THE CYTOLOGY OF

HALICLONA OCULATA

(DEMOSPONGIAE, HAPLOSCLERIDA)

by

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ABSTRACT

Specimens of the marine sponge ***Haliclona oculata*** were collected from Passamaquody Bay, New Brunswick. Once transported to Montréal, Québec, the sponges were fixed and embedded in paraffin for light microscopy and the cells were chemically dissociated, fixed and imbedded in Spurr resin for transmission electron microscopy. Based upon the presence or absence of a nucleolus, the types of cytoplasmic inclusions and cell size examined, seven cell types are found in all specimens sampled: archeocytes, choanocytes, pinacocytes, sclerocytes, spongocytes, granular cells and spherulous cells. The dimension of each cell, nuclei, nucleoli and cytoplasmic inclusions are measured and analysed statistically. The characteristics and possible function of the cell types are discussed. The isolation of archeocytes with four Ficoll gradients proved to be unsuccessfull. Cells with various inclusions were precipitated with the archeocytes when centrifuged.

RÉSUMÉ

Des spécimens de l'éponge marine ***Haliclona oculata*** ont été récoltés dans la Baie de Passamaquody au Nouveau Brunswick. Une fois tranportées à Montréal, Québec, les éponges ont été fixées et enrobées de paraffine pour être examinées au microscope et les cellules ont été dissociées dans une solution chimique et elles ont été fixées puis enrobées de résine Spurr pour observations au microscope électronique. D'après la présence ou l'absence d'un nucléole, les types d'inclusions cytoplasmiques et les dimensions cellulaires, sept types de cellules sont trouvés dans tous les spécimens échantillonnés: archéocytes, choanocytes, pinacocytes, sclerocytes, spongocytes, cellules granuleuses et cellules spheruleuses. Les dimensions de chaque cellule, nucleus, nucléolus et inclusions cytoplasmiques sont mesurées pour analyse statistique. Les caractéristiques et fonction possible des types cellulaires sont discutées. L'isolation d'archéocytes utilisant quatres gradients de Ficoll a été essayée sans succès. Diverses cellules à inclusions ont été précipitées avec les archéocytes lors de la centrifugation.

ACKNOWLEDGEMENTS

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INTRODUCTION

Sponges are considered to be the most primitive metazoans (Hyman, 1940). The general biology of sponges is for the most part well documented. However, much research remains to be done on the cytology of sponges in order to elucidate the functions of several cell types. Such research could help to clarify the classification of sponges which relies mainly on the content, size and arrangement of spicules and spongin. Hartman (1958) has shown that the latter two criteria vary significantly within the species he examined. Simpson (1968b) has used the presence of special cell types in the distinction of similar sponge species. Knowledge of the cell population may also help establish phylogenetic relationships between different sponges.

Wilson (1907) first attracted the interest of researchers on the cytology of sponges when he discovered that marine sponge cells, dissociated by pressing a piece of sponge through a bolting cloth, reaggregated to form a functional sponge. He also noted that when the cells of two different sponge species were mixed, the cells segregated into their respective species before they reaggregated. Although Curtis (1962) observed some non-specificity between certain sponge species, strain specificity was demonstrated by Van de Vyver (1971) in fresh water sponges. The presence of a specific aggregation factor was examined by Humphreys (1963) and

Moscona (1963). They showed that this factor was released in the absence of calcium and magnesium in the culture medium. Subsequent research identified the ultrastructure of the aggregation factor as a sunburst structure of high molecular weight (see Henkart *et al*, 1973 and Müller and Zahn, 1973). Lowenstein (1967) demonstrated the importance of such an aggregation factor as well as the presence of calcium and magnesium in cell communication.

Since Wilson's initial observations, the identification of cell types has led to some conflicting results. The sponge specimens used in several studies were often in the form of rejuvenating explants or cell aggregates. Certain cell types identified in such material showed different characteristics from similar cell types observed in adult specimens (Simpson, 1968b). In addition, there is no consensus on the number of cell types generally present in various adult sponges. Many cells with inclusions have ambiguous functions and relationships with respect to the stem cell: the archeocyte.

Research by Borojovic (1966) has shown that archeocytes, as widely hypothesized previously, are totipotent and can differentiate into other cell types necessary in the formation of a functional sponge. His observations also suggested that the differentiation was irreversible. The material used in his research was taken from larvae, therefore the archeocytes examined were true embryonic cells.

There was no direct evidence as yet of the totipotency of archeocytes from an adult sponge. De Sutter and Van de Vyver (1979) successfully isolated archeocytes from a fresh water sponge and demonstrated their capacity to differentiate into a functional sponge. Their research presented direct evidence of the totipotency of archeocytes but the sponges used in their study were grown from gemmules and were only one week old. Therefore, they may not have been fully developed adult sponges.

The objectives of the research reported here were: 1) to identify each cell type of an adult sponge with the use of electron microscopy observations and ultrastructural measurements; 2) to compare the results obtained with previous observations to determine the possible function of each cell type; 3) to attempt isolation of archeocytes from other cell types to demonstrate directly their totipotency.

The north-Atlantic demosponge ***Haliclona oculata*** (Pallas, 1766 and Grant, 1841; in Hartman, 1958) was chosen for investigation on the basis of its availibility and its use as an experimental animal in cell aggregation studies.

MATERIALS AND METHODS

Haliclona oculata specimens were collected in April, June and August 1984. They were dredged in the vicinity of Deer Island (45° lat. N, 67° long. W); at the mouth of Passamaquody Bay, New Brunswick, Canada. Two sponges were sampled and fixed in epoxy as outlined below at Huntsman Marine Laboratory, St-Andrews, N.B. for controls. Selected healthy specimens were transported by air to Montréal, Québec, in plastic bags filled with oxygenated sea water which were kept in an ice-filled styrofoam cooler. At McGill University, the sponges were transfered to a closed aquarium filled with artificial sea water (Humphreys, 1963: NaCl, 24.7; KCl, 0.7; $CaCl_2$, 1.0; $MgCl_2$, 4.7; $MgSO_4$, 6.3; $NaHCO_3$, 0.18 gr/l H_2O, pH 7.2) at constant temperature of 4°C. They remained healthy for 1 to 3 weeks after which they rapidly decayed. All histologic procedures were carried out within a week of the shipment. The health of the sponges and their water filtering capacity were assessed by the presence of a pinacocyte or dermal layer and the presence of flagellated chambers when inspected under light microscopy.

All specimens were identified by spicule analysis. Sponge tissue was dissolved in nitric acid and the remaining spicules were washed and placed in a grided petri dish. The length and width of spicules were measured under an Olympus compound microscope with an ocular micrometer accurate to

0.5μm.

Sections of 2 cm length cut from branches of specimens collected in August were fixed for 1 hour with 3.5% glutaraldehyde in 0.4M NaCl and 0.1M sodium cacodylate (pH 7.4) at room temperature (Reed and Cloney, 1979; modified). The fixed branches were twice rinsed for 15 minutes in 0.2M sodium cacodylate buffer, dehydrated in an alcohol series, cleared in xylene and embedded in paraffin. Sections of the sponge stalk were also fixed and embedded.

The paraffin blocks were sectioned on a microtome with a disposable metal razor blade. The 10μm sections were mounted on glass slides and were stained with Delafield's hematoxylin for general cell features, Best's carmine stain (Pearse, 1968) for glycogen, Schiff's periodic acid test (Pearse, 1968) for mucosubstances, and PAS-Alcian blue (Pearse, 1968) for differentiation of acid and basic mucopolysaccharides. The sections were examined by light microscopy under oil immersion objective.

Two cell populations were sampled from each of two healthy sponges collected in April, June and August. The cells were chemically dissociated according to the procedure of Humphreys *et al* (1960) and Humphreys (1963). Two grams of blotted sponge tissue were immersed in 80 ml of calcium-magnesium-free artificial sea water (CMF-SW) at 0°C

(Humphreys, 1963: NaCl, 27.0; KCl, 0.8; Na_2SO_4, 1.0; $NaHCO_3$, 0.18 gr/l H_2O, pH 7.2). The tissue was cut into $2mm^3$ pieces, soaked for 30 minutes in CMF-SW and squeezed through a 25μm mesh plankton netting into 80 ml of fresh cold CMF-SW. The resulting suspension was centrifuged for 2 min at 1000 rpm in an IEC-7 centrifuge. The cell pellets were resuspended in fresh, cold CMF-SW and adjusted to a concentration of approximately 10^6 cells/ml (estimated with a hemacytometer chamber).

A 3 ml sample of the cell suspension was diluted to 15 ml with CMF-SW and centrifuged for 2 minutes at 1000 rpm. The cell pellets were fixed for 1 hour with 3.5% glutaraldehyde in 0.4M NaCL and 0.1M sodium cacodylate buffer (pH 7.4) at room temperature. They were twice rinsed for 15 minutes in a 0.2M solution of the buffer and then post-fixed for 1 hour using 1% osmium tetroxide in 1.25% sodium bicarbonate at pH 7.2. The cell pellets were finally dehydrated in an alcohol series, cleared in propylene oxide and embedded in Spurr (1969) epoxy resin. The blocks were cured for 8 hours in an oven at 60°C.

The blocks were sectioned at 0.1μm on a Porter-Blum ultramicrotome MT-2B with either glass or diamond knife. The sections were floated onto uncoated 400 mesh copper grids and stained for 45 minutes in saturated aqueous uranyl acetate at 60°C and for 10 minutes in Reynolds (1963) lead citrate at

room temperature. The sections were viewed and photographed with a Phillips 400 transmission electron microscope operating at 80 Kv.

To isolate archeocytes, the method of de Sutter and Buscema (1977) was employed. Sponge cells were first dissociated (Humphreys, 1963; modified). A 15 ml cell suspension was prepared as outlined above and it was agitated for 12 hours with a magnetic stirrer in a 40 ml beaker kept at 0°C. Three ml of the cell suspension were vigorously resuspended 15 times with a pasteur pipette into a test tube to break up remaining cell clumps resulting in a suspension of single cells (checked with a hemacytometer chamber). Cell permeability was tested with 1 ml of cell sample mixed in 3 ml of saturated trypan blue. A monoionic polymer of sucrose, Ficoll was purchased from Pharmacia. Gradients made with this product permit the separation of cells with different cell size when centrifuged. Four discontinuous Ficoll gradients of 10, 8, 6 and 4% each made with 8 ml of cold CMF-SW were layered in succession in a 40 ml centrifuge tube. A 1 ml cell sample was carefully pipetted on top of the gradients. The suspension was centrifuged for 5 minutes at 2000 rpm or 750 g instead of 3000 g used by de Sutter and Buscema. The top gradients were removed and the entire 10% fraction was collected with a pipette. The cells were then washed in artificial sea water, pelleted and fixed as outlined above.

Cells photographed by electron microscopy and observed by light microscopy were categorized by the presence or absence of a nucleolus, cell size and the presence and types of inclusions. The length and width of both cell and nucleus, the largest diameter of the nucleolus and the various cytoplasmic inclusions were recorded. If the nuclear membrane of a cell was cut obliquely, cell measurements were usually not recorded since this indicated the cut was not in the mid-section of the nucleus. However, some spongocytes, granular cells and spherulous cells were still measured without a mid-section cut through the nucleus to record the size of their inclusions.

An elementary statistical table, a normal probability test, a general linear model test and a non-parametric analysis of variance were computed for each cell variable using the Statistical Analysis System (SAS) software package on McGill computer facilities.

OBSERVATIONS AND RESULTS

General Features of *Haliclona oculata*:

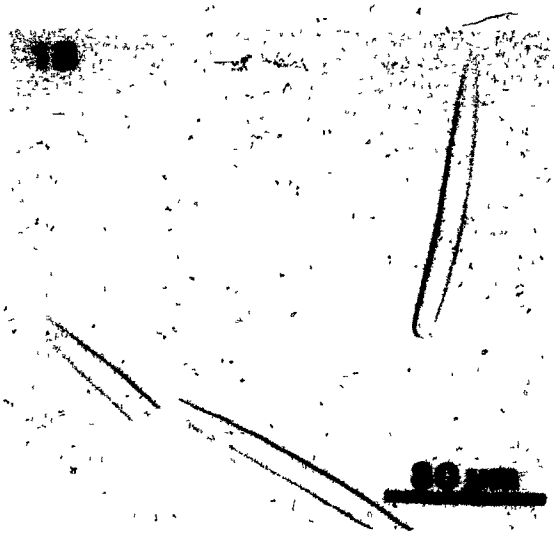
The specimens collected varied in gross morphology. Some had a few slender long branches while others had many short thick ones (plate 1A, left to right). Their color in healthy conditions varies from beige to brick red. The healthy sponges sampled from April to August all contained active choanocyte chambers and a continuous pinacocyte layer. The mean length and width of oxea and style spicules (plate 1B) from a typical specimen employed in this study are listed in table 1. The specimens fixed and embedded at the field station included both whole pieces of sponges as well as isolated cell samples. The cells in both instances show better preservation with the sponge kept in its natural sea water. The cells fixed from specimens kept in artificial sea water exhibit most characteristics of their respective cell type but the Golgi bodies are lacking in some cells.

Table 1: Spicule measurements of 102 oxeas and 13 styles from one specimen. The mean and extremes are listed with the standard deviation in parentheses. Hartman (1958) has measured oxeas from *H. oculata* of the Bay of Fundy.

SPICULE	LENGTH	WIDTH
OXEA	97.4-133.3-258.8μm (±20.6)	3.9-9.7-12.8μm (±1.6)
STYLE	168.3-191.4-209.6μm	8.9-10.2-11.8μm
OXEA (Hartman,1958)	103-128-165μm (±13.4)	7.3-10.1-12.8μm (±1.3)

Plate 1A: Specimens of ***Haliclona oculata*** showing variation in gross morphology.

Plate 1B: Spicules from a typical specimen collected. Note the definite rounded end of the style.


50 mm

Thick sections of whole branches from specimens collected in August show larval development to be widespread in both specimens collected. The stalks of all sponges collected proved to be devoid of cell populations, but they contained gemmule-like spongin-bound bodies. Thick sections from these bodies reveal that cells are present but the small dense nucleus is masked by many semi-translucent granules. The cells were not investigated by electron microscopy since the dense spongin hindered thin sectioning. Incubation for 7 days at room temperature of 10 isolated bodies in two petri dishes filled with artificial sea water produced no differentiation of the tissue within the bodies or hatching.

Cell Type Description:

Based on cell size, the presence of a nucleolus and the types of inclusions, the following cell types are described as present in the *H. oculata* population of New Brunswick (table 2): archeocytes, choanocytes, pinacocytes, sclerocytes, spongocytes, granular cells and spherulous cells. The cell terminology is taken from Simpson (1984).

Table 2: General description of the cell types of ***H. oculata***. Mitochon.: mitochondria; RER: rough endoplasmic reticulum.

CELL	NUCLEOLUS	INCLUSIONS	MITOCHON.	RER	GOLGI
ARCHEOCYTE	present	many large phagosomes	present	present	present
CHOANOCYTE	absent	few small phagosomes	few	few	absent [1]
PINACOCYTE	absent	few large phagosomes	few	few	present
SCLEROCYTE	present	1 large brittle inclusion	present	present	present
SPONGOCYTE	may be present	many small inclusions	present	present	present
GRANULAR CELL	rare	many granular inclusions	few	present	present
SPHERULOUS CELL	rare	many large inclusions	rare	rare	absent

1: not seen in this study

Staining of Paraffin Sections:

The results of the different staining procedures on thick section are listed in table 3. The hematoxylin stain provided for general cell shape and major organelle identification. Glycogen is present in archeocytes, sclerocytes and in spongocytes. Archeocytes and spongocytes have mucosubstances in their cytoplasm and sclerocytes have little or none. Choanocytes and pinacocytes have acid mucopolysaccharrides while the mucopolysaccharides of archeocytes, sclerocytes and spongocytes are basic. Because of the poor resolution of thick sections under oil immersion, it was not possible to differentiate between spongocytes and granular cells. Spherulous cells did not readily take up any of the stains.

Table 3: Histochemical results. Gran. c.: granular cell.

STAIN / CELL	BEST'S CARMINE FOR GLYCOGEN	PAS FOR MUCOSUBSTANCES	PAS-ALCIAN BLUE FOR MUCOPOLYSACCHARIDES
ARCHEOCYTE	+	+	BASIC MUCOPOLY.
CHOANOCYTE	-	-	ACID MUCOPOLY.
PINACOCYTE	-	-	ACID MUCOPOLY.
SCLEROCYTE	+	±	BASIC MUCOPOLY.
SPONGOCYTE (GRAN. C.) SEE TEXT	+	+	BASIC MUCOPOLY.
SPHERULOUS CELL	-	-	-

Electron Microscopy:

All cell types described from electron micrographs are found simultaneously in every sponge collected during the entire sample period. The mean cell measurements are listed in table 4. Some cells could not be categorized because the sections lacked nucleoli and their cytoplasms did not contain characteristic inclusions or vacuoles. These cells may be in an undifferentiated state and were not included in this study.

Table 4: Mean cell measurements (μm). Nuc.l.: nucleus length, nuc.w.: nucleus width, nucleo.: nucleolus, incl.: inclusions.

CELL/DIMENSION	LENGTH	WIDTH	NUC.L.	NUC.W.	NUCLEO.	INCL.
ARCHEOCYTE	12.1	10.1	5.4	4.8	1.8	1.8
CHOANOCYTE	6.6	5.5	2.5	2.2	x	1.0
PINACOCYTE	13.4	11.2	4.6	3.9	x	1.4
SCLEROCYTE	13.6	10.8	5.4	4.5	1.8	2.1
SPONGOCYTE	12.0	9.6	4.0	3.3	x	0.6
GRANULAR C.	12.2	9.9	3.2	2.8	x	1.0
SPHERULOUS C.	13.8	11.8	3.3	3.0	x	1.2

The major distinction for archeocytes is the presence of a prominent nucleolus (plate 2A,B). The cytoplasm is often filled with phagosomes of other cells and cell debris though these vary greatly in number. The mitochondria are 0.5μm in diameter or smaller, easily recognized by the characteristic lamellae. They are numerous in archeocytes and have the same morphology in all cell types. The rough endoplasmic reticulum (RER) in archeocytes as in other cell types is lined with ribosomes on the outer surface of the membrane. The Golgi bodies are not well developed having a few small saccules. Spongin "A" bundles are found in the cytoplasm of some archeocytes (plate 2C,D). The fibrils within are 20nm in diameter and they display an axial periodicity of approximately 20nm.

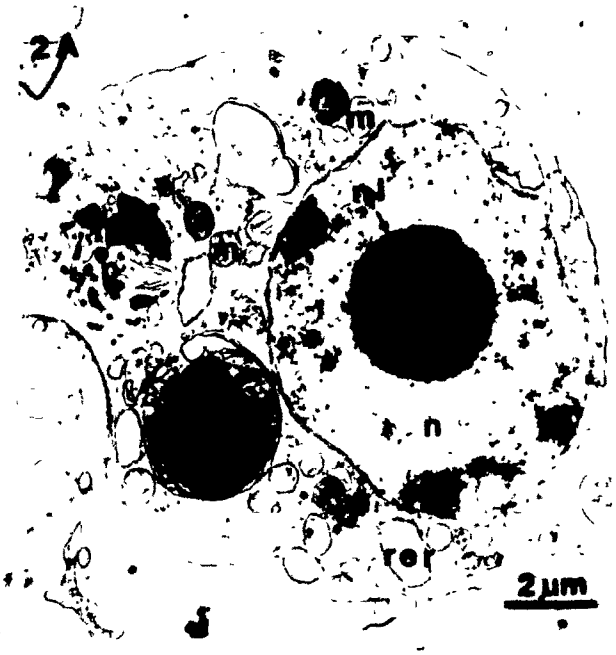
Choanocytes are recognized by their small size, anucleolate nucleus and flagellum where still intact (plate 3A-D). The nucleus is small and lined with chromatin at its periphery although the chromatin sometimes forms a nucleolus. A few small inclusions (cell debris?), mitochondria and RER are present. The flagellum has small lateral projections of its membrane and typical 9+2 microtubule arrangement (plate 3D). It is surrounded by numerous microvilli of 0.1μm in diameter (plate 3C).

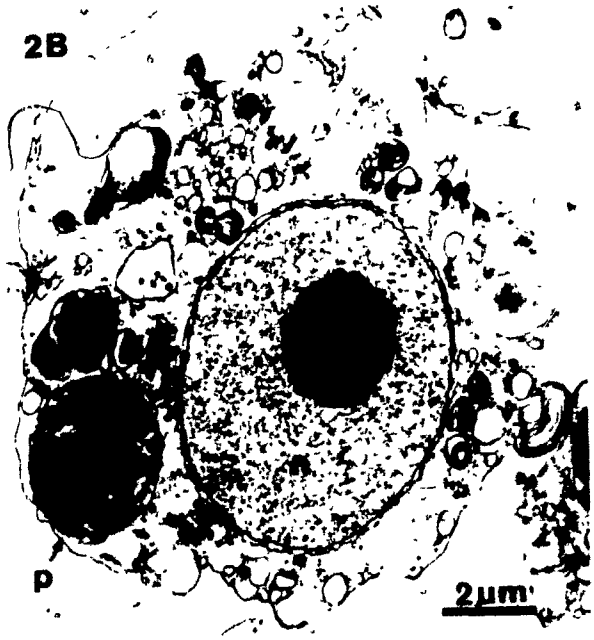
Plate 2A: Micrograph of an archeocyte from a sponge specimen kept in artificial sea water showing the major cell organelles. n: nucleus, nl: nucleolus, p: phagosomes, m: mitochondria, rer: rough endoplasmic reticulum.

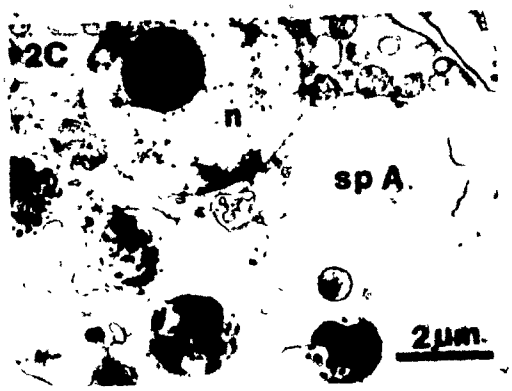
Plate 2B: Archeocyte from a control cell sample of a sponge kept in natural sea water. Note the appearance of the nucleus as compared to 2A; the cell organelles show better preservation. G: Golgi bodies.

Plate 2C: Archeocyte containing a spongin "A" bundle (sp A).

Plate 2D: High magnification of an isolated spongin "A" bundle. Some axial periodicity is visible (arrow).


2A
m
n
n
rer
2µm


2B
p
2µm


2C
n
sp A
2µm.

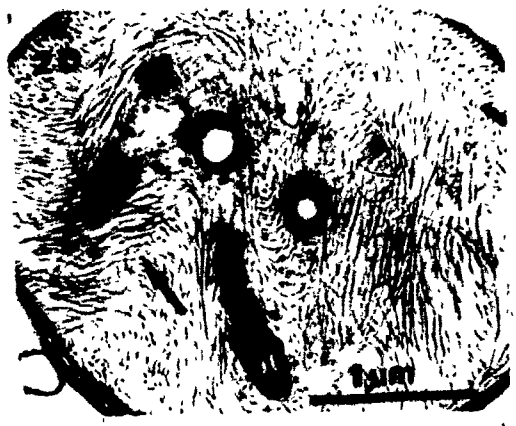
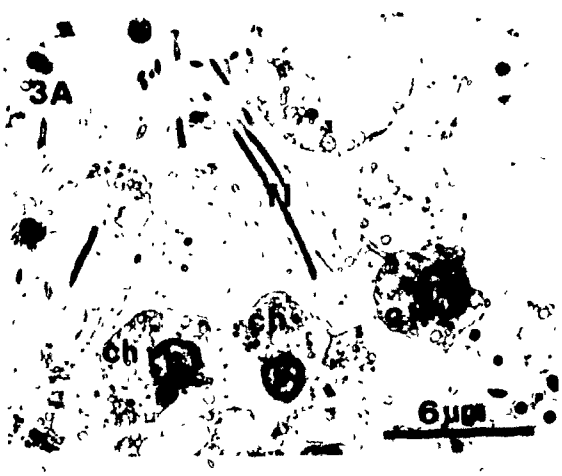

2D
1µm

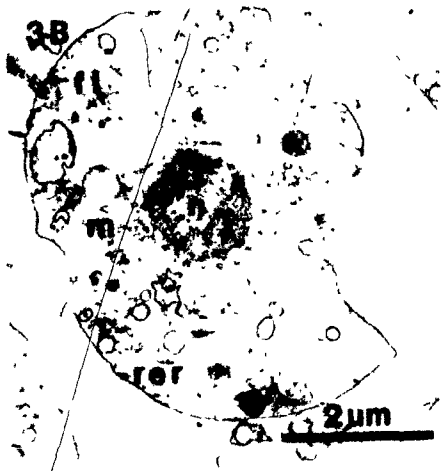
Plate 3A: Micrograph of a group of choanocytes (ch) which have retained their flagella (fl).

Plate 3B: Single choanocyte at higher magnification showing the major cell organelles.

Plate 3C: Apical end of a choanocyte with the basal part of the flagellum and some microvilli (mv) which form the collar. The inclusion (i) appears phagosome-like.

Plate 3D: High magnification of a flagellum showing the two central microtubules (mt) and small membrane projection (arrow).


3A
ch
ch
6µm


3B
m
rer
2µm


3C
m
2µm


3D
0.2µm

The pinacocytes are one of the largest cells in the species. Their cytoplasm has many small (< 0.5μm) empty vacuoles and few if any phagosomes although multivesicular bodies are regularly found (plate 4A,B) and mitochondria and RER are present. Golgi bodies are prominent only in the control samples (plate 4C) where the cells display a long and flattened shape (plate 4D) and are not dissociated from the skeleton.

Sclerocytes resemble archeocytes in that they share a prominant nucleolus and similar cell dimensions (plate 5A). However, they contain very few phagosomes, their cytoplasm is more vacuolated and each has one or more large vacuoles containing electron opaque, brittle material often in the form of a small spicule (plate 5B,C,D). Mitochondria and RER are present. The Golgi body is poorly developed.

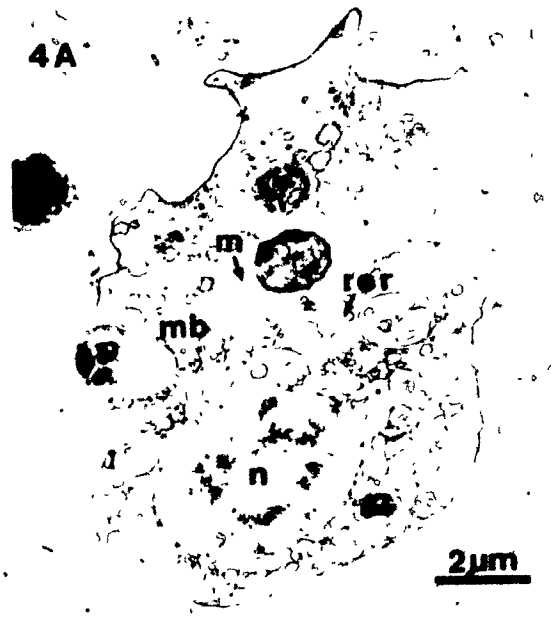
The spongocytes are recognizable by the presence of uniform inclusions, 0.6μm in mean diameter, filling the cytoplasm (plate 6A,B). These inclusions are membrane bound and osmiophilic. At high magnification they appear regularly banded (plate 6C), the width of each band being 7nm with a more pronounced band at 28nm intervals. The nucleus is sometimes nucleolate, mitochondria are present and the rough endoplasmic reticulum is abundant throughout the cytoplasm. A Golgi body is present in the cells fixed at the field station(plate 6D). Small bundles of spongin "A" are also

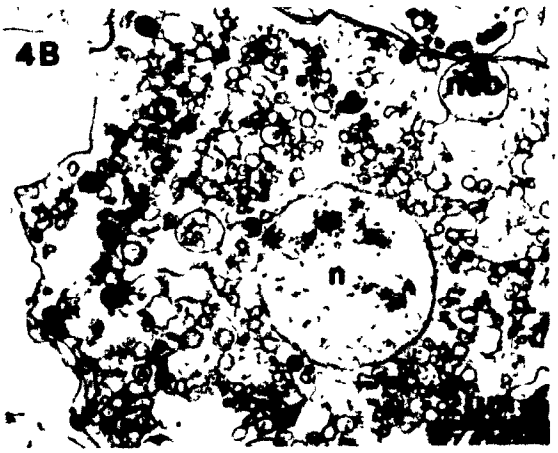
Plate 4A: Micrograph of a pinacocyte with anucleolate nucleus, phagosomes and multivescular bodies (mv).

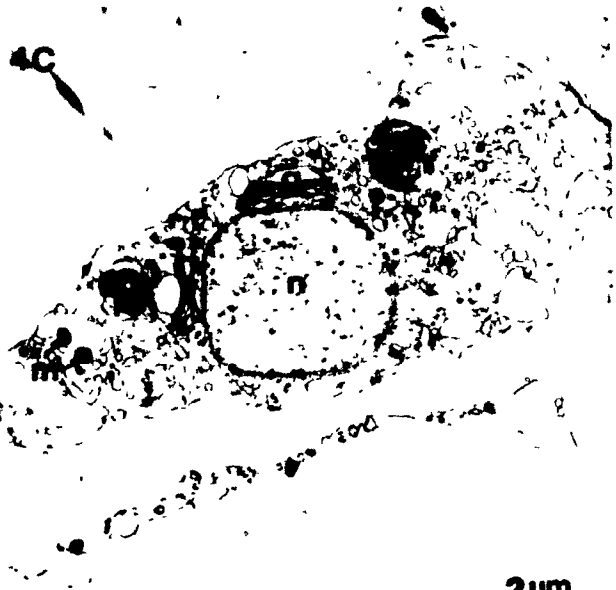
Plate 4B: Pinacocyte with multivesicular bodies present in the cytoplasm. Note the vacuolated cytoplasm in 4A and 4B.

Plate 4C: Pinacocyte from a control sample of an intact sponge branch. The Golgi bodies are well developed and the cell shape is elongate.

Plate 4D: Long cytoplasmic extensions of pinacocytes from a control sample making intimate contact with adjacent cells (arrows).


4A
m
rer
mb
n
2µm


4B
mb
n


4C
n
m
2µm

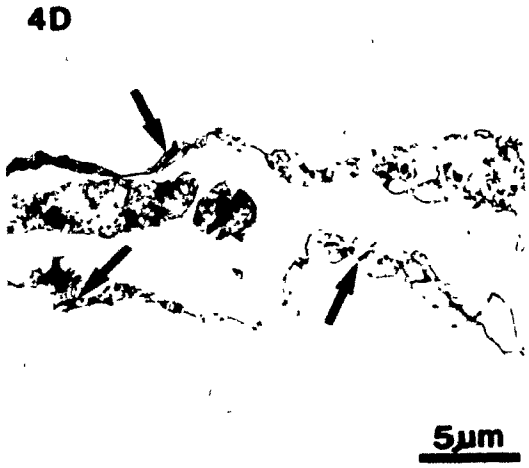
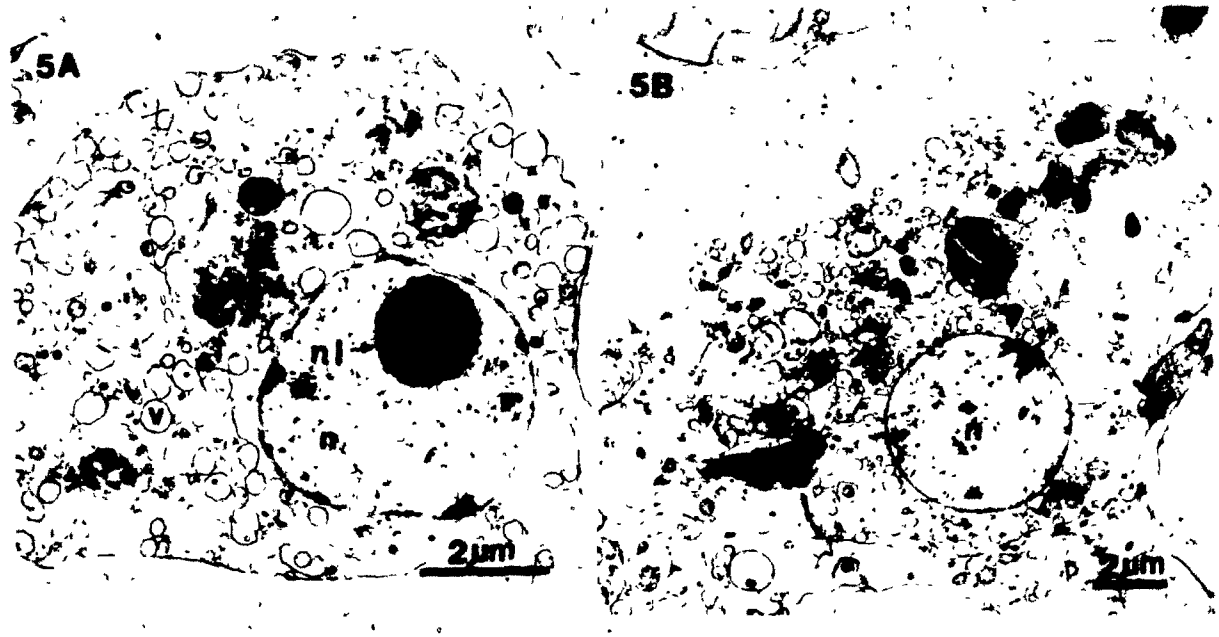

4D
5µm

Plate 5A: Micrograph of a sclerocyte showing a nucleolated nucleus, brittle material (s) and many small vacuoles (v). Note the lack of phagosomes.

Plate 5B: Sclerocyte with a vacuole containing spicule-like, brittle material. The nucleolus is not seen in this micrograph.

Plate 5C: High magnification of an inclusion within the vacuole of a sclerocyte. Some vacuoles contained a single spicule-like inclusion but most sclerocyte vacuoles examined contained many fragments.

Plate 5D: High magnification of an inclusion within the vacuole of a sclerocyte.


5A
nl
n
v
2µm
5B
n
2µm

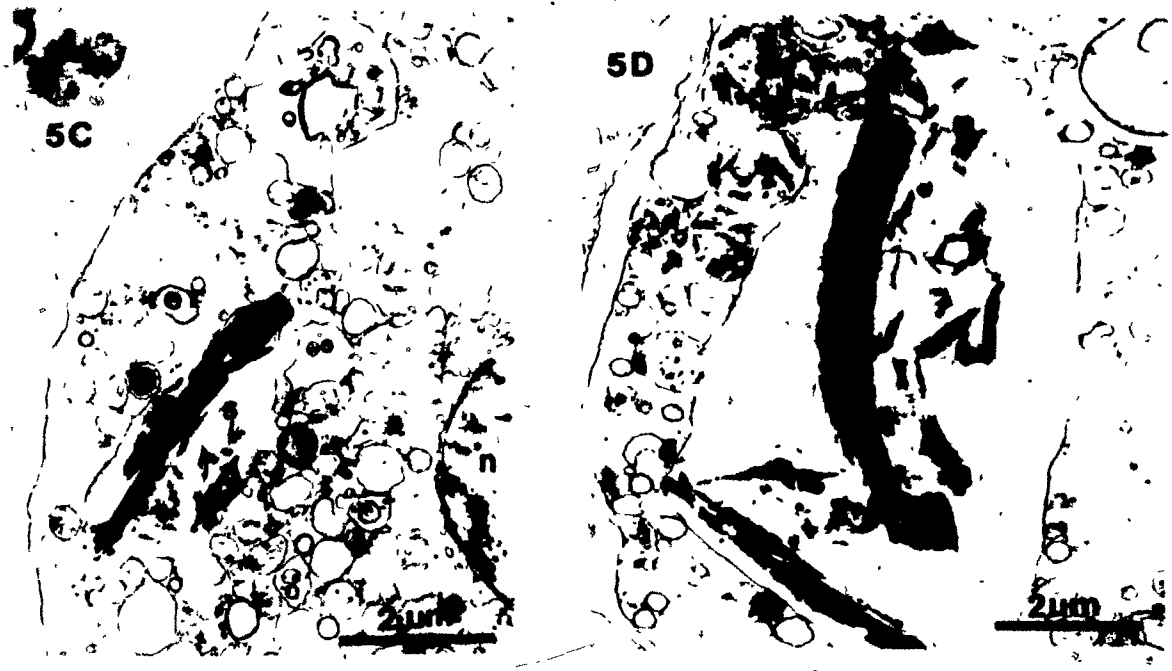

5C
n
2µm
5D
2µm

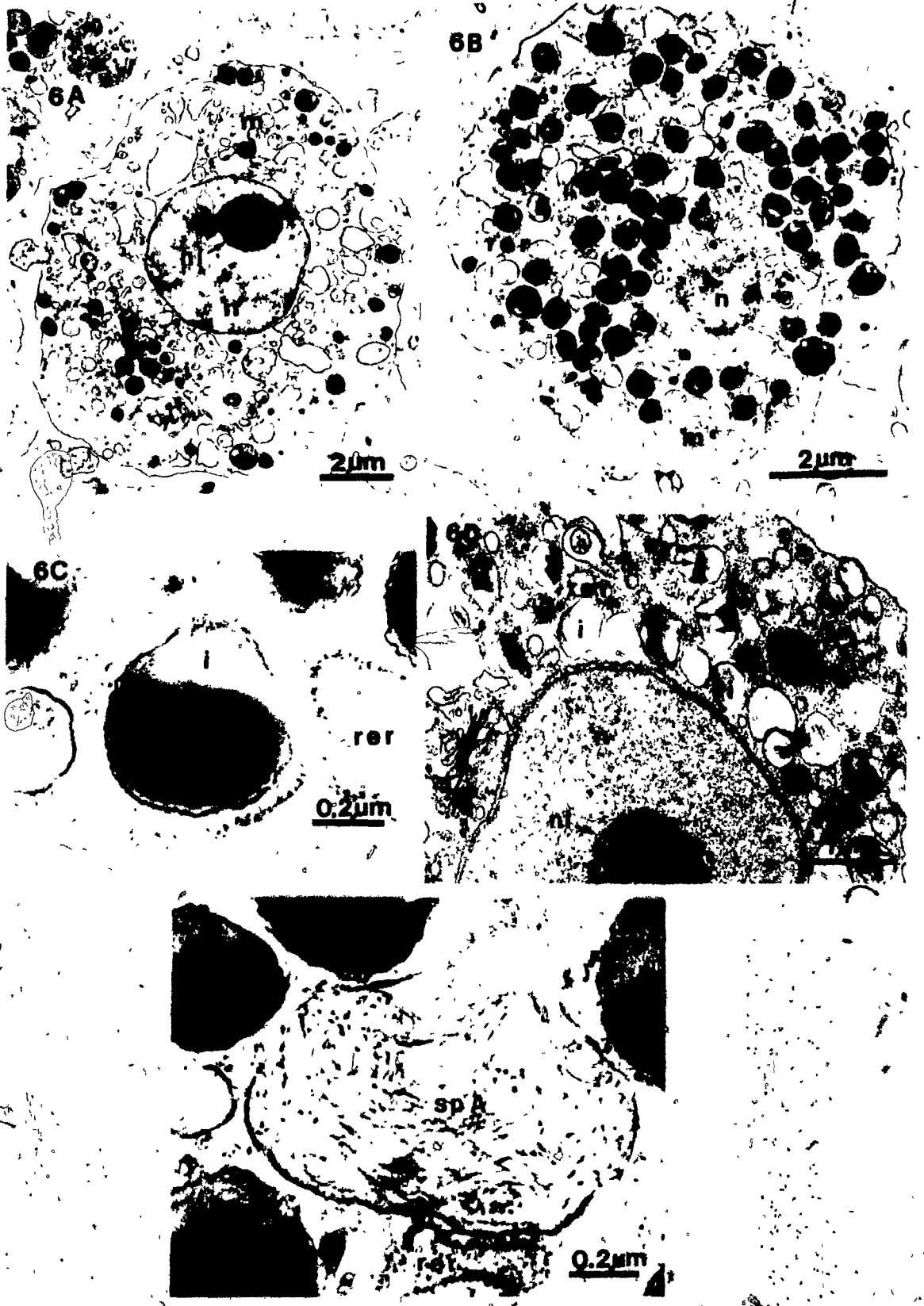
Plate 6A: Micrograph of a spongocyte containing a nucleolate nucleus and many small osmiophilic inclusions (i)

Plate 6B: Spongocyte filled with osmiophilic inclusions. The cytoplasm has many mitochondria and the rough endoplasmic reticulum is abundant throughout.

Plate 6C: High magnification of a spongocyte inclusion. Each light and dark band is approximately 7nm in width. The dark bands are more pronounced at 28nm intervals.

Plate 6D: Spongocyte from a control cell sample. In this micrograph, the inclusions are very light in contrast and some banding is visible (arrows).

Plate 6E: High magnfication of a spongin "A" bundle amidst the spongin "B" inclusions of a spongocyte.


6A
m
n
n
2µm
6B
n
2µm
6C
i
rer
0.2µm
6D
i
n
sp A
rer
0.2µm

found in the cytoplasm of some spongocytes (plate 6E).

The granular cells are similar to spongocytes. Their inclusions, not as electron dense as those in the spongocytes, are larger with a mean diameter of 1.0μm (plate 7A,C). At high magnification the dense material in some inclusions is also seen to be regularly banded (plate 7B). The width of each band is approximately 3nm with some larger bands of 7nm. A nucleolus is rarely found. Some mitochondria and RER are present. Free ribosomes are particularly abundant (plate 7C,D) and a Golgi body is present only in cells fixed in the field (plate 7C) which also contain many large vacuoles.

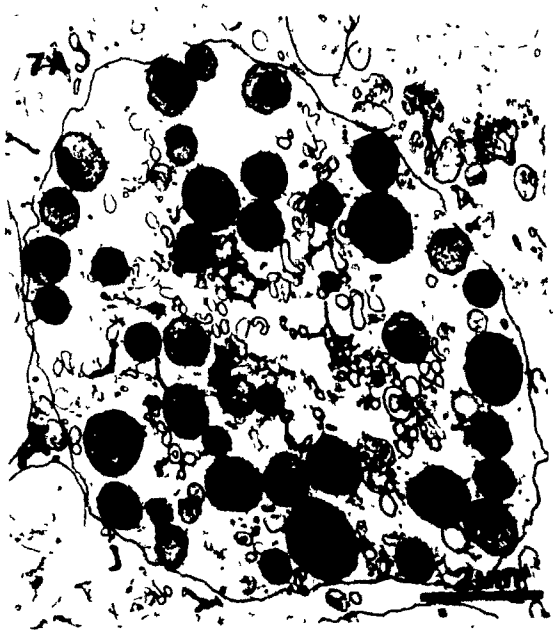
The spherulous cells are typically filled with large spherules (plate 8A,C). The spherules (mean diameter: 1.2μm) contain little material and are electron translucent. Some have small dense granules within them. At high magnification these granules reveal a regular banding of 7nm in band width (plate 8B;D). Other spherules contain fibrils of 7nm in diameter aligned in the form of a crystalline body with a diamond shape (plate 9A). Some crystalline bodies exhibit staggered edge layers suggesting growth by regular accretion to the lattice (plate 9B,C). A nucleolus is rarely present. A few mitochondria and RER are found but no Golgi body has been detected. The configuration of spherulous cells is the least altered by the artificial sea water

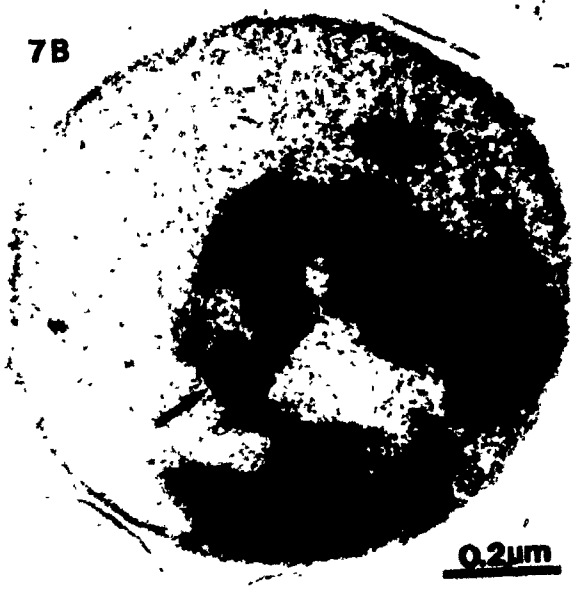
Plate 7A: Micrograph of a granular cell kept in artificial sea water. Besides the granules, there is a lack of cell organelles; the nucleus in this instance was not sectioned and only a few mitochondria and ribosomes (r) are apparent.

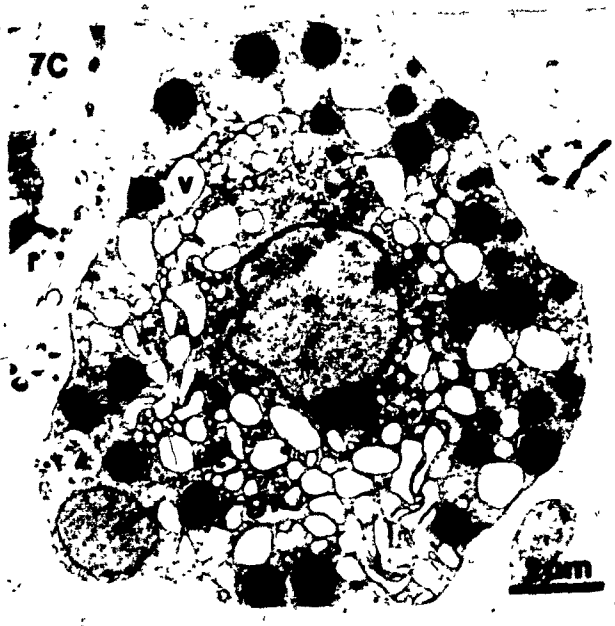
Plate 7B: High magnification of a granule from a cell kept in artificial sea water. The regular banding visible in many of these inclusions also forms into ring patterns (arrow)

Plate 7C: Granular cell from a control cell sample. The inclusions here are of the same size as in 7A but much more opaque. A nucleus, Golgi bodies and ribosomes are clearly visible as are large vacuoles which sometimes alter the cell shape.

Plate 7D: High magnification of a small inclusion from a granular cell kept in natural sea water. The banding of the dense material is not regular but appears fibrilar in nature. The ribosomes are lightly visible due to the high electron density required to take such micrographs.


7A


7B
0.2µm


7C
V

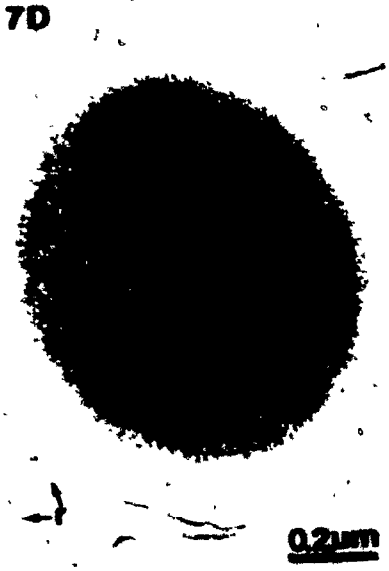
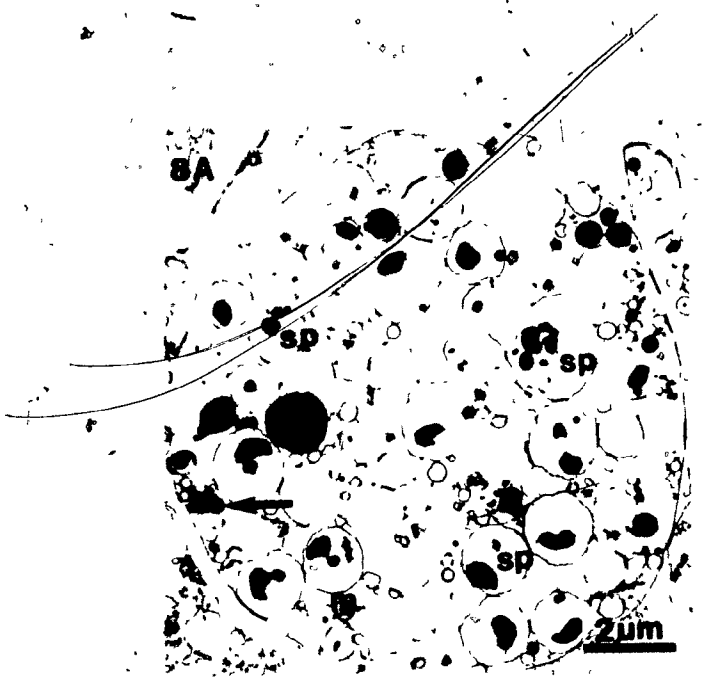

7D
0.2µm

Plate 8A: Micrograph of a spherulous cell containing a variety of inclusions. Most spherules (sp) have some dense material within them and there are smaller inclusions present as well (arrow).

Plate 8B: High magnification of the small inclusion in 8A. A regular banding is visible much as in the spongocyte inclusions.

Plate 8C: Spherulous cell with different inclusions and spherules. One of the inclusions (arrow) appears like a spongocyte inclusion.

Plate 8D: High magnification of the inclusion in 8C. The banding pattern closely resemble that of a spongocyte inclusion.


8A
sp
sp
sp
2µm


8B
0.2µm

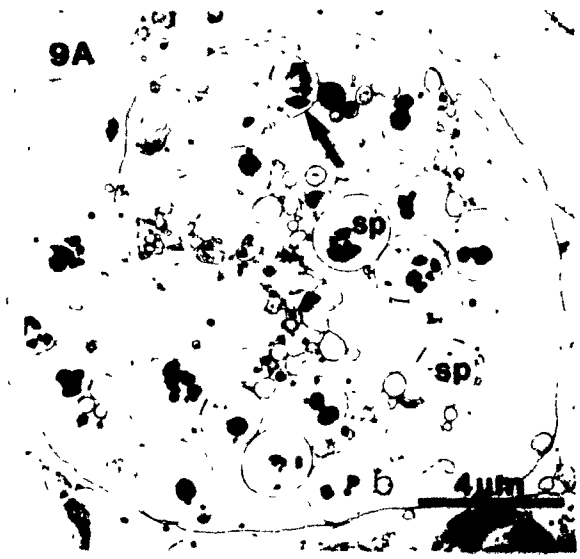

8C
sp
sp
2µm


8D
0.3µm

Plate 9A: Micrograph of a spherulous cell with diamond-shaped sections of crystalline inclusions within some spherules (arrow).

Plate 9B: High magnification of the spherule in 9A. The diamond-shaped inclusion is made of a crystalline array of fibril-like material aligned at two different angles. Each fibril measures 7nm in diameter.

Plate 9C: High magnification of a spherule from a spherulous cell kept in natural sea water. The pattern of the inclusion within the spherule is identical to that of cells kept in artificial sea water (9A,B).


9A
sp
sp
b
4µm


9B
sp

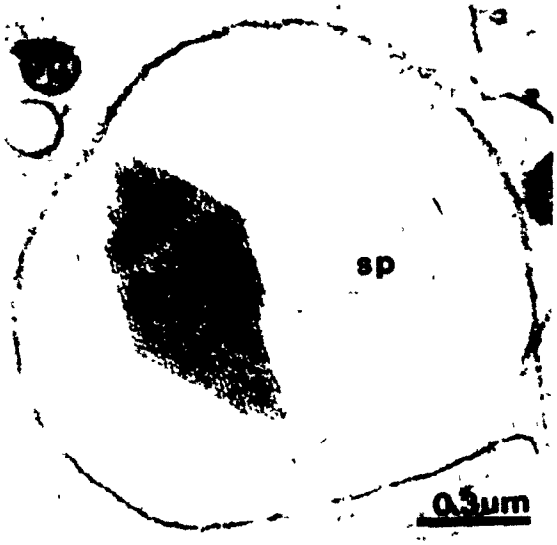
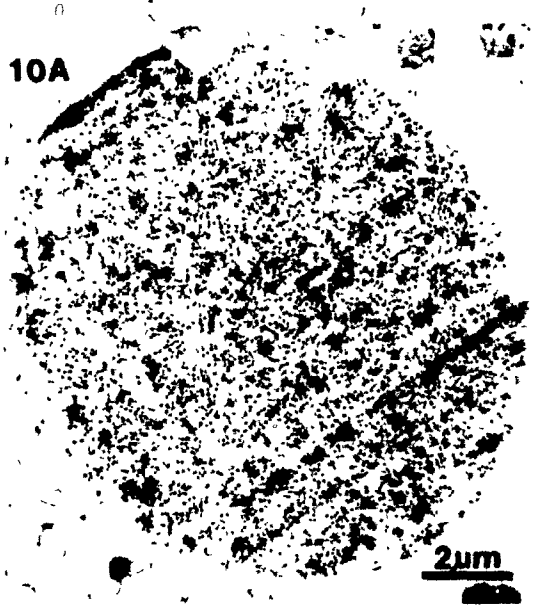

sp
0.3µm

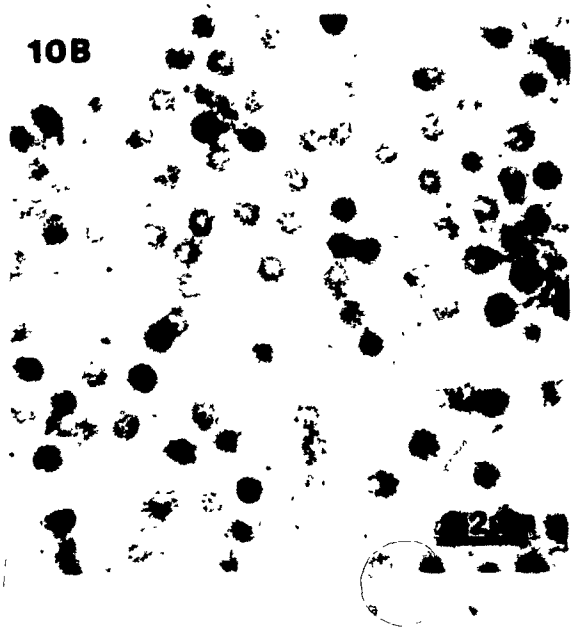
Plate 10A: One of two large inclusions found in a specimen collected in August. The inclusions were not surrounded by any other cell membrane. Many small bodies fill the inclusion.

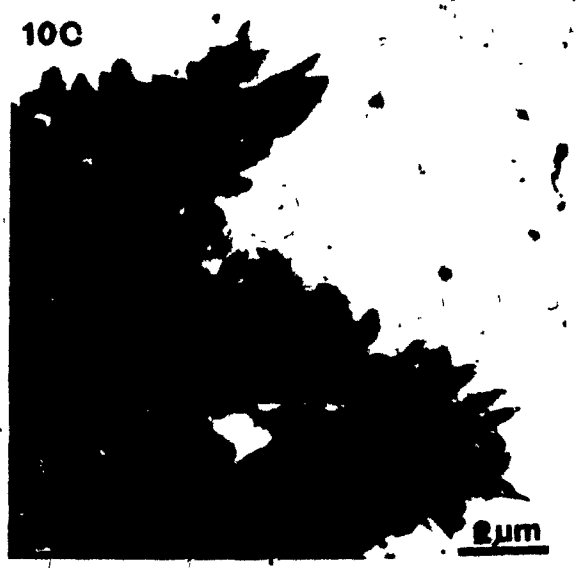
Plate 10B: High magnification of the small bodies within the inclusion in 10A. Some bodies appear hollow while others are electron-opaque.

Plate 10C: Micrograph of a spongin "B" fragment in a cell sample. Long uniform fibers as well as branching fibers (this micrograph) were observed.

Plate 10D: High magnification of the spongin "B" fragment in 10C. No regular banding or axial periodicity is discernable.


10A
2µm


10B


10C
2µm


2µm

Two large round inclusions filled with what may be microtubule-like bodies were found in one of the specimens collected in August (plate 10A). The inclusions measured 12.5μm in diameter and the small bodies were 68nm in diameter (plate 10B). As will be discussed below, these inclusions may represent evidence of a rare globoferous cell type in this species. Some spongin "B" fragments were also observed and photographed (plate 10C,D). High magnification of these fragments reveal a wavy pattern of the spongin fibrils. The diameter of individual fibrils is 7nm.

Statistical Analysis:

An elementary statistics table and a normal probability test were computed for each dimension of each cell type. The archeocyte dimensions are normally distributed except for the inclusions (table 5). Their mean cell size is 12.1 x 10.1μm with a large mean nucleus size of 5.4 x 4.8μm. The choanocyte dimensions are positively skewed toward smaller sizes with a few large cells (table 6). Both cell and nucleus mean cell dimensions are roughly half those of archeocytes. The inclusions are likewise small. The normality of the distribution of dimensions of other cell types could not be assessed properly due to small sample sizes. These normalities are listed in parentheses in the statistical tables. Pinacocytes have a larger cell size (13.4 x 11.2μm) than archeocytes but their nucleus size of 4.6 x 3.9μm is smaller (table 7). Their inclusions are relatively large. Sclerocytes closely resemble archeocytes in mean cell dimensions(table 8). Except for a greater cell length in sclerocytes, they have a comparable cell width and their nucleus size is nearly identical. The mean diameter of the nucleolus of both cell types is the same. The inclusions of sclerocytes are the largest of any cell type. Spongocytes have a cell size comparable to archeocytes but their nucleus is smaller (table 9). Although the sample size of spongocyte inclusions was large, their distribution is not normal but very leptokurtic indicating the inclusions have a uniform

size. Granular cells are also comparable to archeocytes in cell size yet again their nucleus is much smaller (table 10). The distribution of the inclusions is negatively skewed and platykurtic indicating a wide variety of sizes. The spherulous cells have a mean cell size of 13.8 x 11.8µm making them the largest cell element present in *H. oculata* (table 11). The nucleus, often crowded by spherules, is small. As in spongocytes, the distribution of the inclusions (spherules) is leptokurtic thus suggesting they have a predominant size in spherulous cells as well.

Table 5: Elementary statistics of archeocytes (µm). Nuc.l.: nucleus length, nuc.w.: nucleus width, nucleo.: nucleolus, incl.: inclusions, P: normal: probability for a normal distribution.

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	NUCLEO.	INCL.
SAMPLE SIZE	49	49	49	49	49	100
MEAN	12.1	10.1	5.4	4.8	1.8	1.8
STANDARD DEV.	2.0	1.9	0.9	0.8	0.4	0.8
VARIANCE	3.9	3.5	0.8	0.7	0.1	0.6
SKEWNESS	0.5	0.3	0.9	1.1	0.7	1.5
KURTOSIS	0.4	0.5	0.4	1.1	0.4	1.7
MINIMUM	8.7	6.5	3.7	3.6	1.1	0.6
MAXIMUM	18.2	15.7	7.7	7.3	2.9	4.2
RANGE	9.5	9.2	4.0	3.7	1.8	3.6
P: NORMAL(%)	96	97	92	90	95	83

Table 6: Elementary statistics of choanocytes (μm).

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	INCL.
SAMPLE SIZE	61	61	61	61	32
MEAN	6.6	5.5	2.5	2.2	1.0
STANDARD DEV.	1.5	1.3	0.7	0.6	0.3
VARIANCE	2.3	1.7	0.5	0.3	0.1
SKEWNESS	1.0	1.1	0.9	0.9	0.1
KURTOSIS	0.7	1.3	0.6	1.2	-1.1
MINIMUM	4.4	3.6	1.6	1.3	0.5
MAXIMUM	11.1	9.9	4.4	4.0	1.5
RANGE	6.7	6.3	2.8	2.7	1.0
P: NORMAL(%)	88	89	88	88	92

Table 7: Elementary statistics of pinacocytes (μm).

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	INCL.
SAMPLE SIZE	12	12	12	12	8
MEAN	13.4	11.2	4.6	3.9	1.4
STANDARD DEV.	2.2	2.2	0.9	0.8	0.3
VARIANCE	5.0	4.8	0.9	0.6	0.1
SKEWNESS	-0.2	0.4	0.8	0.3	-0.4
KURTOSIS	-1.3	-0.6	-0.7	-0.5	-1.3
MINIMUM	9.8	8.4	3.4	2.6	1.0
MAXIMUM	16.6	15.4	6.3	5.3	1.8
RANGE	6.8	7.0	2.9	2.7	0.8
P: NORMAL(%)	(95)	(95)	(89)	(95)	(90)

Table 8: Elementary statistics of sclerocytes (μm).

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	NUCLEO.	INCL.
SAMPLE SIZE	12	12	12	12	7	16
MEAN	13.6	10.8	5.4	4.5	1.8	2.1
STANDARD DEV.	3.8	2.6	1.2	0.8	0.4	1.0
VARIANCE	14.5	7.0	1.5	0.6	0.2	1.0
SKEWNESS	1.1	0.6	1.1	0.8	0.1	0.8
KURTOSIS	1.1	1.2	0.8	0.1	-2.3	0.5
MINIMUM	9.5	6.3	4.2	3.4	1.3	0.8
MAXIMUM	22.2	16.5	8.2	6.1	2.3	4.4
RANGE	12.8	10.2	4.0	2.7	1.0	3.6
P: NORMAL(%)	(90)	(97)	(89)	(94)	(85)	(94)

Table 9: Elementary statistics of spongocytes (μm).

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	INCL.
SAMPLE SIZE	11	11	11	11	252
MEAN	12.0	9.6	4.0	3.3	0.6
STANDARD DEV.	1.2	0.9	0.9	0.8	0.2
VARIANCE	1.4	0.9	0.8	0.7	<0.1
SKEWNESS	-0.5	-0.5	-1.0	-0.5	4.6
KURTOSIS	-0.9	-0.5	1.4	0.3	44.3
MINIMUM	9.9	8.0	1.9	1.8	0.3
MAXIMUM	13.5	11.0	5.2	4.4	2.3
RANGE	3.6	3.0	3.3	2.6	2.0
P: NORMAL(%)	(93)	(92)	(95)	(90)	81

Table 10: Elementary statistics of granular cells (μm).

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	INCL.
SAMPLE SIZE	10	10	8	8	184
MEAN	12.2	9.9	3.2	2.8	1.0
STANDARD DEV.	2.0	2.6	0.5	0.5	0.3
VARIANCE	4.1	6.6	0.3	0.2	<0.1
SKEWNESS	1.3	1.1	-1.6	0.4	0.6
KURTOSIS	1.4	0.3	3.4	2.1	-0.2
MINIMUM	10.0	7.0	2.0	2.0	0.5
MAXIMUM	16.5	15.0	3.7	3.6	1.7
RANGE	6.5	8.0	1.7	1.6	1.2
P: NORMAL(%)	(87)	(89)	(85)	(94)	84

Table 11: Elementary statistics of spherulous cells (μm).

STAT./DIM.	LENGTH	WIDTH	NUC.L.	NUC.W.	INCL.
SAMPLE SIZE	12	12	11	11	168
MEAN	13.8	11.8	3.3	3.0	1.2
STANDARD DEV.	1.7	1.7	0.8	0.9	0.4
VARIANCE	2.6	2.9	0.7	0.7	0.2
SKEWNESS	-0.6	-0.8	0.7	1.1	1.7
KURTOSIS	-0.1	0.0	2.3	2.5	4.0
MINIMUM	10.5	8.3	1.9	1.8	0.4
MAXIMUM	16.0	13.9	5.1	5.0	3.3
RANGE	5.5	5.6	3.2	3.2	2.9
P: NORMAL(%)	(96)	(94)	(92)	(90)	81

Multiple regressions were calculated to test for a statistically discernable difference between the means of each cell type dimensions (table 12). The mean cell length and width of most cell types are not statistically different; only choanocytes are significantly smaller than all other cell types. The cell width of spongocytes and spherulous cells are also statistically different from each other and spongocyte inclusions are statistically discernable from all other cell type inclusions. The mean nucleus length and width of most cell types differ statistically. This permited certain cell type groups to be established. Thus archeocytes and sclerocytes have no discernable difference between their nucleus dimensions. This is also true for the nucleus dimensions of spongocytes, granular cells and spherulous cells. However, these two cell type groups are statistically different from other cell types. Furthermore, the mean diameter of the inclusions in archeocytes and sclerocytes are not statistically different.

Table 12: Multiple comparison of the means of cell types dimensions. Cell type groups are not significantly different. Note: the nucleolus of archeocytes and sclerocytes are not significantly different.

LENGTH	WIDTH	NUC.L.	NUC.W.	INCL.
all cell types except choanocyte	all cell types except choanocyte	archeocyte and sclerocyte	archeocyte and sclerocyte	archeocyte and sclerocyte
	spongocyte + spherulous cell	spongocyte granular + spherulous cell	spongocyte granular + spherulous cell	granular + spherulous cell

The ratios of variances between two cell type dimensions are listed in table 13. The significance level to accept the null hypothesis (no statistical difference) is taken at 95%. Choanocytes were not included in this analysis because of their size difference. All variances from the dimensions of archeocytes and sclerocytes except for cell length do not differ statistically from each other. The variances of pinacocytes for all cell dimensions except inclusions are statistically different from variances of archeocyte cell dimensions. Archeocytes, spongocytes and granular cells have comparable variances for cell length and width only.

Table 13: Analysis of variance (F values). An asterisk (*) denotes the variance ratios which are not significantly different. Note: The variances of the nucleolus of archeocytes and sclerocytes are not significantly different.

CELLS/DIM.	LENGTH	WIDTH	NUC.L.	NUC.L.	INCL.
ARCHEOCYTE SCLEROCYTE	3.53	1.17*	0.00*	1.12*	1.47*
ARCHEOCYTE PINACOCYTE	4.07	3.48	8.47	10.52	1.98*
ARCHEOCYTE SPONGOCYTE	0.01*	0.68*	24.59	29.59	588.95
ARCHEOCYTE GRANULAR C.	0.00*	0.05*	50.02	44.23	182.91
ARCHEOCYTE SPHERUL. C.	7.11	9.11	50.90	41.06	68.62
SPONGOCYTE GRANUL. C.	0.02*	0.16*	4.90	2.48*	376.48
SPONGOCYTE SPHERUL. C.	8.00	14.95	3.56	0.61	431.31
GRANUL. C. SPHERUL. C.	4.20	4.46	0.10*	0.48*	37.63

Spherulous cells are statistically different in their variances for cell length and width to the variances of archeocytes, spongocytes and granular cells. The variances of the nucleus length and width for spherulous cells and granular cells are not statistically distinctive.

Cell Separation on Ficoll Gradients:

All attempts to obtain pure isolates of archeocytes with Ficoll gradients were unsuccessfull. Two fractions which should have contained only archeocytes if cell separation here followed the process of de Sutter and Buscema (1977) using fresh water sponges, when examined contained archeocytes, sclerocytes, spongocytes, granular cells and some undifferentiated cells. No choanocytes and very few pinacocytes were present. Varying the time period and/or the speed of centrifugation yielded no improvement in the separation of archeocytes from other cell types. Most cells remained in the upper fractions when the time period was short or the centrifuge speed low. The cells were pelleted to the bottom of the centrifuge tube when the time period was longer or the speed increased. The results are discussed below.

DISCUSSION

Haliclona oculata is a common Atlantic sponge on the eastern coast of Canada and the United States. While other sponges from these regions such as ***Microciona prolifera*** have been exhaustively studied, the cytology of ***H. oculata*** has remained largely unexplored. Moreover, the cytology of sponges in general is still at an early descriptive stage despite the advances in electron microscopy. There are close to 30 cell types which have been described, yet many of these cell types have ambiguous definitions and their functions are only speculative. Simpson (1984) gives a complete review of all cell types thus far described. This study concentrates on the cell types examined in ***Haliclona oculata*** and how they compare in general to those found in other siliceous marine sponges. Although Pomponi (1974) studied several ***Haliclona*** species, her observations were made with light microscopy and accurate comparison is not possible. Garrone (1969b) has looked extensively at ***Haliclona rosea*** but he described mostly the spongocytes. The observations on spongocytes presented in this study agree with the results obtained by Garrone.

General:

Healthy sponges collected during the sample period had active choanocyte chambers and a pinacocyte layer. Temperature records of Passamaquody Bay for this period

showed the water temperature to be 4°C or higher. No collection was made during the winter months but recorded temperatures of less than 2°C in winter may induce the sponges to assume a semi-dormant overwintering stage as was shown by Simpson (1968a) in ***Microciona prolifera***.

The specimens kept in a closed aquarium did not survive more than 3 weeks. No bacterial or algal food was provided and this is undoubtebly a major factor. Rasmont (1963) demonstrated the importance of feeding in the fresh water sponge ***Ephydatia fluviatilis***. The addition of streptomycin may also have helped check bacterial growth in the aquarium. However, when fixed within the first week of captivity, the cells showed most of the characteristics of the cells fixed in the field although the latter cells in some instances had more Golgi bodies and all cell types were better preserved. Live specimens kept at Huntsman Marine Laboratory survived beyond a month of observation in an open aquarium constantly flushed with fresh unfiltered sea water from Passamaquody Bay. Therefore, the artificial sea water used alone may not be sufficient to sustain normal sponge activity due to the limited availibility of trace minerals and nutrients.

The spicule size for oxeas recorded in this study is representative of ***H. oculata*** from the Bay of Fundy as reported by Hartman (1958) but the presence of proper styles has never been documented. They occur in all specimens

examined. They make up less than 10% of the skeletal spicules and they are larger in mean length and width than the oxeas. Their location within the skeletal network is not known (random ?).

Many developing larvae were present in specimens collected in August. Topsent (in Hartman, 1958) also reported in the early 1900's the occurrence of larvae in ***H. oculata*** taken from the coast of France during the month of August.

The gemmule-like spongin-bound bodies found in the stalk of all sponges collected confirm the observations made by Topsent (1888) and Fell (1974) in ***H. oculata.*** The incubation of these bodies also duplicates the negative results obtained by Fell. The bodies may be gemmules formed when the sponge was in its early growth stages in order to survive adverse conditions. Once the sponge is fully grown, the gemmules, imprisoned within the stalk appear to become impotent. The limited observations from thick sections did not permit the accurate characterization of the cells within the bodies. Further observations are required to determine the source of the cells found in these bodies and their longevity.

The chemical dissociation of the sponge permitted a larger sample of the cell population to be fixed and examined at one time, unhindered by the spicules and perispicular spongin. Cell permeability to trypan blue after such

procedure was low (<1%) indicating high retention of cell viability. Some cells fixed within the skeleton and embedded in Spurr were examined but the thin sections were of poor quality and extensive observations were not possible due to the score marks left by the spicules. An alternative to this problem would be the desilification of the sponge skeleton within the epoxy blocks as done by Mackie and Singla (1983).

Disadvantages of the chemical dissociation are that some cells involved in skeleton secretion may still be fused to the skeletal network while larger cells may rupture when passed through a 25μm plankton netting. Therefore, the sample size of each cell type cannot be representative of their proportions within the sponge. The cell position with respect to the rest of the sponge is also altered yet the results produced in this study show that accurate identification can be made with dissociated cells.

The fixative used by Cloney and Reed (1979) incorporates 27 gr/l NaCl with the glutaraldehyde which is the approximate concentration used in the artificial sea water of Humphreys (1963). Garrone (1969b) used 18 gr/l in his fixative whereas Boury-Esnault (1973) added no supplemental salts. The cells fixed in the field gave consistent results as did the cells fixed at the McGill laboratory. The osmolarity was not tested and minor adjustments may help in improving the fixative.

Cell Types of *Haliclona oculata*:

1. Archeocytes:

Archeocytes are considered to be the stem cells or the embryonic reserve for all other cell types in the sponge. There is some direct evidence of their totipotency in fresh water sponges (Buscema *et al*, 1980). Furthermore, the gemmules of fresh water sponges contain binucleolate archeocytes which when hatched, differentiate to form an adult sponge. The primary function of archeocytes when undifferentiated is to phagocytose cell debris and food particles trapped by the choanocytes. As outlined previously, they are characterized by a nucleolate nucleus and large phagosomes.

Galstoff (1925) described the archeocytes of ***Microciona prolifera*** as cells of 7-9μm in diameter with a large nucleolus and 1-6μm granules. Wilson and Penney (1930) studying the same species found archeocytes of similar size with a nucleus of 4μm, a nucleolus of 2μm and inclusions of 0.5-4.0μm. Simpson (1963) and Bagby (1972) recorded similar observations for ***M. prolifera*** but the nucleolus averaged only 1.0μm in diameter. Lévi (1956) reported archeocytes of 8μm with a nucleus of 3-4μm in ***Halisarca dujardinii***. Borojovic and Lévi (1964) described in ***Ophlitaspongia seriata*** archeocytes with a nucleolus of 0.7μm and phagosomes of

0.5-3.0μm in diameter. The mitochondria were 0.4-0.5μm in diameter and RER and Golgi bodies were present. Simpson (1968b) studied many ***Microciona*** species as well as other genera. The archeocytes varied in cell size from species to species, but all cells had an average nucleus size close to 4.0μm and a nucleolus of 1.0μm. It must be mentioned here that many of the observations of Simpson and other workers were made from explants or cell aggregates. Therefore, the cells are more likely to be actively dividing and thus smaller in size than adult cells. The cytology of ***Haliclona viridis***, ***H. rubens*** and ***H. variabilis*** has been studied by Pomponi (1974). The archeocytes of these species are much smaller than reported here. Their cell size averaged 8 x 6μm with a nucleus of 3.5μm.

The archeocytes of ***H. oculata*** have an average cell size of 12 x 10μm and a nucleus of approximately 5μm in diameter. Hartman (1958) noted that spicules from northern specimens of ***H. oculata*** were significantly larger than southern specimens. It is therefore possible that cells from northern specimens are also larger than southern specimens. This would explain the difference in cell and nucleus size between ***H. oculata*** from the east coast of Canada and other species from the east coast of the United States. The inclusions of archeocytes in ***H.oculata*** contain acid mucopolysaccharides as in other ***Haliclona*** species reported by Pomponi. Spongin "A" bundles are common in the cytoplasm of archeocytes. Gross *et al*

(1956) studied in detail the ultrastructure of spongin "A" in ***H. oculata***. The spongin fibrils measured 20-25nm in diameter and they had an axial periodicity of 63nm. The fibrils examined in this study are also 20nm in diameter but the axial periodicity discernable is only 20nm. However, Gross *et al* did find some interbands measuring 20nm in the spongin "A" of ***H. oculata***.

2. Choanocytes:

The survival of any sponge depends on its water filtering capacity. The main function of choanocytes is to maintain a water current through the sponge canals by flagellar undulation. This brings food particles in and waste is carried out. Food matter is trapped by the choanocytes and it is either digested by the cell or transferred to surrounding archeocytes for intracellular digestion.

Galstoff (1925) found anucleolate choanocytes of 3μm in diameter in ***Microciona prolifera***. Wilson and Penney (1930) measured its nuclear diameter as 2μm which was confirmed by Simpson (1963) in studying the regeneration tissue of ***M. prolifera***. The choanocytes of other ***Microciona*** species were examined by Simpson (1968b) and their cell size ranged from 3.5 to 7.0μm in diameter. However, all nuclei measured close to 2μm. Tuzet (1932) found a nucleolus in the choanocytes of two ***Reneria*** species as did Lévi (1956) in the choanocytes of

Halisarca dujardinii. Garrone (1969a) studied the choanocytes of *Haliclona rosea*. They were 3-4μm in diameter and contained mitochondria, RER and a Golgi body. Pomponi examined other *Haliclona* species and found choanocytes averaging 3.5μm in diameter in each species and all contained acid mucopolysaccharides in their inclusions as is reported here.

The average cell size of choanocytes in *H. oculata* is quite large at 6μm. Yet the average nucleus size of 2.5 x 2.3μm is comparable to values reported for choanocytes in other marine siliceous sponges. On some occasions, a nucleolus could be seen in the center of the nucleus but most choanocytes observed had chromatin lining the nuclear inner membrane. Although reported in other sponges, the presence of a Golgi body in the choanocytes of *H. oculata* has not been determined. Mitochondria and RER are common and phagosome-like inclusions are present suggesting choanocytes can ingest particulate food.

3. Pinacocytes:

Pinacocytes line the exterior surface of the sponge as well as the water canals running through the sponge and they act as a barrier to outside elements. Pinacocytes may also be involved in food capture. Willenz and Van de Vyver (1984) have shown that the pinacocytes of *Ephydatia fluviatilis* can

ingest latex beads as well as ***Escherichia coli*** bacteria.

Galstoff (1925) first examined the pinacocytes of ***Microciona prolifera***. According to his observations, the pinacocytes measured 3-5μm in diameter and they had a clear protoplasm. However, Wilson and Penney (1930) reported that the pinacocytes of the same species were syncytial with a nucleus of 3μm in diameter. This was later refuted by Simpson (1963) and Bagby (1970). Lévi (1956) found a nucleolate nucleus of 2.5-3.0μm in the pinacocytes of ***Halisarca dujardinii***. Although Simpson (1968b) also found a nucleolus in the pinacocytes of some ***Microciona*** species, these occured only in regenerating explant tissue and not in the adult sponges. The nucleus in each species averaged 4μm in diameter. Bagby (1970) also recorded an average nucleus size of 4μm for the pinacocytes of ***M. prolifera***. The cells contained a few mitochondria, RER and Golgi bodies. Multivesicular bodies and phagosomes were found in some cells. Bagby (1972) noticed that the flattened shape or club projection (T-shaped) of the pinacocytes was lost once the sponge was dissociated; the cells became more or less spherical. Boury-Esnault (1973) reported that apart from fresh water specimens, all Haplosclerida (to which ***H. oculata*** belongs) had T-shaped pinacocytes. However, Pomponi (1974) found no pinacocytes in the ***Haliclona*** species she studied but a "protoplasmic layer" was present in ***H. viridis***.

In the dissociated sponge tissue of ***Haliclona oculata,*** pinacocytes have no definite shape. However, when examined from intact sponge samples, most pinacocytes are extremely flat and elongated. They have a greater cell size than archeocytes in dissociated cell samples. Their nucleus is anucleolate and its size of 4.6 x 3.9μm is comparable to the nucleus in the pinacocytes of other genus. There are some mitochondria, RER and phagosomes as reported in other sponges. The phagosomes can be as large as those in archeocytes lending support to the phagocytic role of pinacocytes as suggested by Willenz and Van de Vyver. The Golgi bodies were observed only in pinacocytes which were taken from sponges fixed in the field as controls. This shows the inability of the artificial sea water used alone in the laboratory to sustain normal cellular activity for long periods of time.

4. Sclerocytes:

The spicules of the sponge skeleton are secreted by sclerocytes. Hartman (1958) examined in detail the features of the spicule skeleton in ***H. oculata*** and the reader is refered to this study for further information on the skeleton of the sponge. There are some citations of sclerocytes in other siliceous marine sponges but they are often described simply as archeocyte-like.

Tuzet (1932) first recognized spicule-secreting cells in ***Reneria elegans*** and ***R. simulans***. She described the "silicoblastes" as nucleolate cells with a granular protoplasm, Golgi body and mitochondria. Lévi (1963) studied the sclerocytes of ***Mycale contarenii*** and reported the presence of a small nucleolus, 0.15μm ribosomes and 1.5-2.0μm vacuole containing a spicule but no endoplasmic reticulum or Golgi bodies were found. There are however suggestions that these cells may be spongocytes (Reiswig, personal communication). The ribosomes in bundles reported by Lévi are similar in description to the RER observed in spongocytes from ***H. oculata*** (this study) and ***H. rosea*** (Garrone, 1969b). Simpson (1963) found in ***Microciona prolifera*** archeocytes which contained spicules. The cells measured 11-19 x 5-12μm with an average nucleus and nucleolus size of 3.7 and 1.0μm respectively. Garrone (1969b) described the sclerocytes in ***Haliclona rosea*** as nucleolate cells with many mitochondria and microtubules. There were no sclerocytes described in the ***Haliclona*** species studied by Pomponi (1974).

The sclerocytes examined from ***H. oculata*** are indeed archeocyte-like. The cell dimensions of the two cell types are nearly identical. However, the make-up of the cytoplasm in the sclerocytes is different. There are few phagosomes, the cytoplasm is vacuolated with one large vacuole secreting the spicule and there is little RER. The Golgi body as in the archeocytes is poorly defined.

5. Spongocytes:

The spicules of the sponge skeleton are bound together with spongin. This material is secreted by spongocytes and possibly other cell types such as granular cells and spherulous cells discussed elsewhere.

Wilson and Penney (1930) first recognized the presence of spongocytes in ***Microciona prolifera*** but they gave no description of the cells. Tuzet (1932) studied the spongocytes of two ***Reneria*** species. The cells measured 9x10μm and contained a nucleus with a regressing nucleolus. A Golgi body and mitochondria were present. Lévi (1960) described the "ameobocytes spongoblastes" of ***Ophlitaspongia seriata*** as cells measuring 10 x 2-4μm with a nucleolate nucleus of 2-3μm. Simpson (1963) examined the cell types of ***Microciona prolifera*** and he observed nucleolate cells filled with cytoplasmic inclusions. The cells were in close contact with spicules and histochemical results suggested the inclusions contained spongin material. Garrone and Pottu (1973) reported on the spongocytes of ***Haliclona elegans.*** The cells had a nucleolus and a finely granular cytoplasm filled with 0.8μm inclusions which contained amorphous and fibrillar material. The authors remarked that these cells had well developed endoplasmic reticulum.

The structural and chemical analysis of spongin was

undertaken by Gross *et al* (1956) to establish the relationship of spongin with the connective tissues of higher phyla. They found in ***Haliclona oculata*** as in the other sponges examined, two types of spongin. The spongin "A" bundles contained long non-branching fibers of 20-25nm in diameter which showed an axial periodicity of 65nm. Branching spongin "B" fibers were made of microfibrils less than 10nm in diameter which were embedded in an amorphous material. The authors occasionally observed an axial periodicity of 65nm in the fibers. The axial periodicity of both spongin fibers was comparable to the axial periodicity of the collagen tissue from the Vertebrae phyla. Chemical analysis of the spongin "A" and "B" from ***Spongia graminea*** revealed the presence of the same amino acids and sugars found in mammalian collagen though their proportions varied from the latter. Garrone (1969b) studied both spongin fibrils from ***Haliclona rosea.*** The perispicular spongin "B" fibrils were 5-10nm in diameter and showed an axial periodicity of 65nm. Spongin "A" fibrils were 20nm in diameter and their axial periodicity was 25nm.

The observations of this study support previous description of spongocyte cells. In ***Haliclona oculata,*** these cells were often nucleolate and their cytoplasm was filled with inclusions of 0.6μm in diameter. As reported in ***Haliclona elegans,*** the spongocytes of ***H. oculata*** have well developed endoplasmic reticulum distributed throughout the cytoplasm. A Golgi body was found predominantly in the

spongocytes of specimens fixed in the field where active secretion and growth of the sponge is certainly enhanced. Garrone and Pottu (1973) speculated on the origin of spongin "A" and "B" from a single cell type. The present study reports the first observations of such an occurence in the phylum of ***H. oculata***. Spongin "A" fibrils are often present in archeocytes but some spongocytes also have small bundles of spongin "A" which are slightly larger than their spongin "B" inclusions. The fibrils have a diameter of 20nm and an axial periodicity of 20nm. Fragments of the perispicular spongin were examined in some cell samples. The Spongin "B" fibrils have a diameter of 7nm. No axial periodicity was discernable from these fragments although better preparation of the material by Gross *et al* did reveal an axial periodicity of 65nm, Structured bodies as reported by Garrone and Pottu were not observed in the few fragments of perispicular spongin "B" from ***H. oculata***. The inclusions of the spongocytes were regularly banded when viewed under high electron density. Each band measured approximately 7nm with a more pronounced band at 28nm intervals. There can be little doubt that these inclusions are precursors to the spongin "B". Most inclusions contained amorphous material from which the bands appeared to form. Observations on granular cells and spherulous cells suggest that the bands themselves are the spongin "B" fibrils and not their axial periodicity since no fibrils were found to display such a periodicity. The structured bodies reported in ***H. elegans*** by Garrone and Pottu

(1973) have a similar appearance to the inclusions in the spongocytes of ***H. oculata***. However, further research is warranted, particularly on the secretion of the inclusions, to learn the exact nature of these bands with respect to the ultrastructure of spongin "B" fibrils.

6. Granular Cells:

There are several citations of granular cells in sponges but as Simpson (1984) acknowledges, they are not well characterized. It is the author's belief that the observations reported here show specific characteristics of the granular cells in ***H. oculata*** which will help in determining the function of these cells. The following discussion compares the granular cells of other species with those of ***H. oculata*** and it takes into consideration the suggestion made by Garrone (1978) that granular cells, microgranular cells and gray cells may secrete collagen fibrils.

Tuzet (1932) described in two ***Reneria*** species cells with small granules and cells with large granules. She believes the cells with small granules to be equivalent to the gray cells of ***Microciona prolifera*** as reported by Wilson and Penney (1930). The cells with large granules measured 12-13μm, the nucleus had an irregular nucleolus, the granules were 1μm in diameter and mitochondria and a Golgi body were

present. Lévi (1956) found in ***Halisarca dujardinii*** cells with granules which he called "cellules fuchsinophiles". The cells were 10μm in diameter, the nucleus was nucleolate and there were many round or rod-shaped inclusions of 1μm. Simpson (1968b) reported on the granular cells of two ***Reneria*** and ***Lissodendoryx*** species. Their nuclei were anucleolate and averaged 2.5μm in diameter. Their inclusions were 0.5-1.7μm in diameter. Pomponi (1974) observed granular cells in ***Haliclona variabilis*** only and two ***Callyspongia*** species. The cells in each species were approximately 10μm in diameter and a nucleolus was often present. Microgranular cells have been reported in several sponges but the size of the inclusions (0.5μm) suggests these cells may have been spongocytes.

The granular cells of ***H. oculata*** were the most altered by the artificial sea water. As compared to granular cells from control samples fixed in the field, the cells fixed in the laboratory had fewer cytoplasmic organelles and their inclusions were not as homogeneous having often both granular and fibrillar materials. The nucleus, sometimes nucleolate, was 3.2 x 2.8μm. The fibrillar material showed regular bands of 3nm in width with some larger bands of 7nm visible. A Golgi body was found only in the cells fixed in the field. Mitochondria and RER were present. The fibrillar material bears a close resemblance to the material in the inclusions of spongocytes. The amorphous material in the inclusions of granular cells from control samples showed some banding. The

reason for the consolidation of the material in the inclusions of granular cells kept in artificial sea water is not known.

Gray cells were studied by Wilson and Penney (1930) in ***Microciona prolifera.*** The cells measured 14-10 x 7μm with an anucleolate nucleus of 2-5μm and many granules of 0.5-1.0μm in diameter. Tuzet (1932) reported on cells with small granules making an analogy to the gray cells studied by Wilson and Penney. The cells measured 8-10μm and the nucleus had an irregular nucleolus. Simpson (1968b) studied several ***Microciona*** species. The gray cells in each species varied in cell size but all nuclei were anucleolate and measured 2.0-3.5μm. The cytoplasmic inclusions were 0.2-1.1μm in diameter. Histochemical analysis indicated the presence of glycogen. Borojovic and Lévi (1964) and Lévi (1966) observed rosettes of 0.18μm, beleived to be glycogen, in the gray cells of ***Ophlitaspongia serieta, Mycale contarenii*** and ***Pachymastima johnstoni.*** Bagby (1972) examined the cell population of ***Microciona prolifera.*** The gray cells had a nucleus of 1.5-2.5 x 2.0-4.5μm with a nucleolus of 0.5-0.6um. The inclusions measured 0.3-0.6 x 0.6-1.0μm. Glycogen granules were present as were ovoid mitochondria of 0.4 x 0.3μm. Golgi membranes were occasionally seen close to the nucleus. Boury-Esnault (1977) studied the gray cells in several sponge species. The inclusions were of a constant size within each species. They measured from 0.1 to 1.5μm.

The author noted that when the sponge ***Polymastia mamillaris*** was kept in hypoxic conditions by continuous bubbling of nitrogen; the gray cells accumulated glycogen rosettes. Although she characterizes gray cells as having numerous osmiophilic inclusions and glycogen rosettes, the glycogen content of these cells may depend on environmental conditions as she has shown.

No glycogen rosettes were observed in the spongocytes or granular cells of ***H. oculata***. However, histochemical results show the presence of glycogen in the spongocytes. Since granular cells could not be differentiated from spongocytes under light microscopy, the presence of glycogen in granular cells can only be considered speculative at the present.

Apart from the presence of glycogen in gray cells (subject to environmental conditions ?), there are no clear distinctions established between the gray cells and the granular cells. The inclusions of both cell types cited in the literature are often very similar in size although the gray cell inclusions are said to be smaller. Therefore, it is possible that both cell types are closely related and may secrete collagen fibrils as suggested by Garrone (1978). Further investigation of the inclusions in gray cells is needed to determine their exact nature.

7. Spherulous Cells:

There are many reports of spherulous cells in sponges. These cells have a great variety of inclusions and the reader is referred to Simpson (1984) for a complete review. However, many studies have shown that spherulous cells contain antibiotic substances. Bretting (1979) has reported the presence of lectins in the spherulous cells of an ***Axinella*** species. He suggested these lectins could play a role in the immunological response of the sponge toward other species. Spherulous cells in general are believed to have a secretory function and observations from this study support this view.

In ***H. oculata,*** these cells are characterized by the presence of a dozen or more large spherules which can fill the entire cell. The nucleus is rarely nucleolate. There are very few cell organelles and no Golgi bodies were found. The inclusions can be completly electron translucent or they may contain amorphous amd fibrillar material. The fibrillar material is similar in appearance to the material in the inclusions of spongocytes. Furthermore, fibrils of 7nm in diameter are clearly distinguishable in some spherules and may correspond to the spongin "B" fibrils of ***H. oculata.*** This provides strong evidence to support the secretion of spongin by spherulous cells as suggested by Garrone (1978).

8. Globoferous Cell Inclusions:

No globoferous cells per se were found in the cell samples of *H. oculata* but two large uniform inclusions were examined and are believed to be from globoferous cells. The inclusions are similar to those reported by Borojovic and Levi (1964) from *Ophlitaspongia serieta*. The inclusions contained microtubule-like bodies which measured 18-20 x 70nm. In *H. oculata*, these microtubule-like bodies were 68nm in diameter. It is presumed that a cross section of the inclusions was observed, therefore there is no data on the length of the microtubule-like bodies at the present if they are indeed such a structure. No globoferous cells have been reported in other *Haliclona* species. The function of these inclusions is not known.

Statistics:

The various statistical analysis of this study permitted further definition of the cell types. Although a great deal of variation in the measurements can be expected due to the very small cross- sections taken, this new approach will help to eliminate ambiguities in the dimensions of different cell types. Hence, from the measurements recorded, it is concluded that archeocytes and sclerocytes are physiologically closely related whereas choanocytes and pinacocytes differ significantly from all other cell types. Finally,

spongocytes, granular cells and spherulous cells have different inclusions but their nuclei are of similar dimensions.

Cell separation on Ficoll Gradients:

Observations on the differentiation of archeocytes into other cell types have been reported. However, these are limited to transformation into choanocytes (Rasmont and Rozenfeld, 1981) and pinacocytes (Bagby, 1972), and little is known of the transformation of archeocytes into the various cells with inclusions. Recent studies have shown that spherulous cells contain antibiotic substances. Therefore, cells with inclusions may have different functions. This study did not concentrate on the biochemical make-up of the cells and observations here indicate that spongocytes, granular cells and spherulous cells may secrete collagen fibrils. Could antibiotic elements be present with the collagen? Further work on the differentiation of these cells is needed to determine their function and the source of their different inclusions in various species.

Agrell (1951) attempted to separate the cell types of ***Halichondria panicea*** on sucrose gradients. He described only three types: nucleolate cells, anucleolate cells and flagellated cells. The nucleolate cells which were separated, when incubated, differentiated into a functional sponge.

However, there is no evidence to suggest the cells Agrell was able to isolate were exclusively archeocytes. Burkart and Burger (1977) studied the cell population of ***Microciona prolifera.*** They made a rough cell type separation using Ficoll gradients but as with Agrell, they did not distinguish different nucleolated cells. De Sutter and Van de Vyver (1977) isolated the archeocytes of the fresh water sponge ***Ephydatia fluviatilis*** with multiple Ficoll gradients. The archeocytes, when incubated, formed a functional sponge. De Sutter and Buscema (1977) devised a method to isolate archeocytes from the same sponge using 4 Ficoll gradients and Buscema et al (1980) made observations on the differentiation of the isolated archeocytes with electron microscopy. These observations constitute the only direct evidence of the ability of archeocytes to differentiate into other cell types of a sponge but most specimens used were grown from gemmules for one week only. Indeed, de Sutter and Tulp (1981) working with the same material and similar methods admitted finding many archeocytes which contained vitelline platelets. These structures are characteristic of gemmular archeocytes, therefore they cannot be considered adult sponge cells as previous observations indicated. Furthermore, only 4 cell types are present in such juvenile sponges: archeocytes (gemmular?), choanocytes, pinacocytes (often nucleolate) and sclerocytes (archeocyte-like). Finally, Van de Vyver and Buscema (1981) noted that sponges grown from isolated archeocytes oversecreted spicules and contained fewer

choanocyte chambers than normal sponges. This could be caused by the lack of food and cell debris available to isolated cells.

The isolation of archeocytes from the cell population of ***H. oculata*** was not successfull. Although many modifications of the method used by de Sutter and Buscema (1977) were tested, the best results obtainable were not those desired. Many nucleolate cells were present in the bottom cell fraction but they included spongocytes, granular cells and sclerocytes as well as other undifferentiated cells. Further separation with this method may not be possible on such simple gradients since there are more cell types present in the adult sponge and many of these have similar cell dimensions. No top gradients were collected for examination. Since choanocytes were absent from the bottom gradient, it is infered that these cells were separated in the top gradients. Hence, the Ficoll gradients were effective in separating the small cells only. The use of CMF-SW to make the Ficoll gradients could also have affected the density of the different gradients. Naturally, the density of marine sponge cells in an adult specimen is likely different from that of cells from a juvenile fresh water sponge and the use of other Ficoll concentrations may improve on the results obtained here. The isolated cells when examined, were also in a poor state of preservation. Yet, the cell's permeability to trypan blue was insignificant after the dissociation procedure. The

centrifugation of the cells after such dissociation could be the cause of some cell deterioration.

In conclusion, the isolation of archeocytes from an adult sponge cell population will likely require a more extensive array of Ficoll gradients or successive centrifugation to acquire the desired cell fraction. Moreover, this study has shown the presence of at least 7 cell types in adult specimens of ***H. oculata***. Evidence is presented to support the secretory role of granular cells and spherulous cells. The observations indicate for the first time that these cells may secrete spongin "B" fibrils as do spongocytes. Finally, the statistical analysis of cell dimensions has further defined each cell type from the cell population of ***Haliclona oculata***.

SUMMARY

1. Specimens of the sponge ***Haliclona oculata*** (Demospongiae, Haplosclerida) were collected from Passamaquody Bay, New Brunswick in April, June and August 1984 and healthy specimens were transported back to Montréal, Québec.

2. The sponges, kept in artificial sea water, were chemically dissociated in a calcium-, magnesium-free medium. Two samples of the resulting cell suspension from each of two specimens were fixed in gluteraldehyde and osmium tetroxide, dehydrated and embedded in Spurr epoxy resin. Some samples were also fixed and embedded in paraffin.

3. Thick sections from the paraffin blocks were stained for glycogen, mucosubstances and mucopolysaccharides and examined under light microscopy. Thin sections from the epoxy blocks were stained for contrast with uranyl acetate and lead citrate. They were examined and photographed with a transmission electron microscope.

4. The various dimensions of the cells and cell inclusions were measured from electron micrographs. The cells were categorized into types by the presence of a nucleolus, cell size and the inclusions present. Statistical tests were made to further define each cell category.

5. Seven cell types were found in adult tissue of ***H. oculata*** throughout the sample period. They are: archeocytes, choanocytes, pinacocytes, sclerocytes, spongocytes, granular cells and spherulous cells. Globoferous cells may also be present in this sponge as two inclusions similar to those reported in globoferous cells of other species were found.

6. The isolation of archeocytes from the other cell types was attempted using Ficoll gradients. A cell sample was centrifuged through four Ficoll gradients (4%, 6%, 8% and 10%) and the bottom gradient (10%) was collected, washed with artificial sea water, fixed and embedded in Spurr. This gradient contained not only archeocytes but sclerocytes, spongocytes and granular cells as well as some undifferentiated cells.

7. The results of this study are discussed in comparison to previous observations made on similar material from other sponges. A hypothesis is presented for the function of the various cells with inclusions.

BIBLIOGRAPHY

Agrell I., 1951. Observations on cell differentiation in sponges. Ark. Zool. **4** (2): 519-525.

Bagby R.M., 1970. The fine structure of pinacocytes in the marine sponge *Microciona prolifera* (Ellis and Sollander). Z. Zellforsch. **105**: 579-594.

Bagby R.M., 1972. Formation and differentiation of the upper pinacoderm in reaggregation masses of the sponge ***Microciona prolifera*** (Ellis and Sollander). J. Exp. Zool. **180**: 217-225.

Borojovic R., 1966. Étude expérimentale de la différentiation des cellules de l'éponge au cours de son dévelopement. Dev. Biol. **14**: 130-153.

Borojovic R. et C. Lévi, 1964. Étude au microscope électronique des cellules de l'éponge ***Ophlitaspongia serieta*** (Grant) au cours de la réorganisation après dissociation. Z. Zellforsch. **64**: 708-725.

Boury-Esnault N., 1973. L'exopinacoderme des Spongiaires. Bull. Mus. Nat. Hist. Nat.(Paris), ser. 3, **117**: 1193-1206.

Boury-Esnault N., 1977. A cell type in sponges involved in the metabolism of glycogen. Cell. Tiss. Res. **175**: 523-539.

Bretting H., 1979. Purification and characterization of sponge lectins and a study of their immunochemical and biological activities. In: C. Lévi and N. Boury-Esnault (Eds). Biologie des Spongiaires. Colloq. Internat. C.N.R.S., Paris **291**: 247-255.

Burkart W. and M.M. Burger, 1977. Studies on cell population from the marine sponge ***Microciona prolifera*** separated by Ficoll gradients. Bio. Bull. **153**: 417.

Buscema M., de Sutter D. and G. Van de Vyver, 1980. Ultrastructural study of differentiation processes during aggregation of purified sponge archeocytes. W. R. Arch. **188**: 45-52.

Curtis A.S.G., 1962. Pattern and mechanism in the reaggregation of sponges. Nature **196**: 245-248.

de Sutter D. and M. Buscema, 1977. Isolation of a highly pure archeocyte fraction from the fresh water sponge ***Ephydatia fluviatilis***. W. R. Arch. **183**: 149-153.

de Sutter D. and A. Tulp, 1981. A method for large scale sponge cells separation: 1 g sedimentation (cellular composition of the fractions obtained). Biol. Cell. **40**: 63-68.

de Sutter D. and G. Van de Vyver, 1977. Aggregative properties of different cell types of the fresh water sponge ***Ephydatia fluviatilis*** isolated on Ficoll gradients. W. R. Arch. **181**: 151-161.

de Sutter D. and G. Van de Vyver, 1979. Isolation and recognition properties of some definite sponge cell types. Dev. Comp. Immunol. **3**: 389-397.

Fell P.E., 1974. Diapause in the gemmules of the marine sponge ***Haliclona loosanoffi***, with a note on the gemmules of ***Haliclona oculata***. Bio. Bull. **147**: 333-351.

Galstoff P.S., 1925. Regeneration after dissociation (An experimental study on sponges) I. Behavior of dissociated cells of ***Microciona prolifera*** under normal and altered conditions. J. Exp. Zool. **42**: 183-222.

Garrone R., 1969a. Une formation paracristalline d'ARN intranucléaire dans les choanocytes de l'éponge ***Haliclona rosea*** (O.S.)(Démosponge, Haplosclérides). C. R. Acad. Sci. Paris **269**: 2219-2221.

Garrone R., 1969b. Collagène, spongine et squelette minéral chez l'éponge ***Haliclona rosea*** (O.S.)(Démosponge, Haplosclérides). J. Microsc. **8**: 581-598.

Garrone R., 1978. Phylogenesis of connective tissue. Morphological aspects and biosynthesis of sponge intercellular matrix. S. Karger. 250 pp.

Garrone R. and J. Pottu, 1973. Collagen biosynthesis in sponges: Elaboration of spongin by spongocytes. J. Submicr. Cytol. **5**: 199-218.

Gross J., Sokal Z. and M. Rougvie, 1956. Structural and chemical studies on the connective tissue of marine sponges. J. Histochem. Cytochem. **4**: 227-246.

Hartman W.D., 1958. Natural history of the marine sponges of southern New England. Bull. Peabody Mus. Yale **12**: 1-155.

Henkart P., Humphreys S. and T. Humphreys, 1973. Characterization of sponge aggregation factor. A unique proteoglycan complex. Biochem. **12**: 3045-3050.

Humphreys T., 1963. Chemical dissolution and *in vitro* reconstruction of sponge cell adhesions. I. Isolation and functional demonstration of the components involved. Dev. Biol. **8**: 27-47.

Humphreys T., Humphreys S. and A.A. Moscona, 1960. A procedure for obtaining completly dissociated sponge cells. Bio. Bull. **119**: 294.

Hyman L.H., 1940. The invertebrates. Vol.1. McGraw-Hill, New York.

Lévi C. 1956. Étude des ***Halisarca*** de Roscoff: Embryologie et systématique des Démosponges. Ark. Zool. exp. Gén. **93**: 1-184.

Lévi C., 1960. Reconstitution du squelette de l'éponge ***Ophlitaspongia serieta*** (Grant) à partir de suspensions cellulaires. Cah. Biol. Mar. **1**: 353-358.

Lévi C., 1963. Scléroblastes et spiculogénèse chez une éponge silicieuse. C. R. Acad. Sci., Paris **256**: 497-498.

Lévi C., 1966. Le glycogène chez les spongiaires. C. R. Soc. Biol. **3**: 651-652.

Lowenstein W.R., 1967. On the genesis of cellular communication. Dev. Biol. **15**: 503-520.

Mackie G.O. and C.L. Singla, 1983. Studies on Hexactinellid sponges. I. Histology of ***Rhabdocalyptus dawsoni*** (Lambe). Phil. Trans Roy. Soc.(London) **301**: 365-400.

Moscona A.A., 1963. Studies on cell aggregation: Demonstration of materials with selective cell-binding activities. Proc. Nat. Acad. Sci.(USA) **49**: 742-748.

Müller W.E.G. and R.K. Zahn, 1973. Purification and characterization of a species-specific aggregation factor in sponges. Exp. Cell Res. **80**: 95-104.

Pearse A.G.E., 1968. Histochemistry: Theoretical and applied. Vol. I. Little, Brown and Compagny, Boston. 759pp.

Pomponi S.A., 1974. A cytological study of the Haliclonidae and the Callyspongiidae (Porifera, Demospongiae, Haplosclerida). In: F.W. Harrison and R.R. Cowden (eds.). Aspects of sponge biology. pp. 203-213.

Rasmont R., 1963. Le rôle de la taille et de la nutrition dans le déterminisme de la gemmulation chez les Spongillides. Dev. Biol. **8**: 243-271.

Rasmont R. and F. Rozenfeld, 1981. Étude microcinématographique de la formation des chambres choanocytaires chez une éponge d'eau douce. Ann. Soc. Roy. Zool. Belg. **111**: 33-44.

Reed C.G.and R.A. Cloney, 1979. Brachiopod tentacles: Ultrastructure and functional significance of the connective tissue and myoepithelial cells in ***Terebratalia***. Cell Tiss. Res. **185** (1): 17-42.

Reynolds E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. **17**: 208-211.

Simpson T.L., 1963. The biology of the marine sponge ***Microciona prolifera*** (Ellis and Sollander) I: A study of cellular function and differentiation. J. Exp. Zool. **154**: 135-151.

Simpson T.L., 1968a. The biology of the marine sponge ***Microciona prolifera*** (Ellis and Sollander) II: Temperature related annual changes in functional and reproductive elements with a description of larval metamorphosis. J. Exp. Mar. Biol. Ecol. **2**: 252-277.

Simpson T.L., 1968b. The structure and function of sponge cells: New criteria for the taxonomy of Poecilosclerid sponges (Demospongiae). Bull. Peab. Mus. Nat. Hist.(Yale) **25**: 1-140.

Simpson T.L., 1984. The cell biology of sponges. Springer-Verlag, New york Inc. 662pp.

Spurr A.R., 1969. A low viscocity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31-43.

Topsent E., 1888. Note sur les gemmules de quelques silicospongeuses marines. C. R. Acad. Sci. Paris **106**: 1298-1300.

Tuzet O., 1932. Recherches sur l'histologie des éponges ***Reneria elegans*** (Bow.) et ***Reneria simulans*** (Johnston). Arch. Zool. exp. gén. **74**: 169-192.

Van de Vyver G., 1971. Mise en évidence d'un facteur d'aggrégation chez l'éponge d'eau douce ***Ephydatia fluviatilis***. Ann. Embryol. Morph. **4**: 373-381.

Van de Vyver G. and M. Buscema, 1981. Capacitées morphogènes des cellules d'éponges dissociées. Ann. Soc. Roy. Zool. Belg. **111**: 9-19.

Willenz P. and G. Van de Vyver, 1984. Ultrastructural localization of lysosomal digestion in the fresh water sponge ***Ephydatia fluviatilis***. J. Ultrastruct. Res. **87** (1): 13-22.

Wilson H.V., 1907. On some phenomena of coalescence and regeneration in sponges. J. Exp. Zool. **5**: 245-258.

Wilson H.V. and J.T. Penney, 1930. The regeneration of sponges from dissociated cells. J. Exp. Zool. **56**: 73-148.