it to expand again. Hecht (1918a) has further shown that in *Ascidia* the test on the right side is thinner than on the left. In this way the test bends easily on the right during muscle contraction while on the left side it forms an elastic framework to assist return of body shape. The situation in the stolidobranchs, where the gut is reflected forwards and in most cases the test is tough but the musculature complete on both sides, does not appear to have been examined.

Within this framework the types of movement available to ascidians are limited and restricted to : (1) opening and closing of the siphons and, under strong stimulation, retraction of the siphons; (2) expansion and contraction of the mantle wall; contraction of the wall results in expulsion of water from the branchial and atrial cavities through the siphons; (3) longitudinal contraction of the whole body which in *Ciona* results in uniform shortening of the body. In *Ascidia* it tends to produce a bending of the anterior end of the animal toward the right-hand side; this is entirely due to the unequal distribution of muscle and the rigidity of the test.

Two principal types of activity and response in ascidians can be identified, namely direct responses and crossed reflexes. Direct responses are the result of external stimulation of the animal either on the general surface of the test or around the siphons, and result in siphon closure, contraction of the mantle musculature and expulsion of water from the branchial and atrial cavities. If the external surface is stimulated near one of the siphons, that siphon will close first and the other siphon a moment later. This type of response is primarily elicited by mechanical stimulation of the body surface.

The cross reflex type of response is elicited by stimulation of the interior of either siphon, the branchial tentacles and the interior of the mantle cavity adjacent to the atrial siphon. Stimulation of the interior of either siphon results in the following series of events. (1) closure of the opposite siphon, (2) contraction of the mantle wall and (3) closure of the stimulated siphon. The important feature of this activity is that if the branchial siphon is stimulated it is the atrial siphon which closes first followed by mantle contraction causing expulsion of water through the branchial siphon and thereby " flushing out " any foreign matter which may be the source of irritation. Only then does the branchial siphon close. If, on the other hand, it is the atrial siphon or mantle cavity which is stimulated the branchial siphon closes first so that water is ejected through the atrial siphon. This type of reflex is primarily elicited by mechanical stimulation (Hecht, 1918b ; Day, 1919) but Hecht also reports that ascidians are sensitive to chemical, thermal and osmotic stimulation as well as to light.

The nervous mechanisms underlying these responses and reflexes have been studied by a number of workers of whom the most important are Jordan (1908), Polimanti (1911), Kinoshita (1910, 1911), Hecht (1918b), Fedele (1923b, 1937b), Ten Cate (1928), Bacq (1935a) and Hoyle (1952). The earlier work is reviewed by Hoyle who also carried out a series of very precise experiments on *Phallusia mammillata* and *Ascidiella aspersa* aimed at clarifying the nature of the integrative mechanism involved in direct responses. He stimulated the animals by means of electrodes placed in the test and recorded the responses of the animals by a mechanical monitoring of siphonal activity. This method of stimulation may be criticized on the grounds that sense cells are not common in the test except immediately around the siphons and hence considerable resistance must be overcome in the test material. This is emphasized by the fact that in *Phallusia* a stimulus strength of the order of 100 V was required in order to elicit a response and 25 V in *Ascidiella aspersa*. Nevertheless the nature of the results obtained suggests that the techniques were not at fault. From these investigations Hoyle found that the type of response initiated in an animal varied considerably and was dependent on the previous history of the animal and the stage it has reached in the cycle of spontaneous squirting. Spontaneous rhythms are discussed elsewhere (p. 21) and in *Phallusia* contractions of the mantle occur about once every 6 to 9 minutes. Electrical stimuli given just before or after a spontaneous contraction may elicit an abnormal series of responses and in addition there are refractory periods when the animal does not respond at all to artificial stimuli. Providing these variations are taken into consideration it is

possible with the techniques used by Hoyle to show that the ascidian nervous system has the following characteristics :

(1) Single stimuli evoke only a minimal response but two successive shocks produce a strong contraction (Fig. 27*a*).

(2) Above the threshold the strength of a stimulus is unimportant and the response is dependent on the frequency of stimulation. With successive shocks there is increasing contraction of the siphon until it is fully closed and withdrawn (Fig. 27*b*).

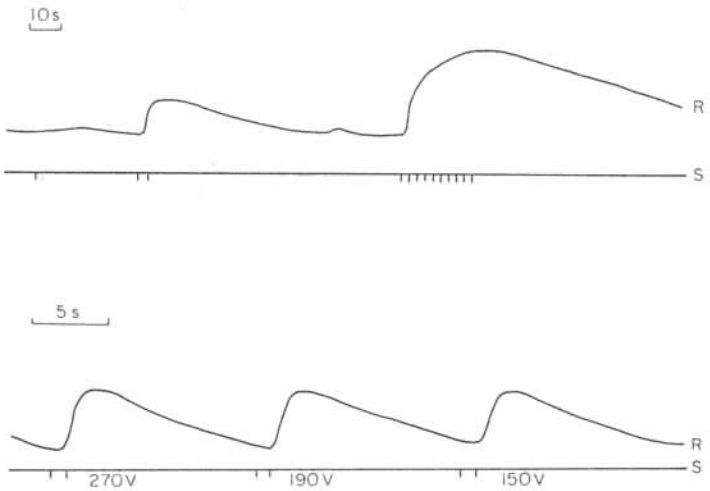


FIG. 27. (a) Responses (R) of the branchial siphon of *Ascidiella aspersa* to a single shock, to a pair of shocks and to a consecutive series of ten shocks (S). (b) Responses (R) of the branchial siphon of *Phallusia mammillata* to pairs of stimuli (S) at various voltages above the threshold (145 V). (Redrawn from Hoyle, 1952.)

(3) The types of response elicited under these conditions of external stimulation are independent of control by the cerebral ganglion. After extirpation of the ganglion both branchial and atrial siphons respond strongly to electrical stimuli.

We can deduce from this that these ascidians exhibit an all or nothing through conduction system which is subject to facilitation very much as is found in actiniarians. There is, however, at the present time no conclusive evidence in favour of the existence of a nerve net system (for a discussion of this see Fedele (1937a,b) and Bullock and Horridge (1965)). No matter what is the nature of the excitable system the

functional significance is clear. Ascidians must frequently be subjected to minor mechanical or other stimuli which are of little concern to the animal but persistent stimuli, regardless of strength, evoke a defensive response. According to Hoyle the first small response is a slight closing of the siphons, the second and large contraction is the contraction of the mantle musculature and squirting of water from the branchial sac. Subsequent contractions represent siphon closure and withdrawal. One of the features of the system is that it is easily exhausted and immediately after one burst of stimulation it responds feebly to a fresh burst. Persistent stimulation results in relaxation of the animal and a period of relative inexcitability. Hoyle considered the possibility that such exhaustion might involve a junction transmitter, either by progressive depletion of the transmitter concerned or by its accumulation at the junction and failure of the system to remove it. He concluded that both possibilities are unlikely and suggested that fatigue might be in the muscle system itself. There is still much controversy over the nature of synaptic transmitters in ascidians but recent work points towards a cholinergic system in which cholinesterase activity is low (Florey, 1963, 1967). Such a system might permit the accumulation of transmitter substance at the junction and give rise to fatigue (*vide infra*).

Hoyle's experiments throw considerable light on the nature of the ascidian response mechanism and its ability to elicit fast withdrawal responses by the development of facilitation in the nervous system. However, his work throws no light on the most important of all nervous responses—the crossed reflex in the siphons, and its nervous control.

There has been no detailed investigation of the nervous control of the crossed reflex and our understanding of it is limited to the results obtained when the cerebral ganglion is removed as has been done by Hecht (1918b), Day (1919), Yamaguchi (1931), Bacq (1934b, 1935a), Das (1948a) and Florey (1951). All of these workers are agreed that ablation of the ganglion destroys or impedes the cross reflex but does not interfere with the direct response. In view of the nature of the innervation of the siphons from the ganglion (p. 89) this result is not surprising, but removal of the ganglion also results in a general loss of tonus in the siphonal muscles, increased tonus in the mantle musculature and more frequent squirting (Hecht, 1918b; Day, 1919; Bacq, 1934b). Florey (*loc. cit.*) considered that the outer layers of circular muscle only are innervated by central nerve connections and that inner muscle layers have only peripheral connections through a nerve net. In the absence of the ganglion stimulation of one siphon will still result in closure of the other through such connections.

F. *Synaptic transmission*

Several workers have attempted to identify the chemical nature of the substances involved in synaptic transmission, their research being focused on an attempt to determine whether cholinergic nerves are present in the system. The problem was first tackled by Bacq (1935b, 1935c, 1939, 1941, 1947) who pointed out that if cholinergic nerves are present it should be possible to identify acetylcholine or related substances in extracts of nervous tissue and to identify cholinesterase in whole tissue or muscle extracts. In addition nervous transmission or muscle action should be inhibited by curare, atropine and similar blocking agents, while the response mechanism should be attenuated in the presence of eserine which inhibits cholinesterases. He was unable to identify any acetylcholine in ascidian extracts and cholinesterase was only found in small quantity in whole animal extracts of *Ciona intestinalis* or in the muscle of one species studied, *Styela plicata*; eserine had no effect on *Ciona intestinalis* and *Halocynthia papillosa* and Bacq concluded that cholinergic nerves were absent in these animals. Similarly no effect on muscle action occurred in the presence of curare. (For summaries of his findings see Bacq, 1941 and 1947.)

Durante (1956) reported the presence in embryos of *Ciona intestinalis* of cholinesterases in the muscle system only and not in the nervous system, but she found that these enzymes disappeared completely at the time of metamorphosis. Scudder and Karczmar (1966) also working with *C. intestinalis* used histochemical techniques to seek cholinesterases and obtained positive results but the enzyme appeared diffusely spread in tissues other than nerves and was in very low concentrations. Furthermore, while this substance hydrolyzed acetylcholine it was unaffected by normal inhibitors of cholinesterases and therefore must be different from the normal cholinesterases. They did also report finding a cholinesterase in the ganglion of *C. intestinalis* but came to the rather curious conclusion that it was probably derived from detritus in the neural gland which was homogenized with the ganglion. We shall see later (p. 112) that it is most unlikely that detritus would accumulate in the neural gland.

Scudder *et al.* (1966) used the siphonal closing mechanism as an indicator of muscle activity and tested the effects of acetylcholine and anticholinergic agents by direct injection into the surrounding water. Their results led them to believe that cholinergic nerves were absent, but there is nothing in their experimental techniques to suggest that the substances presented had an opportunity to reach the neuromuscular system involved.

Florey (1963) criticized Bacq's approach on the grounds that he had homogenized whole animals and not the nervous tissue. Florey removed the ganglion from *C. intestinalis* and made extracts of it. He reported concentrations of acetylcholine in the order of 20 to 120 μg/g wet weight of ganglion and concluded that such high concentrations must indicate a preponderance of cholinergic nerves. This is supported by his finding that the muscles contract in the presence of acetylcholine, although the effect is not potentiated by eserine. However, he did find that cholinesterase activity in the muscles is low and suggested that this might explain the rather slow rate of relaxation of ascidian muscles following contraction. However, there does not seem to be any good evidence to confirm that muscle relaxation is slow. The re-expansion of a whole ascidian after retraction is slow, but this is due, at least in part, to the weakness of the opposing muscle system.

In a later paper Florey (1967) describes some additional careful experiments in support of the presence of cholinergic nerves. He made isolated muscle preparations and found that they responded to acetylcholine by contraction and were sensitive to concentrations of the drug down to 10^{-6} to 10^{-7} g/ml. This reaction was blocked by atropine, *d*-tubocurarine and other known cholinergic blocking agents. In another experiment Florey stimulated a muscle preparation and found that it released quantities of acetylcholine equivalent to 10^{12} molecules per impulse. The released acetylcholine was assayed by means of a molluscan heart preparation (Fig. 28). In a separate experiment Florey stimulated electrically a muscle preparation which had been pinned in the middle to the bottom of a wax dish. The preparation responded to repetitive stimulation by slow contractions, but this response was totally abolished in the presence of *d*-tubocurare. Thus the same agent which blocks the action of applied acetylcholine also blocks neuromuscular transmission and further substantiates the conclusion that *C. intestinalis* possesses cholinergic nerves.

Florey's experiments are very convincing, but nevertheless in the light of Bacq's experiments and the findings of Scudder and Karczmar there still remains an element of uncertainty concerning the presence of cholinergic nerves in ascidians. (See also p. 52 for a discussion of acetylcholine in cardiac regulation.)

Passing reference may be made here to the presence in ascidians of 5-hydroxytryptamine, another possible neuro-transmitter. Welsh and Moorehead (1960) found very low levels of 5-hydroxytryptamine in the cerebral ganglia of *Chelyosoma* sp. but not in *Molgula manhattensis*.*

* Welsh and Moorehead named their ascidian *Chelyosoma productura*. No such species appears to exist and it is apparently a mis-spelling of *C. productum* Stimpson.

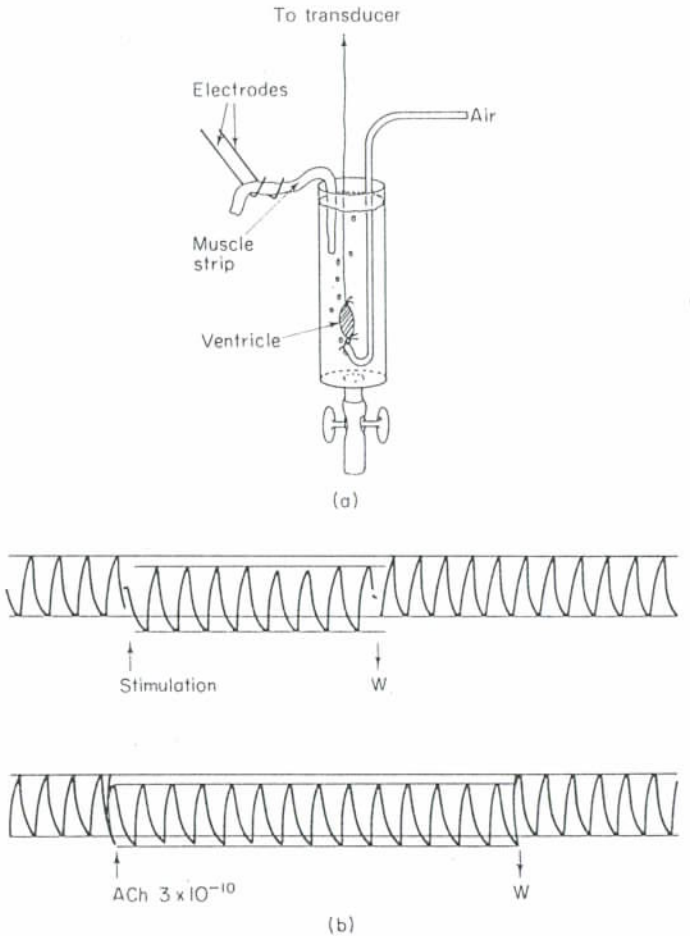


FIG. 28. Diagram of the experimental set-up (a) used to demonstrate the release from *Ciona* muscle strips of an acetylcholine-like agent into the saline medium bathing a *Protothaca* ventricle. Records of the ventricular beat are shown in (b). The recorder was stopped for one minute during and after stimulation of the muscle strip and for 30 seconds during the periods of washing and during and after the application of acetylcholine. The frequency changes observed are of the order of 10%. Horizontal lines have been drawn over the records to facilitate recognition of the change of muscle tone. The concentration of acetylcholine is given in terms of g/ml. W = Washing. (Reproduced with permission, from Florey, 1967.)

Welsh and Loveland (1968) found no trace of the substance in the ganglion of *Ciona intestinalis* but found it in the gut (0·49 μg/g wet wt of tissue), pharynx (0·12 μg/g), endostyle (1·67 μg/g) and heart (0·53 μg/g). They suggest that it may not have been revealed in the ganglion if it occurred in very small traces. The high levels in the endostyle are puzzling but Welsh and Loveland suggest that both here and in the pharynx it may play some role in ciliary co-ordination. They believe that in the oesophagus the 5-hydroxytryptamine may be localized in the chromaffin cells identified by Lison (1933) and Gerzeli (1963) and point out that such a localization would be comparable to that found in vertebrates. Whether or not this is true it is clear that the localization of 5-hydroxytryptamine in these ascidians is not such as to suggest that it is an important neurotransmitter. On the other hand, its localization in pharynx, endostyle and oesophagus, the areas of maximum ciliary activity in ascidians, may be particularly significant. (For a discussion of 5-hydroxytryptamine and other transmitters, see Florey, 1961.)

G. *Neurosecretion*

In order to prove a neurosecretory function in any animal it must be possible to demonstrate the production of a secretion in nerve cells, that this secretion is liberated from the cell or the terminal point of its axon and that it directly or indirectly activates an effector organ or metabolic process in the body. No such function has been unequivocally demonstrated in ascidians, but several workers have reported the presence of granules in nerve cells and axons of the cerebral ganglion (Dawson and Hisaw, 1964; Thiebold and Illoul, 1966; Lane, 1968; Chambost, 1969), although Scharrer (1953) was unable to demonstrate such granules in *Ciona intestinalis*.

In a detailed study of ten species of ascidians, Dawson and Hisaw found granule bearing cells amongst other nerve cells at the periphery of the cerebral ganglion. The granular material stained with Halmi's (1952) modification of Gomori's (1950) aldehyde fuchsin, but the appearance of the granules was irregular and inconsistent, perhaps indicating cyclical activity. Two quite distinct types of granular cell were identified, one in pleurogonid ascidians, the other in enterogonid forms. In the Pleurogona there are large secretory cells in which the granular material is dispersed throughout the cell and stains purple; the granular material is sometimes partially displaced in the cell by a vacuole. In the Enterogona the granular material stains green, and is always paranuclear and there are no vacuoles. There is no proof that these granules are concerned with true neurosecretion, but if they are their most likely function would be associated with reproduction. It is

therefore particularly interesting that different types of granule bearing cell should occur in Pleurogona and Enterogona. In the Pleurogona the gonads are found on the mantle wall and as far as we know have no connection with the dorsal cord (*vide infra*) but in Enterogona the gonad is found within the visceral loop and appears to have the terminal bulb of the dorsal cord embedded within it. In passing we may note that Dawson and Hisaw commented that "the best preparations (in *Chelyosoma*) were obtained in animals with mature gonads", so lending credence to the idea that there may be some relationship between the granules and sexual activity. Thiebold and Illoul found similar granules, stained with aldehyde fuchsin and with Alcian Blue, in the cerebral cortex of *Ciona intestinalis*. They located faint traces of similar granules in fibres of the cerebral medulla thus suggesting axon transport. These workers also compared their preparations with similar ones from the bivalve *Mytilus galloprovincialis* L. and concluded that the concentration of granular material in the ascidian must be very low in comparison to the mussel. Similar granules in *Ciona intestinalis* have also been found by Chambost (1969). Lane (1968, 1972) concluded from electron micrographs that the granules were formed from the Golgi body and that acid phosphatases were also present in the cells.

There is thus very clear evidence for the presence of granular material in cortical cells of the cerebral ganglion of all species examined but we have no idea as to what function it may perform, whether it may be the precursor of a substance transported to axonal terminations, secreted within the ganglion or whether it may have some other function. There is indeed nothing at all to prove that they are true neurosecretions. Clark (1966) has pointed out that all neurones produce a variety of vesicular secretions and in advanced animals there may be a high degree of structural specialization in the nervous system which permits the neurosecretory system to be readily identified. In animals with simple nervous systems this is not so. Clark concludes: "An element of doubt must therefore remain about all cells with stainable inclusions that are discovered in the central nervous system of primitive animals."

XII. The Neural Gland

A. *Structure and cyclic activity*

The neural complex of ascidians consists of the nerve ganglion (p. 87) and a neural gland lying in the mantle wall between the two siphons and surrounded by a haemocoelic space. In the majority of ascidians the ganglion lies dorsally to the gland and closely apposed to it, but in stolidobranchs the gland is dorsal to the ganglion. Because of

the close anatomical and embryological association between them it seems reasonable to conclude that there may also be a functional connection.

The neural gland was first described by Hancock (1868) and has been a subject for study and controversy ever since; the older literature has been reviewed by Pérès (1943) and Godeaux (1964a). The gland consists of a series of folds or evaginations arising from the lower side of a duct or canal which leads from the dorsal side of the pharynx inwards toward the nerve ganglion (Fig. 25). The opening of the canal is usually expanded into a ciliated funnel which is often folded to form either a simple C-shaped aperture or sometimes a more complex spiral structure and usually forming a slight projection, the dorsal tubercle. The dorsal tubercle then represents the ciliated opening of the neural gland and it is important to recognize its position relevant to other structures of the branchial sac (Fig. 29). It opens dorsally immediately anterior to the junction of the peripharyngeal grooves and the dorsal lamina, and immediately inside the branchial siphon posterior to the branchial tentacles. It is thus perfectly placed either to "test" material coming in to the branchial sac or to discharge any exocrine secretion into the beginning of the mucus-wrapped food cord.

According to Godeaux (1964a), Pérès (1943) and Georges (1971) the duct from the gland is lined by a cuboidal epithelium which is ciliated for only a part of its length. This epithelium continues along sides and top below the nerve ganglion while on the ventral side it proliferates the glandular epithelium.

It is now generally agreed that the epithelium of the gland undergoes cyclical changes of activity in which the gland changes from a loose reticulated form (Fig. 30*a*) to an expanded compact form (Fig. 30*b*) resulting from vacuolization of the epithelial cells and increased turgor throughout. Although morphological changes of this sort have been known for a long time (Metcalf, 1900; Butcher, 1930) the first comprehensive account is that of Pérès (1943). According to this author in the reticulate phase the lobes of the gland are sufficiently flattened for large blood spaces to exist between neighbouring lobes but these are substantially reduced as the gland becomes turgid. Pérès claims that starting from a simple cuboidal epithelium there is rapid multiplication of cells which slough off into the lumen of the gland where they assume a star shape, put out pseudopodia and form a network between the free cells and those of the intact epithelium. Some of these cells become vacuolar and phagocytic and are augmented by blood phagocytes which penetrate through the epithelium to the lumen. Both types of vacuolar cell phagocytize other cells in the lumen and as more cells proliferate

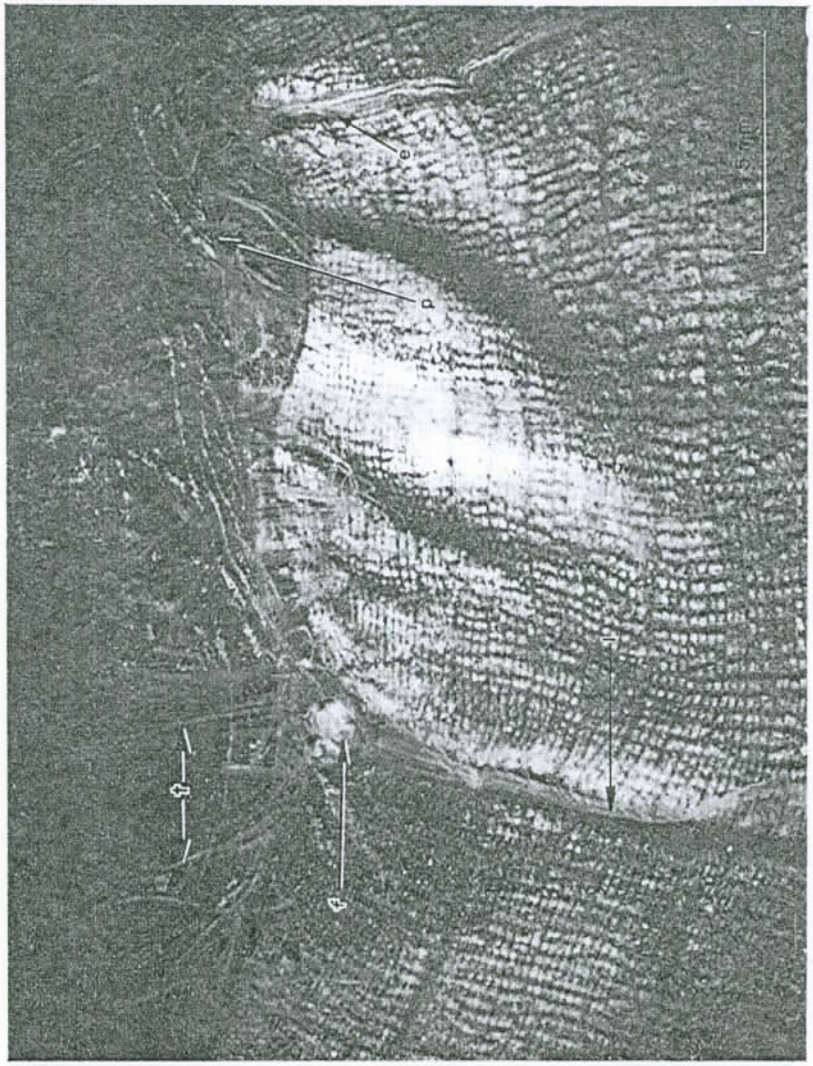
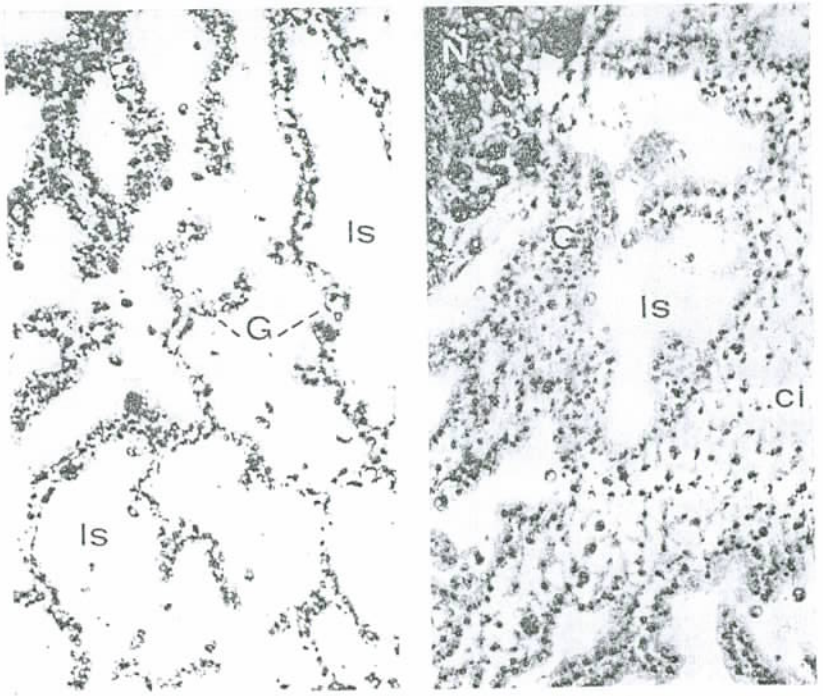


FIG. 29. The positional relationships of the branchial tentacles (t), endostyle (e), peripharyngeal groove (p), ciliated funnel (f) and dorsal lamina (l) in *Ascidia interrupta*.



(a) (b)

FIG. 30. The neural gland of *Ciona intestinalis* showing (a) the reticulated phase and (b) the compact phase. G, glandular tissue; ls, blood space; ci, star-shaped cells accumulating in the lumen of the gland. (Reproduced with permission, from Georges, 1971.)

and turgidity of the gland increases there is a discharge of cellular material from the gland. Pérès is emphatic that the ciliary currents are such that material can be carried out of the gland to the pharynx, and he recognizes three stages in the cycle: (1) The reticulate stage with empty gland; (2) proliferation, sloughing and vacuolization; (3) discharge of the gland and return to the reticulate phase.

More recently the fine structure of the epithelial cells and their cycle of activity has been studied with the aid of electron microscopy by Swan and Olsson (1965), Chambost (1969), Georges (1967, 1970, 1971) and Lane (1971). The epithelium is separated from the neighbouring blood sinus not only by its own basement membrane but also by an outer connective lamella in which fine fibres may be discerned. Swan and Olsson reported cells, in the lamella, containing numerous membrane bound granules with a mean diameter of about 300 mμ, and small aggregations of ribosome-like particles. They considered that these cells might be secretory.

There is general agreement between Georges and Lane that the intact epithelial cells are about 5 μ high with a rounded nucleus and are held together laterally by desmonemes. Basally the cell membrane lacks folding, is not microvillous and is not associated with dense aggregations of mitochondria as might be expected in excretory cells. There are numerous small vesicles in the cell, some optically clear to the electron microscope, others more dense and apparently enclosing fibrillar material. Similar vesicles were noted by Swan and Olsson who believed that the contained material was due to phagocytosis. The Golgi-body is relatively small with a few flattened saccules and some small vesicles, thus suggesting that the cell at this stage is not in active secretion. The endoplasmic reticulum has irregular vesicles.

When the gland enters the compact phase the epithelial cells swell and become vacuolated and by loosening of the desmonemes are separated from one another and freed into the lumen of the gland. The content of the vacuoles is uncertain but according to Lane (1971) thiamine pyrophosphatase can be demonstrated in some of the Golgi saccules and Golgi vesicles while acid phosphatase is found in these as well as in other vacuoles. The presence of acid phosphatase suggests a lysosomal origin for at least some of the vacuoles. Lane also reported that in the highly vacuolated phase distension of the rough endoplasmic reticulum might indicate active protein synthesis producing hydrolytic enzymes in the vacuoles.

The evidence so far confirms the fact that the epithelium of the neural canal undergoes periodic changes from a simple cuboidal state (the reticulated phase of the gland) to an enlarged highly vacuolar state (the compact phase of the gland) culminating in sloughing of the epithelium and breakdown of the cells which must thus release in the lumen of the gland the contents of their vesicles. Several authors (Pérès, 1943; Godeaux, 1964a; Georges, 1971) report that large phagocytic cells, probably of vascular origin, also invade the lumen of the gland at this time, passing through the connective lamella and insinuating themselves between the epithelial cells. Both Pérès (1943) and Georges (1971) have found a circadian rhythm in this cycle but while Pérès found a single cycle in 24 hours in the Mediterranean, Georges

reports that at Roscoff, the same species (*Ciona intestinalis*) has two cycles in 24 hours.

There are differing opinions concerning the fate of the cells discharged into the lumen of the gland. Pérès (1943), who believed that the gland was primarily phagocytic, insisted that the ciliary currents in the canal were such as to create an outward movement down the centre of the canal carrying material from the gland to the pharynx. This is supported by Millar (1953a) who showed that cilia around the periphery of the ciliated funnel draw water inwards. The currents only make a shallow penetration before being directed out again by cilia of the median border. These beat outward and drive water away from the funnel and thus the water flow is such as to wash cells out of the funnel. However, Georges (1970, 1971) is equally emphatic that the ciliary currents of the canal beat inward from pharynx to gland and considers that the contents of the cells may diffuse into the blood stream and thus permit the gland to function as an endocrine organ. She likens the connective sheath around the gland to the tunica propria of the insect prothoracic gland and suggests that it may be intimately concerned with the passage of material from gland to blood stream.

B. *Origin of the neural gland*

Before considering the function of the neural gland it is relevant to examine its embryological origin and its relation to the nerve ganglion. According to Elwyn (1937), who also reviews the older literature, the anterior position of the neural tube after closure of the neuropore divides longitudinally into two tubes. The right side immediately gives rise to the sensory vesicle of the larva while the tube on the left side becomes the " neurohypophysis " and will later give rise to both neural gland and nerve ganglion. The " neurohypophysis " is at first blind at both ends but the anterior end grows forward and eventually forces an opening into the anterior portion of the pharynx. Elwyn shows micrographs of sectioned material to demonstrate that the resultant duct of the neural gland is entirely of " neurohypophysial " origin and neither pharyngeal endoderm nor stomodaeum contribute any tissues to the gland or duct. Furthermore the opening into the pharynx is complete before the stomodaeum has broken through to the pharynx. The nerve ganglion is formed by proliferation from the dorsal wall of the neurohypophysis except in Stolidobranchiata where it proliferates from the ventral wall.

There is thus a very close link between nerve ganglion and neural gland in development and they remain in close association to one another in adult life. The origins and close association of these two structures has led many workers to consider the neural gland as the homologue of the vertebrate pituitary, but if this is so it can only represent the pars nervosa and not the whole pituitary. Furthermore the vertebrate pituitary is an entirely endocrine structure while the neural gland appears to be primarily exocrine.

C. *Functions of the neural gland*

In spite of extensive research by a number of workers we appear still to be very far from understanding the true functions of the neural gland. Reviews on this subject have already been written by Butcher (1930), Huus (1937), Carlisle (1951) and Dodd and Dodd (1966). We have seen in the previous section that on purely embryological grounds it cannot be homologized with the whole pituitary, but only with the posterior lobe. Furthermore the histological evidence presented above suggests an exocrine rather than an endocrine function. Nevertheless numerous attempts have been made to establish the presence of pituitary-like hormones in the neural gland or neural complex (i.e. neural gland plus ganglion). These studies may be divided into those seeking posterior pituitary hormones, such as oxytocin, vasopressin and melanocyte stimulating hormone, and those which seek the presence of adenohypophysial hormones such as gonadotrophin; they are treated separately below.

1. *Oxytocin and vasopressin*

Butcher (1930), on the basis of a single extract of fifty glands from *Ciona intestinalis*, claimed that oxytocin was present in the complex at a strength comparable to mammalian pituitary extracts. Bacq and Florkin (1935, 1946) also reported the presence of oxytocin as well as a vasopressin and a melanocyte stimulating hormone (MSH), but Pérès (1943, 1947a,b) found that oxytocic activity, while admittedly present, was not confined to the neural complex but was found in many other tissues; he concluded that it was probably a histamine and not an oxytocin. Sawyer (1959) also demonstrated the presence of oxytocic activity in both neural complex and in other tissues, but he found sufficient differences in its physiological behaviour to lead him to conclude that it was not actually oxytocin, but a substance which mimics oxytocin. Sawyer was unable to detect any vasopressor activity in neural complex extracts, and found a very weak anti-diuretic activity in *Pyura* only, which he considered to be non-specific.

The most critical study of this problem has been carried out by Dodd and Dodd (1966) who prepared an extract from the neural complexes of about 8000 specimens of *Ciona intestinalis* which was assayed for oxytocin, vasopressin, ADH and MSH. In tests for oxytocin both guinea-pig and rat uterus preparations were used for assay and showed that a smooth muscle contractant is present in the neural complex. Assays on guinea-pig uterus suggested an activity equivalent to 30 ImU of oxytocin, while rat uterus preparations suggested an activity equivalent to only 0·4 ImU. This might be due to the presence of two different active substances acting on the different assay preparations. Furthermore extracts of female gonad, male gonad plus stomach, and branchial region of the ascidian had a similar action on rat uterus, but

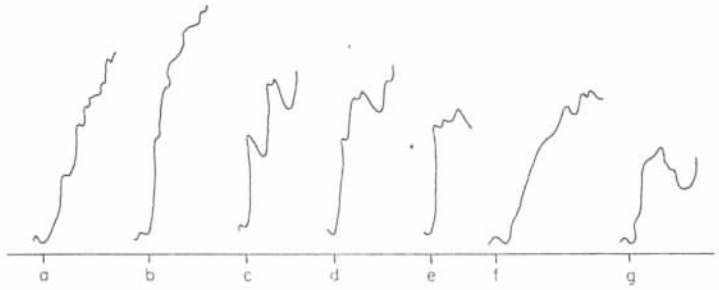


FIG. 31. The existence of a smooth muscle contractant in the neural complex and branchial region of *Ciona* illustrated by the effects of extracts on the guinea pig uterus. a and b, injections of 50 ImU Pitocin; c, d and f, injection of 30 ImU Pitocin; e, injection of 4·0 mg of an extract of neural complex; g, injection of 4·0 mg of an extract of the branchial sac. (Redrawn from Dodd and Dodd, 1966.)

were slightly less potent—approximately 50%, 50% and 70% respectively of the potency of neural complex (Fig. 31). Although a smooth muscle contractant is present in the ascidian neural complex, it is probably not oxytocin itself. Dodd and Dodd give the following reasons for concluding that it is different. (a) The active principle has a much shorter latent period of action than oxytocin (see Fig. 32). (b) It survives storage at pH 7 in the cold for several months, and exposure to air at pH 3·5 at room temperature for a year, procedures which destroy posterior pituitary activity. (c) It is resistant to boiling with caustic soda and incubation with sodium thioglycollate at concentrations which destroy posterior pituitary oxytocin. (d) It is resistant to ultraviolet light.

It seems therefore that the active principle in ascidian neural complex and causing smooth muscle contraction is different from oxytocin

and Dodd and Dodd show evidence that the effects cannot be caused by potassium, acetylcholine, histamine or 5-hydroxytryptamine.

Dodd and Dodd did not find any vasopressor activity in neural complex extracts assayed on rats, but did find that these extracts and those of control tissues, caused an initial fall in blood pressure after injection into rats. This phenomenon was also noted by Bacq and Florkin (1935). Such a depressor activity could be due to histamine but Dodd and Dodd note that the effect is such that if it were due to histamine alone this substance would have to represent 2·6% by weight of the neural gland tissue which is most unlikely.

Finally Dodd and Dodd found no evidence for the presence of an anti-diuretic hormone or a melanocyte stimulating hormone in neural

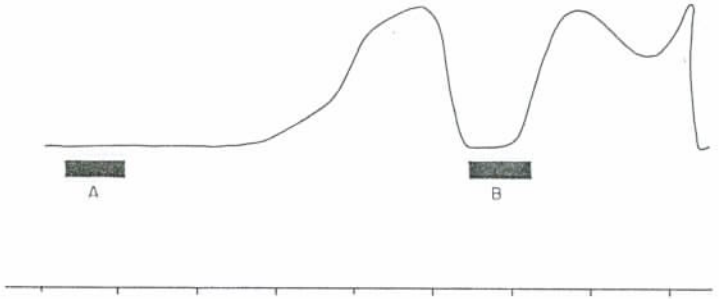


FIG. 32. The difference in latency between extracts of *Ciona* neural complex and Pitocin on the rat uterus. (A) Period of injection of Pitocin (0·8 ImU); (B) Period of injection of neural complex extract (2·5 mg). Time scale 10 seconds. (Redrawn from Dodd and Dodd, 1966.)

complex extracts. The absence of MSH (Fig. 33) is in conflict with the findings of several other authors (Bacq and Florkin, 1935; Abramowitz, 1937; Carlisle, 1950a; Carlisle and Olsson, 1965). Dodd and Dodd used a specially selected strain of *Xenopus laevis* for bio-assay of MSH, in which the reactions of the chromatophores are well understood, and they point out that the reactions of chromatophores in test animals used by these other workers are poorly known and therefore interpretation of bio-assay work in which they are used is very difficult.

Table XIV summarizes the work of Dodd and Dodd in respect of posterior pituitary hormones. It is clear from this table and the precision of their experimental techniques that there is little evidence for the existence in the ascidian neural complex of any of the normal posterior pituitary hormones. There is, however, clear evidence in their work and in the work of other authors cited here, for the presence of a smooth

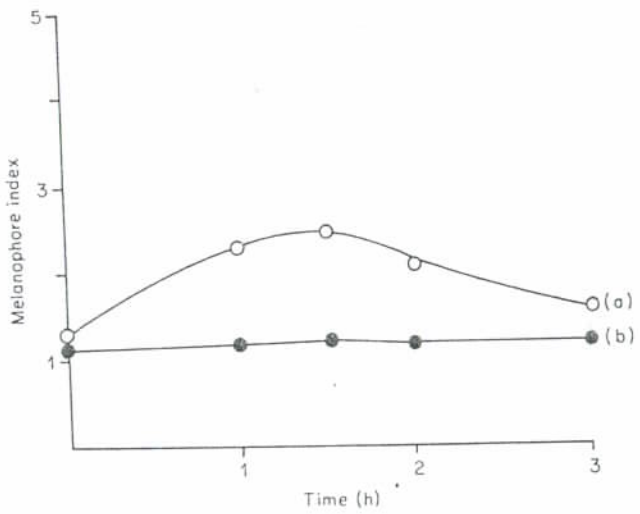


FIG. 33. Time scale graph showing the average expansion in the web melanophores of a group of *Xenopus laevis* after injecting (a) 0·125 μg of International Standard Posterior Lobe Pituitary powder (IS.PLP), (b) 2·5 mg of neural complex of *Ciona*. A melanophore index of 1 is fully contracted, 5 is fully expanded. (Redrawn from Dodd and Dodd, 1966.)

TABLE XIV. MAXIMUM CONCENTRATION OF VARIOUS POSTERIOR PITUITARY HORMONAL TYPES IN EXTRACTS OF THE NEURAL GLAND OF *Ciona intestinalis* COMPARED WITH SIMILAR EXTRACTS OF INTERNATIONAL STANDARD POSTERIOR LOBE PITUITARY (IS.PLP)

Hormonal Type	*Max. conc. in neural gland (ImU/mg)*	*Comparative potency of IS.PLP*	*Bio-assay material*
Oxytocin	7·5	250	Guinea-pig uterus
	0·4	5 000	Rat uterus
Vasopressin	1·0	2 000	Anaesthetized rat
ADH	0·01	200 000	Water-loaded rat
MSH	0·1	20 000	Web melanophores of *Xenopus laevis*

Concentrations in neural gland extract are expressed as International milliunits per milligram. (Data from Dodd and Dodd, 1966.)

muscle contractant which mimics the activity of oxytocin. Because of the small size of the neural gland it is not possible to be certain of working with it alone and most assay work is carried out on the complex of neural gland and ganglion. In consequence we cannot be sure whether the smooth muscle contractant (SMC) resides in the tissues of the neural gland or in the neurosecretory cells of the ganglion (Dawson and Hisaw, 1964; Lane, 1968). Furthermore SMC activity is also found, though less strongly, in other tissues of the animal and it may therefore be a generally distributed substance in ascidian tissues or it may be a hormone maintained in circulation in the blood. It is not clear what function such a substance might serve in the living ascidian, particularly in view of the fact that all ascidian muscle is non-striated. However, it is not beyond the bounds of possibility that it might be specific for one specialized muscle and maintain tonus in the sphincter guarding the genital opening and preventing premature release of sperm and eggs (see below). This would necessitate a mechanism for the inhibition of secretion and relaxation of the muscle at spawning.

2. *Gonadotrophins*

In spite of the fact that the embryological evidence demonstrates that there is no stomodeal component in the formation of the neural gland and that therefore it can have no morphological homology with the vertebrate anterior pituitary a great many workers have sought evidence for the presence of gonadotrophins in the neural complex. The resulting information produces a confusing and conflicting array of data. Some of the confusion probably arises from a misunderstanding of the ovarian cycle and spawning behaviour of ascidians. Pérès (1954) has drawn attention to the fact that in sexually mature ascidians, and he refers specifically to *Ciona*, the ovarian cycle can be divided into two parts, an intercycle and a period of germinal growth. During the intercycle the germinal epithelium is reduced and a somatic epithelium expands; oogonia are rare and the remaining oocytes break down. When the period of germinal growth commences there is a rapid development of oogonia and production of oocytes which are discharged into the lumen of the gonadial cavity and complete their maturation there or in the oviduct. Egg production then becomes a continuous process and the eggs are stored up in the oviduct which prior to spawning becomes greatly distended. It seems then that the liberation of gametes at spawning is not a function of increased gonadial activity but must be the result of relaxation of the sphincter guarding the gonopore together with contraction of the mantle muscle to increase the hydrostatic pressure in the mantle cavity and thereby squeeze out the

gametes. These facts must be taken into consideration when interpreting the results of experiments on the hormonal control of reproduction.

Although Huus (1937) did not postulate the presence of a gonadotrophin, and in fact considered the neural gland as the homologue of the posterior pituitary only, he suggested that the gland controlled the reproductive process. He considered that the ciliated funnel would collect stimulating substances (i.e. gametes from neighbouring ascidians of the same species) and these would stimulate a secretion from the neural gland which, transported in the blood stream to the gonads, would stimulate discharge of gametes. In the same year Hogg (1937) using *Polycarpa obtecta* Traustedt carried out the first experiments aimed at identifying a gonad stimulating activity in the neural complex, by injecting extracts into three mice while three controls received injections of saline. Five days later autopsy revealed that on average the gonads of the experimental animals were three times as heavy as those of the controls and the follicles in the former were larger and more numerous than in the latter. Carlisle (1950c), using *Ciona intestinalis* and *Phallusia mammillata*, obtained similar results using three test mice and two controls into which he injected extracts of tissues other than from neural gland. He also reported that extracts of neural gland injected into male frogs caused discharge of sperm. These results are in conflict with those of Benazzi (1939) and Dodd (1955). Benazzi obtained negative results when extracts of neural gland were injected into five mice and he was unable to produce ovulation in newts when injected with extracts of neural complex. Dodd obtained only one positive result from ten mice injected with extracts from neural complexes of *Ciona intestinalis*, and no positive results following similar injections into male *Xenopus laevis*. Dodd suggested that seasonal variation might account for these discordant results but at the same time points out that his material came from ascidians which were sexually ripe and that therefore if gonadotrophin exists at all it should have been revealed in his own experiments.

All of the above experiments were based on the premise that if a gonadial hormone is present in the ascidian neural complex it will have properties similar to those found in vertebrates. But this may not be a reasonable premise and experimentalists may be on much safer ground if they use ascidian material upon which to test their extracts, at least in the first instance.

In an interesting but controversial series of experiments Carlisle (1951) attempted to show that the neural gland and nerve ganglion were intimately associated in the neuro-humoral control of gamete-release and the synchronization of spawning. Following the lead already

given by Huus (*loc. cit.*) Carlisle carried out experiments along the following lines using *Ciona intestinalis* and *Phallusia mammillata* as experimental animals.

(a) Mammalian chorionic gonadotrophin was injected into test animals and distilled water into controls. Regardless of the site of injection the gonadotrophin stimulated discharge of gametes about 20 hours later (see also Carlisle, 1954, with reference to these experiments). If the heart had previously been destroyed and the blood drained, this response (shedding of gametes) only occurred if injection was directly into the neural region but not if injected into the gonad or elsewhere in the body. These experiments could be interpreted to mean that the hormone induces gamete release but does not affect the gonads directly and probably only through the intermediary of the nerve ganglion.

(b) The nerves between the ganglion and the gonads were sectioned and gonadotrophin was then injected into the body as above. Under these circumstances there was no positive response of gamete release but if the gonadial nerve and ganglion were left intact and all other nerves from the ganglion were severed, then there was a response of gamete release. Furthermore if the ganglion was left intact but the neural gland was destroyed there was still a positive response of gamete release when gonadotrophin was injected into the ascidian. These experiments can be interpreted to mean that mammalian chorionic gonadotrophin stimulates the nerve ganglion which in turn stimulates release of gametes.

(c) When instead of using chorionic gonadotrophin a highly concentrated extract of ascidian neural gland was injected (about 100 glands per injection dose) a similar positive response of gamete discharge was elicited.

(d) Gametes were also released when an ascidian was fed with a suspension of gametes of its own species but not if fed with gametes from another species of ascidian.

From these experiments Carlisle concluded that the neural gland functions in the release of gametes in the manner suggested by Huus (*loc. cit.*) but through the following chain of events:

Gametes in the inhalant water current are taken into the gland.
Gametes stimulate the gland to secrete a hormone.
The hormone excites the ganglion.
Nervous pathways from the ganglion through the gonadial nerve stimulate the release of gametes.

While the experimental techniques used by Carlisle appear to have been sound there are several difficulties which make it impossible to

accept his interpretation. In the first place Millar (1953a) and Pérès (1943) insist that the ciliary currents beat material out of the ciliated funnel and not into the gland; Georges (1971) considers that they draw material into the gland and Carlisle claimed to have found gametes in the gland when sectioned after feeding on sperm and ova. Secondly if cross fertilization with other spawning ascidians of their own species is the goal then the response to gametes in the water should be immediate and not have a time delay of 20 hours. Under normal circumstances one would expect the response to involve release of the gonoduct sphincter and contraction of the mantle, rather than increased gamete production. Reference has already been made to the work of Pérès (1954) on the ovarian cycle which suggests that the scheme prepared by Carlisle cannot be correct.

If there is a gonadotrophin present in the neural gland or if the gland in any way controls reproduction we would expect that surgical removal of the gland should disrupt the sexual cycle. Hisaw *et al.* (1966) examined this possibility in *Chelyosoma productum*. The whole or a part of the neural complex was removed from animals after spawning was completed in summer and before growth of gonadial tissue had commenced again. No regeneration of neural tissues occurred and the animals, after extirpation of the complex, were returned to the sea to continue their growth. The ascidians were re-examined at intervals varying from one month to a year and there was no evidence at all that extirpation of the neural complex had interfered with the gonadial growth cycle neither did it modify the subsequent spawning season. However, in interpreting these experiments we must bear in mind that Pérès (1943, 1947) and Lender and Bouchard-Madrelle (1964) report that the neural gland has considerable powers of regeneration. Furthermore these experiments are at variance with those of Bouchard-Madrelle (1967) who reports that extirpation of the neural complex causes a decrease in the number of germinal centres in the ovary and in the number of mature follicles. He also states that removal of all or part of the complex shows that the neural gland controls the number of young oocytes while the ganglion controls the number of mature follicles and the number of oocytes in perivitellogenesis. Experiments carried out by Sengel and George (1966) suggest that the neural gland may also have an inhibitory effect on the spawning process.

Finally in this context reference should be made to the work of Sengel and Kieny (1962, 1963a, 1963b) who cultured the gonads of *Molgula manhattensis in vitro*. Immature and mature gonads were cultured for seven days on various media of sea water and agar with added penicillin. Somatic tissues of the gonads survived well on the unsupplemented medium but the germinal elements only survived if horse serum or chicken plasma was added. Maturation of oocytes would only continue if the gonads were cultured in the presence of explanted neural complex (GNV). This continued maturation of the oocytes did not occur when the gonad was associated with tissues other than GNV. The implication from this work is that proper growth and maturation of the gonads requires some substances present in the neural complex.

There is then some very conflicting evidence concerning the possible role of the neural complex in the control of reproduction in ascidians and until far more critical work has been carried out we cannot resolve this particular problem. One cannot escape from the fact that all of the histological evidence points to the neural gland as being exocrine in function and if any hormonal substances are present in the neural complex they are more likely to exist in the neurosecretory cells of the ganglion than in the neural gland.

3. *Other functions of the neural gland*

It remains to consider what other possible functions may be served by the neural gland. These were reviewed by Butcher (1930) and Carlisle (1951) both of whom concluded that a pituitary-like function was the most probable. A few of the older suggestions merit some comment.

(a) *Mucus secretion* (Roule, 1884). There is no evidence that mucus is secreted by the gland (Butcher, 1930) and there would be no need for it. Mucus or mucus-like substances are secreted by the endostyle and provide all that is required in the food-collecting mechanism.

(b) *Excretory organ* (*E. Van Beneden, quoted by Julin*, 1881); *Organ of phagocytic excretion* (Pérès, 1943). Van Beneden clearly had some sort of kidney in mind when he suggested an excretory function but neither Roule (1884), Kowalevsky (1889) nor Butcher (1930) could demonstrate any such function and recent histological evidence (see above) makes it clear that such a function would be impossible. Pérès (1943), nevertheless, considered that it might be eliminating waste products through a process of phagocytic excretion and that this would explain the periodic sloughing of the glandular epithelium. However, if such a function was necessary it would be surprising to find that evolutionary mechanisms should have resulted in the discharge of these materials into the beginning of the food cord when it might have been discharged the other side of the mantle wall and into the atrial cavity. Butcher (1930) points out that in ascidians of the genus *Phallusia* the

gland does not open into the branchial entrance but into the peribranchial chamber, but these are usually secondary openings and a primary opening in the branchial region still exists. Godeaux (1957) reported that in small aplousobranch ascidians of the family Polyclinidae (= Synoicidae) the neural gland picked up from the water stream and phagocytosed particles of less than 1 μ diameter, but it is not clear what function this might serve. However, this is the size range of ascidian spermatozoa and Godeaux's observations may in fact lend support to Carlisle's (1951) theory that the gland detects gametes in the water.

(c) *Sensory structure*. Hancock (1868) first suggested a special sensory function for the dorsal tubercle, and assumed that it might act to test the incoming water current. Although Hunter (1898) claimed to have found sensory cells amongst the ciliated cells on the funnel, later authors have not found any innervation of this region which would suggest a sensory function (Millar, 1953a). Carlisle (1950b) suggested that in the pelagic tunicate *Salpa* the ciliated pit might act as a receptor organ for judging the density of particles in the inhalant water current. In later papers (Carlisle, 1951, 1953) concerning the whole neural gland he suggests a special sensory function in connection with the detection of gametes in the water: the pathways in this postulated mechanism would be chemical and not nervous.

(d) *Digestive gland* (Roule, 1884). As well as suggesting a mucus secreting role for the gland Roule also believed that it might act as a form of digestive gland. Butcher (1930) tested extracts of the gland for amylase, lipase and protease and concluded that none of these enzymes is present. Apparently no one has considered this possibility since that time and yet it merits further attention particularly in the light of Lane's (1971) suggestion that distension of the rough endoplasmic reticulum in the compact phase of the gland may indicate active protein synthesis and the production of hydrolytic enzymes. There is unequivocal evidence that the neural gland undergoes cyclical changes in which a cellular discharge accumulates in the lumen. If Pérès and Millar are correct in stating that the principal ciliary currents beat out of the gland then some of this material must be being discharged through the ciliated funnel. The ciliated funnel is located at the junction of the two peripharyngeal bands and the dorsal lamina (Fig. 29). It is thus ideally situated for discharging material in a position to be wrapped up in the developing food cord; in many ascidians the anterior end of the lamina forms a folded gutter which leads back from the ciliated funnel. Finally it seems significant that the degree of development of the dorsal tubercle, on which opens the ciliated funnel,

appears to have a direct relationship to the complexity of the food collecting apparatus. In the aplousobranchs the ciliated opening is usually a simple oval aperture; in phlebobranchs there is a tendency towards folding to form a C-shaped aperture; in the more complex stolidobranchs, particularly in Pyuridae, the horns of the C tend to be rolled inward into a spiral structure or to form other complex patterns. The overall effect of such increasing complexity is to increase the length of the ciliated border of the funnel. The organization and position of the ciliated funnel are thus such that it could be used to "inject" cellular material into the beginning of the food cord and the question should be investigated further.

XIII. The Dorsal Strand

In the adult ascidian the duct of the neural gland extends posteriorly as a fine strand of tissue, the dorsal strand or dorsal cord. A short distance behind the gland it leaves the mantle and crosses to the dorsal wall of the pharynx where it runs close above the dorsal lamina. Posterior to the branchial sac in enterogonous ascidians (i.e. *Ciona*) it extends to the gonad where it penetrates into the ovary and terminates abruptly as a slight swelling (Millar, 1953a; Aubert, 1954). In pleurogonous ascidians in which the gonads are in the mantle wall (i.e. *Molgula*) the strand terminates abruptly in the neighbourhood of the digestive diverticula of the stomach (Van Beneden and Julin, 1884). It is generally agreed that this strand is derived from that part of the larval neural tube which also gives rise to the neural gland and ganglion (Willey, 1893; Van Beneden and Julin, 1884; Huus, 1924; Brien, 1927; Berrill, 1950; Millar, 1953a; Aubert, 1954).

Although the strand is derived from embryonic neural tissue there are no nerve cells or fibres in it. In *Molgula manhattensis* Van Beneden and Julin (1884) described the strand as composed of large cells resembling the large cells of the cerebral ganglion and arranged radially around a central core or small cavity. However, these authors pointed out that the cells in *M. manhattensis* were larger than in many other species examined, and Millar (1953a) describes the cells in *Ciona intestinalis* as "quite small, and spindle shaped, being somewhat elongated along the strand". In *Ciona* the whole strand is only 10 μ to 15 μ in diameter. Huus (1924) does not figure the cord in section but his illustrations show rounded cells scattered unevenly in the strand of *Corella parallelogramma* (Muller). Brien (1927) describes them in the following terms for *Aplidium pallidum*: "Les cellules sont identiques à celles du canal excréteur, ou plus exactement aux amoebocytes du sang avec lesquels

elles se confondent facilement ; le cordon dorsal est constitué de cellules indifferenciées. "

Metcalf (1899) claimed that in several species there is fusion between the dorsal strand and the visceral nerve, but the careful work of Maurice (1888), Huus (1924), Brien (1927) and later Fedele (1938), Millar (1953a), Aubert (1954) and Markman (1958) makes it clear that Metcalf was mistaken and that the visceral nerve and the dorsal strand are quite distinct structures. Fedele (1938) recognized that the dorsal strand is not formed of ganglion cells and he considered that the cells which form it are like neuroblasts and give rise to the nerve elements which flank the cord. He observed in *Ciona* that the dorsal strand is formed of such undifferentiated cells which can detach and migrate into the lateral tissues. Around the strand and at some distance from it he detected two layers of multipolar fusiform cells whose processes supply a lateral plexus of fibres forming a sheath around the dorsal strand. Scattered throughout the layer of multipolar cells he recognized pyriform motor cells like those found in the nerve ganglion. Fedele considered that fibres in the fibrous layer connect to the viscera, both branchial sac and intestine. Fedele's observations have since been confirmed by Millar (1953a), Aubert (1954) and Markman (1958).

It is reasonable to conclude from these studies that the dorsal strand is composed of undifferentiated cells surrounded by a fine nerve plexus. In enterogonous ascidians it forms an intimate link between the neural gland and the gonad, but in pleurogonous forms it ends abruptly amongst the viscera. It is difficult to imagine what function might be performed by a structure of this sort. Huus (1924) was unable to find the strand between the cloaca and the gonad and concluded that it gave rise to the gonoduct during development, but Aubert (1954) strongly refutes this on the grounds that in the adult it is possible to demonstrate the entirety of the strand between gland and gonad, that the strand and gonoduct coexist and that the gonoducts arise from the gonad. Brien (1927) considered that in blastogenesis of *Aplidium pallidum* that portion of the strand enclosed in each bud gave rise to the new neural complex and new dorsal strand as well as to gonadial tissue although in a later work he omits reference to the gonads (Brien, 1948). Neither of these two conclusions (those of Huus and Brien) provides an adequate explanation of this enigmatical structure, nor does it seem reasonable to assume as did Millar (1953a) that " the dorsal strand is functionless in the mature animal ". If it was solely concerned with blastogenesis we would not expect to find it in non-budding forms like *Ciona* and *Corella*.

The intimate link between the neural gland and viscera suggests that it might be endocrine in function, perhaps associated with long term metabolic activity or gonadial cycles rather than with the daily life of the animal. In this context we may recall the experiments of Carlisle (1951) in which he injected gonadotrophin into ascidians and stimulated gamete release. When he cut the visceral nerve this response was destroyed, but in cutting the visceral nerve he would almost certainly have also cut the dorsal strand so that we do not know whether the response might have been mediated through the visceral nerve or the dorsal strand. Bouchard-Madrelle (1967) reported that oogenesis is under the control of the neural complex in *Ciona intestinalis* and if this is removed, the number of germinal centres decreases and the young oocytes degenerate. The number of oocytes in perivitellogenesis and that of mature follicles also decrease. Removal of all or a part of the neural complex suggested that the neural gland controls the number of young oocytes and the germinal centres while the ganglion controls the number of mature follicles and oocytes in perivitellogenesis. There is no indication as to how such control may be mediated, but it is possible for the control of oogenesis to be by way of the dorsal strand. However, if that is the case in *Ciona*, it is still not clear how any mediation may occur in pleurogonid forms where the dorsal strand is not in direct contact with the gonad.

The ontogeny of the dorsal strand and its close association with the surrounding visceral fibre complex (p. 91) make it seem more likely that the strand has a functional relationship with the visceral nervous system than with reproductive cycles or other metabolic processes. The strand and surrounding fibres run along the dorsal side of the branchial sac and Fedele (1927, 1938) considered that the fibre complex was associated with nervous control of ciliary activity in the branchial stigmata. Recently Mackie *et al.* (1974) have described nerve fibres going from the fibre complex to the ciliated cells of the stigmata in *Corella* (p. 91). Thus while it is reasonable to associate ciliary activity with the visceral fibre complex the role of the dorsal strand itself remains unexplained. Fedele's (1938) suggestion that the dorsal strand is the site of continuing neuroblastogenesis (p. 124) has not been confirmed by other workers, but nevertheless the close association between fibre complex and strand makes some functional relationship appear likely. Fedele's careful observations on the nervous system of ascidians have been neglected by many authors and deserve closer attention ; his suggestion of continuing neuroblastogenesis may warrant re-examination. Alternatively, the strand may perform a nutritive function in relation to the surrounding nerve cells and fibres much as glial cells do in the nervous systems of other animals.

XIV. Conclusion

The foregoing pages illustrate a number of interesting problems in ascidian physiology, many of them still incompletely resolved. Recent work on the test has revealed the detail of fine structure and the intervention of morula cells in the deposition of complex polysaccharide microfibrils. We still do not understand the precise role of the morula cell in this process and why some ascidians have found it necessary to use vanadium and others iron in the organo-metal complex of the cell. Different groups of ascidians have tests of different organization varying from gelatinous to fibrous and a comparative study of the architecture of the test and its functional significance would be of interest.

Studies on the endostyle over the past ten years have elucidated the fine structure and function of this organ and the type of secretion which it produces. We are beginning to have an understanding of the functional significance of differences in branchial organization and how it relates to food collection, but there is still much work to be done in this field. In particular the role of the papillae in food transport is uncertain and little is known about the mode of functioning of the dorsal lamina and why its organization differs between species. My own work in Jamaica suggests that different ascidians are exploiting different fractions of the food supply available in the environment, but how this is achieved is unknown. There are scattered references in the literature to the possibility of food sorting and selection by the branchial sac, but this has never been confirmed.

There has been little advance in our understanding of digestive physiology although recent studies with electron microscopy have revealed new detail about the nature of the alimentary canal. Some doubt is expressed in this review about the accepted interpretation of food transport and treatment and the role of the pyloric gland; this aspect of ascidian physiology requires new and critical examination. There are also many unresolved problems of metabolism in ascidians particularly as to whether the neural gland contributes any enzymatic material to the food cord and how metabolism proceeds beyond gastric digestion. Recent studies have confirmed earlier suggestions that ascidians are entirely ammonotelic in their nitrogen metabolism but store products of nucleic acid metabolism as urates or higher purines. However, we still do not know whether such storage is a biochemical accident or whether it has particular functional significance.

Considerable advances have been made in recent years in the field of cardiovascular physiology of ascidians. The myogenic nature of the heart and its control by terminal pacemakers is well established as are

many of the the electrical characteristics of the myocardial cells. It now seems likely that the reversal of heart beat, which is common to all ascidians, is the result of fluctuations in the activity of the visceral pacemaker, but we are still totally ignorant of the functional significance of such behaviour. It is suggested in this review that it may be associated in some way with the blood system of the test and blind ending vascular ampullae at the periphery.

Advances in our understanding of the blood itself have been confined to the morula cells and their precursors and little new information has appeared on other aspects of blood and its function. It has been established that the morula cell is derived from amoebocytes through a series of steps including signet-ring and compartment cells and recent work implies that this is associated with the synthesis of the organo-metal compound haemovanadin or in some ascidians a similar compound of iron. These cells are intimately concerned with the production of the test and migrate through the mantle or test vessels into the substance of the tunic where they cytolyse. The organo-metal compound is a reducing agent held in the reduced state inside the cell by the presence of strong acid. At cytolysis the acid is dispersed and the reducing action of the compound becomes available but the precise way in which this activity is used has not been established. The association of these cells with polysaccharide fibres suggests that they may play some significant role in fibre production.

The simplicity of life in ascidians is matched by the apparent simplicity of their nervous system. Ascidian nerves are unresponsive to normal nerve stains and they are difficult to trace, but experimental work has thrown considerable light on their function. The primary role of the nervous system is in the maintenance of the water current. This seems to be achieved through visceral control of the branchial cilia and somatic control of siphons and mantle. While much is now known about separate facets of the nervous mechanism we are still far from an understanding of the overall control of water movement. Siphonal apertures are controlled by a cross reflex mediated through the ganglion but the sensory mechanisms providing for minor adjustments are not fully understood. The precise role of such structures as ocelli and cupula organs remains to be elucidated. The ocelli are not photoreceptors but might be mechano-receptors for monitoring the position of the siphonal lobes. Cupula organs in the atrial cavity may be pressure receptors. Ciliary activity is known to be controlled, at least in part, through the visceral fibre complex and perhaps also through an innervation from the ventral side of the branchial sac, but the relationship between this and the activity of the somatic system is unknown.

Likewise the role of the dorsal strand in relation to the visceral fibre complex remains to be elucidated. There is doubt also about the nature of synaptic transmitters in ascidian nerves and the function of the secretory cells in the ganglion.

The function of the neural gland remains an enigma. It is clear that it is engaged in cyclical activity resulting in the sloughing of cells into the interior lumen of the gland but differences of opinion still exist as to the fate of these cells, and whether or not they are discharged through the ciliated funnel. If discharged they are likely to become wrapped up in the food cord forming on the dorsal lamina. In spite of many attempts to homologize the neural gland with the vertebrate pituitary it seems that any hormonal activity in the gland is at a level comparable to that found in other tissues of the animal and the evidence suggests that it is not a specialized endocrine organ.

XV. Acknowledgements

Much of this review was prepared at the Plymouth Laboratory of the Marine Biological Association during a sabbatical leave. I am particularly grateful to the University of the West Indies and to the Nuffield Foundation for the financial assistance which made this possible; also to the Director of the Plymouth Laboratory for providing me with facilities. Many other people have helped in various ways and particular thanks are due to the Library staff at Plymouth for their constant assistance, to Dr Eve Southward for help with the literature after my return to Jamaica and to Mrs Elaine Fisher for preparing many of the illustrations. I also wish to thank Dr George Mackie and his colleagues and Dr John Croxall for permitting me to read and to quote from their unpublished manuscripts.

Acknowledgement is also made to the following publishers for permission to reproduce original material as indicated: The Wistar Institute Press (Fig. 5); Springer Verlag, New York (Fig. 6); The Marine Biological Laboratory, Woods Hole (Fig. 7); Università di Genova (Fig. 9); Masson et Cie, Paris (Fig. 10); Oliver and Boyd Ltd., Edinburgh (Fig. 13); The Rockefeller University Press (Fig. 14); Microforms International Marketing Corporation, Inc. (Fig. 28); Ragnar Olsson, Stockholm (Fig. 30).

I thank also the authors of these illustrations who have given permission for their reproduction and whose names appear in the relevant captions.

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