IN VIVO AND IN VITRO STUDIES ON AN ENDOZOIC ALGA FROM THE GIANT SCALLOP, PLACOPECTEN MAGELLANICUS (GMELIN)

ROBERT NORMAN STEVENSON

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In vivo And *in vitro* Studies On An Endozoic Alga From The Giant Scallop, *Placopecten magellanicus* (Gmelin)

by

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A Thesis

submitted in partial fulfilment
of the requirements for the degree of
Master of Science
Department of Biology
Memorial University of Newfoundland

St. John's Newfoundland

February 1972

ACKNOWLEDGEMENTS

Above all I wish to express sincere thanks to my supervisor, Dr. G. Robin South, for providing the opportunity to conduct this research and for the constant help and encourgement during the research and thesis preparation. I also thank Dr. South for making it possible for me to travel to the Haskins Laboratory in New York to gain information on culture techniques from Dr. L. Provasoli to whom I am also grateful. Thanks are also extended to Dr. D.L.Taylor for his very informative talks to me on theoretical aspects of algal symbiosis during my short visit to the Haskin's Laboratory.

Mrs. P. Yorke of the M.S.R.L., Mr. E. Rowe, F.R.B.C., Biological Station, St. John's, Mr. J. Merriman and Miss R. Merriman, Photography Department, Camp Hill Hospital, Halifax and especially Mr. R. Ficken the Biology Department photographer are to be commended for their photographic work.

I would also like to thank Dr. C. Davis for his helpful criticism of the thesis during its preparation.

I thank the S.C.U.B.A. divers at the M.S.R.L. and F.R.B.C. for collecting the experimental animals and also the crew of the *A.T. Cameron* for collecting the oceanic seawater for the culture media.

Finally I wish to thank Mrs L. Mosher for the patience and care taken in typing this thesis.

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ABSTRACT

A literature survey indicates that much has been published on algal symbiosis with animals, however, few accounts have been presented in which the algal partner is considered parasitic. Previous reports on the endozoic algae of the giant scallop, *Placopecten magellanicus* (Gmelin), indicates that the relationship is parasitic. This definition is maintained here with the realization that future investigations of both partners are required to fully explain the association.

Description of the algal colonies within the tissues of the giant scallop is presented. Quantitative analysis of the colonies within the mantle tissue indicates a definite distribution pattern. Algal colony concentration was found to be highest within a very narrow delicate strip of tissue, the shell fold, which lies in direct contact with the shell. Colonies were more numerous and larger within the mid-mantle regions of the shell fold tissue. Theories involved in this distribution pattern are presented.

Description of the alga involved in symbiosis is difficult to accomplish completely since there are differences in morphology from *in vivo* and *in vitro*. It is stressed that in newly reported associations it is necessary to describe the alga both from culture and from the animal. Based on morphology *in vivo* and *in vitro* a possible taxonomic position is presented. The alga appears to belong to the Class Chlorophyceae, Order Chlorococcales. Evidence for this position is gained from morphology, reproduction and pigmentation as determined by paper chromatography and spectro-

photometry. Designation to a Family is hampered by discrepencies in reproduction *in vitro* as compared with *in vivo*. The Family Coccomyxaceae and Family Oocystaceae are suggested as possible positions, However, it is pointed out that positive identification of this endozoic alga will have to await electron microscopy studies.

Re-infection experiments demonstrate a change in morphology of the algal cells from the *in vitro* to the *in vivo* habitat as well as demonstrate the tissue and cellular responses of the giant scallop to the algal cells.

Besides the tissues previously reported to be infected by the endozoic algae, colonies were found within the gill filaments illustrating that settlement is possible in tissues where ciliary action occurs. Colonies were also found deep within the adductor muscle and on the anus. Algal cells were observed within blood cells of the circulatory system as well as within phagocytes within the infected tissues.

Theories on the mode of infection are presented based on experimental evidence and literature reports. Initial infection is suggested to be more likely via the digestive system than via mantle penetration as previously suggested. Phagocytosis is suggested to play a major role in the mode of infection and transmission of the algae throughout the scallop tissue as well as spread of the infection to other scallops.

INTRODUCTION

While carrying out growth and population studies on the commercially important giant scallop, *Placopecten magellanicus* (Gmelin), Naidu (1969) reported the incidence of algae in the mantle tissues.

The algae imparted a green coloration to the affected scallops which, upon further investigation, was found to be due to the presence of large numbers of unicellular algae in discrete packets in the mantle and other parts of the host. Naidu and South (1970) reported briefly on the occurrence, suggesting that the responsible algae were zoochlorellae. Naidu (1971) carried out extensive histological studies on the relationship between the algae and their molluscan host and concluded that the algae were parasites and caused considerable stress to the scallop.

An extensive literature exists concerning the occurrence of algae in animals, both in the marine and freshwater environments. Comprehensive reviews on the subject are those of Fritsch (1952), Caullery (1952), Droop (1963), McLaughlin and Zahl (1966), Cheng (1967) and Smith *et al.* (1969). Few reports, however, specifically describe a parasitic relationship. Mortensen and Rosenvinge (1910) reported a parasitic association between unicellular algae and echinoderms. The same authors later (1933) reported the identity of a parasitic unicellular alga, *Coccomyxa astericola* from a sea star host. Davies *et al.* (1964) documented the first case of parasitic

alga (*Prototheca segbwema*) infecting a human host, and Hoffman *et al.* (1965) reported a unicellular green algal parasite, *Chlorella* sp., from a bluegill. Klintworth *et al.* (1968) reported a second case of a parasitic alga infecting man and identified the pathogen as *Prototheca wickerhamii*.

The terminology used to describe relationships between algae and animals, mostly invertebrates, has suffered from considerable confusion in the literature. Naidu (1971) reviewed some aspects of this. It is not the purpose of this investigation, however, to specifically define the true nature of the association between the scallop and its algae, rather to examine the alga itself in some detail. True definition of the nature of the relationship must await more detailed investigation of both partners, especially from a physiological point of view.

Algae occurring endozoically have generally been placed into three rather loose groups, namely zoochlorellae, zooxanthellae, and cyanellae. As previously indicated by Droop (1963), these groupings have no taxonomic significance. It is only recently that workers have begun to establish the true identity of some endozoic algae, the best examples to date being; Freudenthal (1962), *Gymnodinium microadriaticum* from *Cassiopeia*; Parke and Manton (1967), *Platymonas convolutae* from the flatworm *Convoluta roscoffensis*; Taylor (1971a), *Amphidinium chattonii* from *Velella velella* and Taylor (1971b), *Amphidinium klebsii* from *Amphiscolops langerhansi*.

Isolation and identification of the algae involved in any algal/animal association is a necessary prerequisite for subsequent physiological, ecological and nutritional studies, on which basis the relationship can be more fully defined. With the few notable exceptions mentioned above, this first step has not been achieved and relationships have been defined on circumstantial rather than experimental evidence. The difficulty of isolating the algae has been a major stumbling block, since conventional isolation techniques may not be applicable.

Since the identity of the algae in *P. magellanicus* was not established by Naidu and South (1970) and only an initial isolation was attempted, the primary purpose of the present study was to isolate the algae, to describe them in detail both from the host and from culture, and to establish as far as possible their identity and systematic position. The scope of the investigation was then broadened by examining selected aspects of the relationship between the algae and their host, particularly those associated with the possible mode of infection.

For convenience, the parasitic definition given by Naidu (1969) has been maintained here, with the realization that complete justification of this conclusion will depend on future relevant research.

Materials and Methods

Collection and Maintenance of Experimental Animals

Samples of scallops collected from St. Joseph's, Salmonier, St. Mary's Bay (Fig. 1, Area 1) and the Boswarlos Bed, Port au Port Bay (Fig. 1, Area 2) formed the basis of the present investigation. A high incidence of the endozoic algae was previously reported from these populations by Naidu (1969). The St. Joseph's scallops were collected by S.C.U.B.A. divers, while a commercial scallop drag was used in the Boswarlos Bed.

The S.C.U.B.A. divers picked up all scallops encountered during a 30-45 minute dive, and hence a near-random sample was assumed for each collection from the St. Joseph's population. Scallops were placed in plastic buckets partially filled with sea water for immediate road transportation to the Marine Sciences Research Laboratory at Logy Bay.*

Scallops collected by the commercial scallop drag were placed in a submerged wooden holding pen until time for shipment to St. John's. On the day of shipment the animals were air-freighted from Stephenville to St. John's, and transported immediately on arrival to the M.S.R.L. At the M.S.R.L. they were placed in running sea water tanks and left there over-night for a period of recuperation.

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* Subsequently referred to as M.S.R.L.

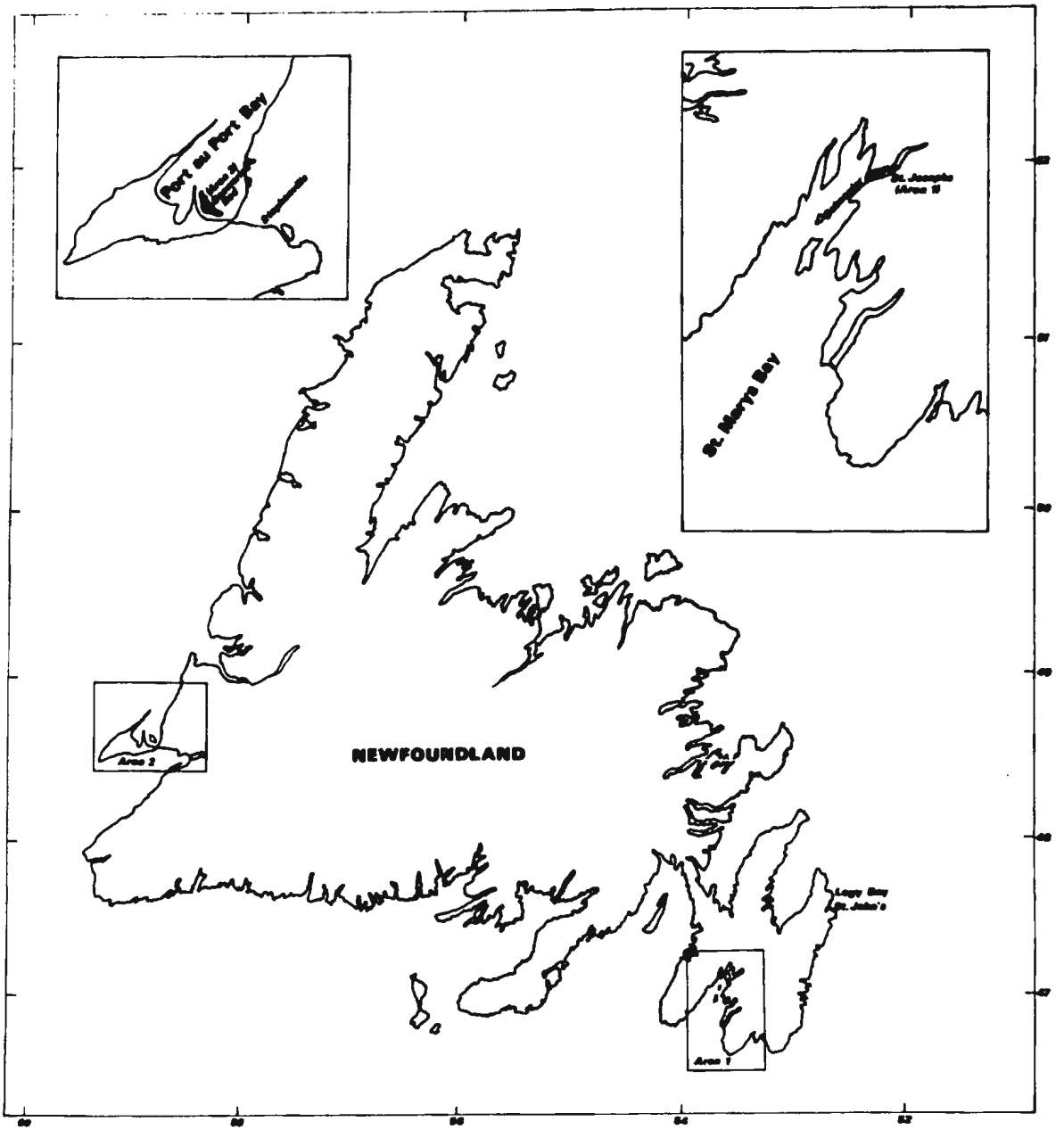

FIGURE 1. Collection areas in Newfoundland. St. Joseph's, Salmonier (Area 1); Boswarlos Bed, Port au Port Bay (Area 2).

After the recuperation period the animals were counted and observed for the presence of the endozoic algae. Infected scallops were detected by visual examination of the exposed mantle tissue for 'green spots'. To facilitate examination the valves of the scallop were pried apart with a putty knife, care being taken not to damage the animal. Each scallop was tagged and designated a number indicating the date and locality of collection, and whether or not it was infected. The infected scallops were placed in separate tanks from the uninfected ones. In some instances, because of lack of tanks, infected animals had to be placed in the same tank as the uninfected animals. When this was necessary the infected molluscs were placed near the out-flow to reduce the possibility of their infecting presumably uninfected ones.

Tanks in which the scallops were placed were drained and cleaned every month and whenever a dead scallop was discovered it was immediately removed from the tank. The flow of fresh sea water was sufficient to maintain a constant temperature within the tanks and also supply the animals with food. Scallops remained alive in the tanks for over a year with no visible stress occurring.

Records were kept during the study on the number of scallops collected, the location, the number of animals infected, the depth at which each sample was collected and the method by which the sample was taken. This data is presented in Table 5.

In vivo Studies

(1) Algal colony distribution and size:

Naidu (1969) reported the areas of algal infection within the scallop tissues and pointed out that the mantle margin was the principal or preferred site of infection. In the present study a systematic investigation was carried out to determine the distribution and size of the algal colonies within the mantle tissues.

Infected scallops were studied and the amount of infection in different parts of the mantle determined subjectively. Preliminary findings warranted a closer quantitative analysis of the distribution pattern.

A ten year old moderately infected female scallop, an eight year old light-moderately infected female scallop, and a seven year old lightly infected male scallop from the St. Joseph's population collected October 20, 1969 were chosen for close preliminary studies on the algal colony distribution pattern. Colony concentration was determined by a modification of the methods employed by Feng (1958) for *Nematopsis* cysts in oysters.

The right valve was removed thus exposing the mantle tissue. An incision 10 mm back from the mantle edge was made along the entire right and left mantle and the strip of mantle tissue removed and measured to the nearest millimetre (Fig. 2,A). The excised mantle tissue was then treated with 10% KOH for 2 - 3 min to digest away some of the animal tissue. The partially digested tissue was then

placed between two microscope slides and observed under low power with a compound microscope. Colonies were counted and measured in three anatomical areas (mantle velum, mantle margin, and shell fold) in each excised segment, (Fig 2B, a,b,c).

It was found that counting colonies over the entire mantle was a very tedious and time consuming task and hence refinement in technique was made to accommodate larger samples. The mantle edge was excised, measured and cut into segments corresponding to 10% of the entire mantle length (Left and Right mantles together) (Fig. 2C). A sample measuring 10 mm long was then cut from the central portion of each 10% segment and treated with 10% KOH. The number of colonies per mm^2 for the sample segments was used as an estimate of the density of colonies per 10% mantle segment. This procedure was carried out on a sample of 10 additional scallops of varying degrees of infection from the St. Joseph's population.

(2) Preparation and examination of algal smears:

Smears were prepared from infected scallops to study the cell size, cell size in relation to reproductivity of the algae, amount of phagocytosis, and for fixation for further cytological examination.

A finely drawn-out pipette was used to extract algal samples from the infected animals. Sites along the exposed mantle edge were chosen since samples could be taken without destroying the animal. Samples from other anatomical areas such as the gills, mantle margin, gonads and adductor muscle were taken when heavily infected

FIGURE 2

Excision and segmentation of mantle tissue for algal colony distribution studies. Scallop with upper valve removed (A) indicating line of incision (I) along the entire mantle length. Segment of mantle placed on microscope slide (B) showing areas in which colonies were counted; mantle margin (a), shell fold (b), and mantle velum (c). Entire excised mantle (C) subdivided into segments representing 10% of the entire mantle length from which samples (s) were cut for study.

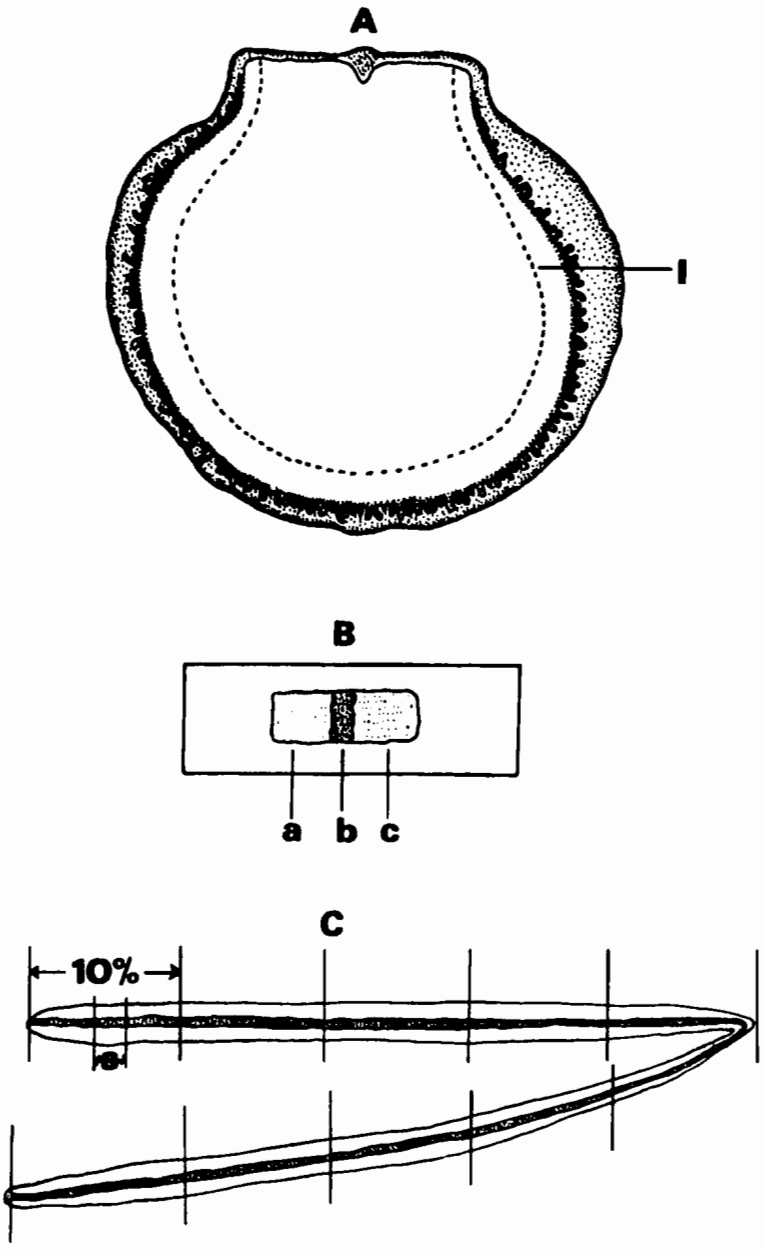

FIGURE 2. Excision and segmentation of mantle tissue for algal colony distribution studies. For explanation see page 9 and text.

scallops were destroyed and the valves removed.

Cell size was determined by measuring 40 cells per smear at random with an ocular micrometer previously calibrated. Using the same smear, reproductivity of the algae was determined by counting the number of cells dividing and not dividing in 10 fields using a 100x oil immersion objective.

Smears were fixed in 90% alcohol, FAA, 10% formalin, 3:1 alcohol: acetic acid, Newcomer Fixative and Bleaching Solution for further cytological examinations.*

(3) Preparation and examination of blood, stomach and fecal smears:

Routine examination of blood, stomach contents and feces contents for the presence of the endozoic algae was carried out on infected and uninfected scallops. Blood was removed from the heart which was exposed by removing the left valve and making a small incision in the pericardial wall. A small puncture was made in the ventricle and the blood removed with a pipette or syringe. The fluid was deposited on a slide and examined immediately under oil immersion or allowed to air dry and fixed and stained with Giemsa Stain. An incision was made in the wall of the stomach and the contents removed and spread thinly on a microscope-slide for immediate examination or fixation for Giemsa Staining. Fecal smears were prepared by collecting fecal pellets from the bottom of the sea water tank or by 'milking' feces onto a slide directly from the live animal with the aid of a pair of

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* Appendix A . . . Preparation of Fixatives.

fine, clean forceps. The fecal smears were studied immediately or fixed for Giemsa Staining.

Animal experimentation

(1) Experimental phagocytosis

Experiments were carried out to determine the phagocytic activity of the animal cells within the blood and digestive tract of the scallop. Finely ground carbon particles were mixed with sea water and injected into the ventricles of uninfected scallops, as described by Stauber, (1950) for oysters. After 24 hours a sample of blood fluid was removed from the heart and observed under oil immersion. Carbon particles were mixed with sea water supplied with freshly collected blood from uninfected scallops in depression microscope-slides fitted with air-tight cover slips (held with petroleum jelly) and refrigerated at $2^{o}C$ for 24 hours, after which samples were studied under oil immersion. Carmine in sea water was injected into the hearts of scallops and mixed with blood in a depression slide and studied after 24 hours. Algae grown in culture were injected into the hearts of uninfected scallops and after 24 hours samples were studied for phagocytic activity. Algal cells in sea water were mixed with freshly collected blood and observed after 24 hours for phagocytic activity.

Algal cells grown in culture were injected into the stomach of living scallops and later, samples from the stomach contents were examined for phagocytic activity. Algal cells from culture were

placed in a quantity of sterile sea water in an aquarium aerated with commpressed air. Scallops were cleaned thoroughly with a wire brush and placed in the aquarium with the algae. After 24 hours samples of the scallops' feces were observed for phagocytic activity.

(2) Artificial infection of scallops

Artificial infection experiments had a four-fold purpose: (1) to investigate the possibility of infecting presumably uninfected scallops with algae grown in culture; (2) to find if there are areas on the mantle where infection is more likely to be established; (3) to study characteristics of the alga during transfer from culture to host, and vice-versa; and (4) to study the responses of the host to the cultured algae.

Algae grown in culture were introduced to the uninfected animal to see if reinfection was possible. This was carried out in several ways:

(a) Algae grown on Chihara-agar plates* incubated for 5 weeks were injected into the mantle tissue of visibly uninfected scallops at different points along the mantle edge with a hypodermic syringe with a No. 10 gauge needle. Three areas on each mantle were chosen as injection sites: Right and left anterior adjacent to mantle fusions; right and left mid-line of mantle; and right and left posterior adjacent to mantle fusions.

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* See page 15

(b) The right valve was damaged at three points along its edge (viz. anterior, mid-line and posterior) and the cultured algae deposited on the exposed tissue.

(c) Algae grown on plates were deposited between the shell and the mantle fold in the right and left mid-line mantles.

(d) Scallops were isolated to sterile sea water aquaria and fed algae grown in liquid culture.

Scallops exposed to the algae were isolated from the other experimental animals and observed every week for the presence of algae. The valves were pried apart and studied closely with the aid of a hand lens under a strong light. When algal deposits were observed, samples were extracted with a finely drawn out pipette for microscopic examination. When the algae could no longer be observed with the hand lens the scallops were destroyed and the mantle tissue originally injected with the algae was excised and sectioned with a freezing microtome for histological examination. One of the scallops in which algal colonies could be observed was also destroyed and sectioned. Fecal, stomach and blood samples were also examined periodically from the experimental animals.

(3) Age determination of scallops

Since many of the infected scallops were heavily covered with epifaunal and epiphytic growths the annual growth rings on the left valves could not be used for age determination. To determine the

age of the scallops the calcareous parts of the resilia were used as in the procedure described by Naidu (1969).

Culture techniques

(1) Source of cultures and subcultures:

Three culture strains of the endozoic algae were studied in the present investigation. Cultures initially used were originally isolated by Dr. G.R. South and maintained by Dr. J.R. Stein at the Department of Botany, University of British Columbia (Naidu and South, 1970).* Two liquid (one old and one young, actively growing) cultures were obtained from Vancouver in early January, 1969. The cultures were growing in a medium formulated by Dr. M. Chihara (Appendix B).** Cultures of the endozoic alga were initiated from two infected scallops by the author; algae isolated from a 7 year old female from the St. Joseph's population; algae isolated from a 9 year old male from the Boswarlos Bed population.

Algal cultures were initiated from live scallops in two ways: (1) Small pieces of scallop tissue containing large numbers of algae were excised and washed thoroughly in 10 rinses of sterile sea water. The 'cleaned' tissue with the algae was then placed in a 25 ml. screw top test tube containing 12 ml. of freshly prepared Chihara Medium and

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* Referred to subsequently as the Vancouver Cultures.

** Referred to subsequently as Chihara Medium.

shaken vigorously for a few minutes. After a few days the animal tissue was removed from the test tube with a sterile glass needle. Cultures were successfully started this way, but were very heavily bacterized.

(2) The mantle tissue containing the algae was excised and washed in 5 rinses of sterile sea water. The tissue was then placed in 200 ml. of sterile sea water in a Waring Blendor and homogenized for 3 minutes. The resultant mixture was strained through 6 layers of cheese cloth to remove the large tissue particles. The strained solution was then centrifuged with sterile sea water for 3 minutes. The process was repeated 6 times while between each centrifugation the cells were washed with sterile sea water. This method proved to be better since larger amounts of the algal cells could be used to initiate cultures, the number of bacteria was reduced because of the repeated centrifugations and washings, and the direct initiation of slope and plate cultures was possible.

(2) Culture media and preparation:

Cultures of the endozoic algae were grown and maintained on 4 culture media which are presented in Appendix B.

Sea water initially used in the preparation of the Chihara and Erdschreiber media was obtained from the running sea water system at the M.S.R.L. It was soon realized, however, that the media made up at different times gave inconsistent results and hence a standard sea water source was desired for the remainder of the study. Since

oceanic sea water is preferred for culture studies, sea water was obtained from the South East Grand Bank, more than 250 miles from shore. The Scientific crew of the Fisheries Research Board of Canada's research vessel, *A. T. Cameron*, returned 12 gallons of the oceanic sea water to the M.S.R.L., which was sufficient for the entire study.

Sea water used in the preparation of the media was either heat treated or autoclaved. The sea water was first filtered through three layers of Whatman No. 1 filter paper to remove much of the organic material and debris. The heat treatment procedure was carried out on three consecutive days. The water was heated to 73°C and held at this temperature for 15 minutes. The next day the sea water was refiltered and reheated to 73°C. The process was repeated on the third day. Sea water which was to be autoclaved was mixed with 200 ml. distilled water per 800 ml. sea water to reduce the salinity and hence reduce precipitation. The sea water was autoclaved in 500 ml. flasks at 15 psi. for 15 minutes. This method, however, proved to be very inconsistent since precipitation often occurred.

The trace metals and elements were autoclaved separately in stock solutions and added to the sea water base with sterile pipettes. The solutions were left over-night to ensure complete dissolution of the chemicals before the pH was tested and altered to the required value with concentrated NaOH.

Even though bacteria-free cultures were not accomplished in this study, care was taken with the cultures and culture vessels to reduce

outside contamination. All glassware used was thoroughly machine-washed with 3 distilled water rinses to ensure complete removal of detergents, and then baked in an oven for 5 hours at 120°C.

In preparation of the liquid cultures 20 ml. of the liquid medium was added to each 20 x 180 mm. screw top test tube with a sterile pipette. Pipette, flask and test tube were flamed at each transfer.

A 1.5% agar preparation of the desired medium was used as a solid substrate for the algae. Powdered agar was added to the liquid medium in 500 ml. flasks and heated gently to dissolve the agar. Eight to 10 ml. of the preparation were added to 16 x 150 or 20 x 180 mm. screw top test tubes with a sterile pipette, to be used as slopes. Twenty ml. of the preparation were added to 20 x 180 mm. screw top test tubes to be used later as plates. Tubes containing the agar preparation were autoclaved for 2 min. at 15 psi. Upon removal from the autoclave tubes containing 8-10 ml. preparation were placed on a 30° incline and allowed to cool, while the tubes containing 20 ml. of the agar preparation were poured immediately into 2 x 10 cm. disposable petri dishes and allowed to solidify.

Biphasic cultures were also prepared. Fifty ml. of the agar preparation were poured into 125 ml. Erlenmeyer flasks and allowed to solidify, after which 25-50 ml. of the desired liquid medium were added.

(3) Maintenance of cultures:

Cultures were initially incubated at a temperature of 15 $\pm$ 0.5°C

in a Psycrotherm incubator (New Brunswick Scientific Corporation) at a light intensity of 1400 lux supplied by 2 white fluorescent tubes (Westinghouse, cool white, high output. F24T12/CW/HO) with an 18-6 hour light-dark sequence. Reserve stock cultures were maintained in a constant temperature room set at 10 $\pm$ 2.0°C with a light intensity of 1500 lux and an 18-6 hour light-dark sequence.

A failure in the temperature control unit of the Psycrotherm in January 1969 resulted in mass destruction of the cultures. New cultures were started from the reserve stocks, but a similar failure in the Psychrotherm in May 1969 resulted in the destruction of the existing subcultures incubated in the unit. Because of the equipment failures subsequent subcultures of the reserve stock cultures were incubated at room temperature with supplementary lighting supplied by two fluorescent tubes giving a light intensity of 1200 lux until more reliable culture chambers could be acquired.

From September 1969 until the conclusion of the study cultures were incubated in a Sherer controlled environmental room Model Cer 68 (Sherer-Gillett Co., Marshall, Mich.) at a temperature of 15 $\pm$ 0.5°C and a light intensity of 3500 lux with an 18-6 hour light-dark sequence.

(4) Culture studies:

(i) Cell size

Average cell size and size-frequencies were determined in relation to age of algal culture and culture type. Smears were prepared from cultures of known age and culture conditions and the cells measured

with an ocular micrometer. Up to 50 cells were measured at random on each smear. Reproductivity of the algal culture was determined by the same methods as prescribed for animal/algal smears. Four or five smears were prepared from each culture and the average reproductivity of the culture determined from the counts.

(ii) Cell shape:

For the cultures studied the cell shapes were determined subjectively. Whenever a smear was prepared for reproductivity studies or cell size studies it was scanned carefully under oil immersion and the different cell shapes were noted. All atypically shaped cells were photographed or drawn with the aid of a *camera lucida*.

(iii) Life Cycle Studies:

Attempts were made to follow the growth and division of individual cells in culture to determine the life cycle of the alga. Small numbers of algae were suspended in liquid Chihara medium in depression microscope slides. With the aid of finely drawn out capillary pipettes 4-10 cells could be picked up from the suspension and deposited on a coverslip. The coverslip, which was supplied with a drop of freshly prepared Chihara medium, was inverted over a plastic ring cemented to a depression slide with a small quantity of petroleum jelly. This hanging drop culture was incubated under normal culture conditions and observed daily for 2 weeks.

(iv) Motility induction studies:

Cultures were subjected to various conditions to try to induce

motility; (1) old cultures were inoculated into fresh Chihara liquid medium; (2) old cultures were used to inoculate fresh Chihara medium supplemented with 1.0% proteose; (3) old cultures were used to inoculate Chihara liquid medium supplemented with 1.0%, 3.0% and 5.0% scallop extract (Appendix C); (4) old cultures were used to inoculate Chihara/agar plates and incubated for 3 days and then resuspended in distilled water; (5) old cultures were exposed to temperature shocks ($0^{o}C$) for periods from 10 minutes to 1 hour; (6) young actively growing and dividing cultures were placed in alternating light and dark (2 hour dark/2 hour light) for periods up to 28 hours and observed every other hour; (7) young cultures were placed in continuous light and continuous darkness and observed every other hour for periods up to 48 hours; (8) young cultures were placed in distilled water for periods from 1 to 24 hours and observed every other hour; (9) young cultures were placed in Chihara liquid medium supplemented with 1.0% proteose and 1.0% scallop extract; (10) young cultures were exposed to temperature shocks (-1.0^{o} and $0.0^{o}C$) periods from 10 minutes to 1 hour and then exposed to higher temperatures than the normal incubation temperature ($15-25^{o}C$) for varying periods of time.

Chromatography and Spectrophotometry

(1) Preparation of endozoic algae:

From scallops:

Endozoic algae were isolated from the scallop tissue according to

a modification of the methods of Muscatine (1967).

The mantle tissue containing the algae was excised and trimmed to remove much of the unwanted supporting tissue. The tissue was cut into small pieces, washed thoroughly in 6 rinses of sterile sea water, transferred with 200 ml. sterile sea water to a Waring Blendor and homogenized for 3 minutes. The resulting suspension was strained through 6 layers of cheese cloth to remove much of the animal debris. The algal mass was concentrated and washed by 6 centrifugation-washing cycles. The resulting wet-packed mass was then ready for pigment extraction.

From cultures:

Chihara-agar plate cultures inoculated with large numbers of algae and incubated at 15°C with a light intensity of 3500 lux and an 18-6 hour light-dark sequence for 5 weeks supplied sufficient quantities of cells for pigment extraction. The algal cells were removed from the agar with a sterile transfer loop and suspended in 10 ml. of sterile sea water in centrifuge tubes. After 3 centrifugation-washing cycles the wet-packed cells were ready for pigment extraction.

(2) Preparation of pigment extracts

Pigments could not be efficiently extracted from the algae using conventional methods. Algal cells suspended in 90% acetone did not yield pigments even after 24 hours. Cells suspended in methanol were

bleached out after 5 hours. Pigments were extracted from cells suspended in 25% chloroform in methanol (V/V) after 3 hours. Difficulty in extraction was attributed to the relatively thick cell walls of the algae. To reduce the extraction time the cells were ultrasonicated.

Approximately 1 ml. of wet-packed cells (either from the scallop or from culture) were suspended in 20 ml. methanol in a 40 ml. beaker and subjected to ultrasonication with an Ultrasonic Power Unit (Wilson Scientific Equipment Limited) for 1 hour.

After the extraction period the suspension was centrifuged for 12 minutes at 2,500 g. to remove the cell residue. The methanol extract was mixed with an equal volume of diethyl ether and washed with 10% NaCl, 5-10 volumes that of the methanol-ether extract, in a 200 ml. separating funnel. The pigments migrated to the ether layer and were easily collected. The ether layer was centrifuged after collection to remove any particles and water droplets. After centrifugation the ether extract was concentrated under a jet stream of nitrogen gas before spotting onto chromatography paper.

The entire process of extraction, washing and concentration was carried out in darkness or subdued light to reduce light decomposition of the pigments.

(3) Paper chromatography:

Ascending paper chromatography was carried out. The apparatus, consisting of 1000 ml. graduated cylinders fitted with air tight

stopper is shown in Figure 3. The solvent consisted of 25% chloroform in light petroleum (60-80°C). Approximately 30 ml. of the solvent were poured into the cylinders. The concentrated pigment extract was spotted on 12" by 1" strips of Whatman No. 3 Mn. chromatography paper using a fine capillary pipette. The spots were dried with a jet of nitrogen gas and the strips placed in the cylinders hung from a paper clip attached to the rubber stopper and allowed to equilibrate for 30 minutes(Figure 3, Equilibration). Following the equilibration period the paper strips were removed from the paper clip and lowered into the solvent (Figure 3, Separation). After 45 minutes the chromatograms were removed from the cylinders, the solvent front marked with a pencil, and hung in a fume hood to dry. After drying the spots were outlined with a pencil and the Rf values were calculated for each fraction.

Chromatograms were run in the dark at room temperature.

When chromatograms were to be used for spectrophotometry they were not allowed to dry but instead utilized immediately after removal from the cylinders.

(4) Spectrophotometry:

Absorption spectra of the pigment fractions were measured with a Beckman Ratio Recording Spectrophotometer, Model DK-A. It was necessary to run four chromatograms simultaneously and elute the pigment spots from each representative fraction in order to produce a pigment

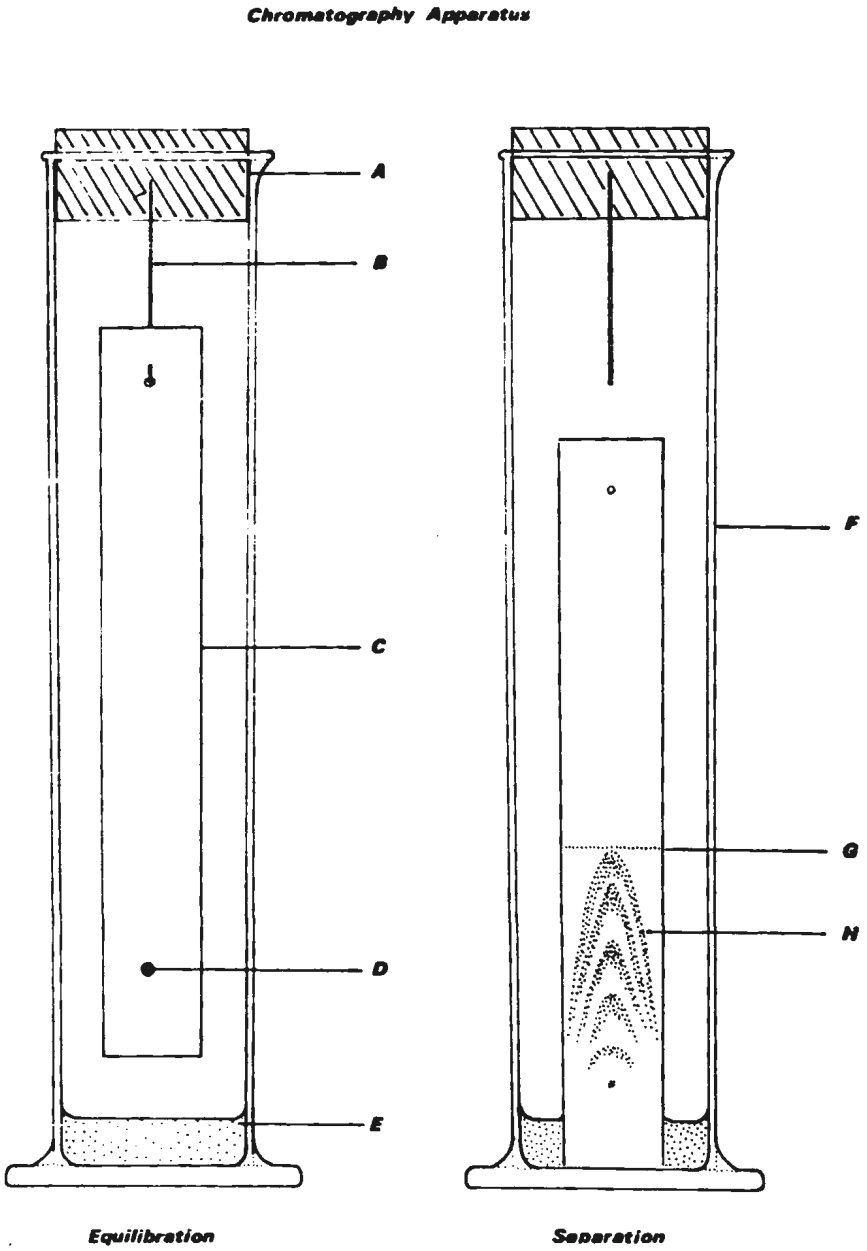

FIGURE 3. Chromatography apparatus illustrating principal parts.

A. Rubber stopper
B. Paper clip
C. Chromatography paper
D. Pigment extract spot
E. Solvents
F. Graduated cylinder
G. Solvent front
H. Separating pigment fractions

concentration high enough to register on the spectrophotometer.

The four corresponding pigment fractions were cut from the chromatograms, divided into small pieces and eluted with ether. The mixture was centrifuged for 3 min. to remove any paper residue, and analysed with the spectrophotometer. The absorption spectrum was plotted over the visible spectrum range (350-780 mμ.). Each of the pigments was analysed in this wav.

RESULTS

Morphology And Reproduction of Endozoic Algae *in vivo*.

1. Occurrence, distribution, size and characteristics of algal colonies.

Naidu (1971) reported the principal areas of algal settlement in the tissues of the giant scallop. He listed them as follows: Left (upper) and right (lower) mantle folds; distal end of gonad; base of the posterior adductor muscle between the muscle and the left valve; base of the posterior adductor muscle between the mantle and the right valve; sloughed off colonies on the inner surface of the shell towards the edge.

Heavily infected scallops are shown in Figures 4 and 6A. Superficial examination of the infected animal reveals that the infection is represented by numerous discrete algal "colonies" or packets lodged within the tissue of the host.

In addition to the areas reported by Naidu (1971) algal colonies were encountered lodged within the tissues of the gill filament, (Figure 5; 6B,C.D.E); deep within the adductor muscle tissue (both posterior and anterior) in heavily infected scallops and on the anus Algal cells were observed in amoebocytes in smears prepared from stomach contents and blood fluid.

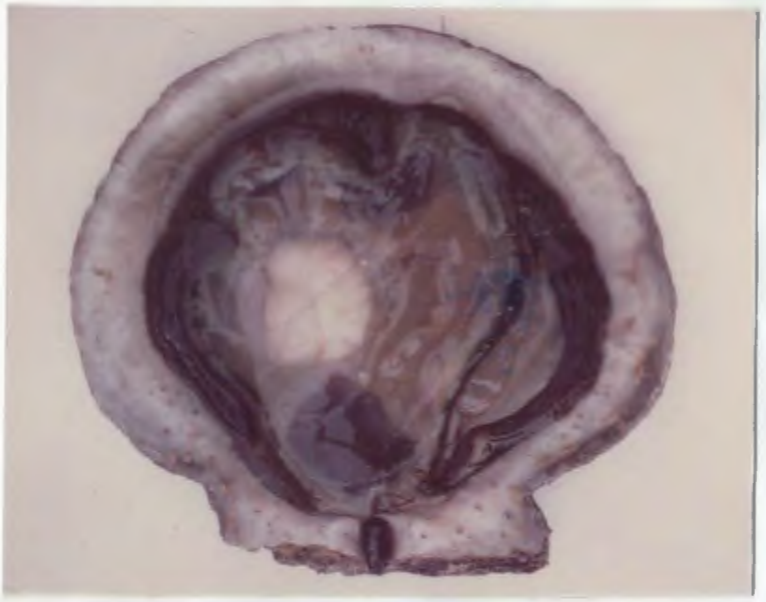

FIGURE 4 A moderately infected giant scallop with the left (upper) valve removed to display the green coloration imparted to the tissues by large numbers of algal colonies (1/2 natural size).

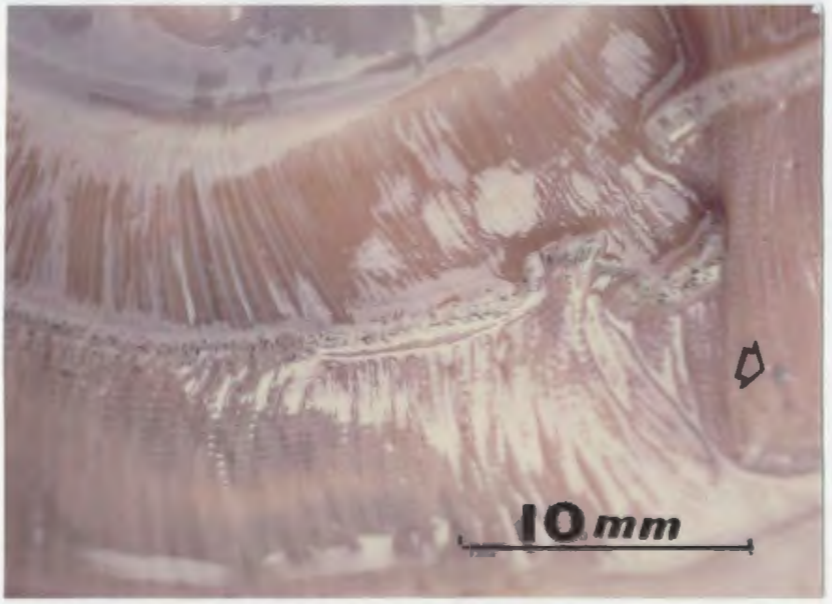

FIGURE 5 Gill filaments of a heavily infected scallop showing the algal colonies dotted along the filaments, and large superficial colony (arrow).

FIGURE 6

Algal colonies within the tissues of heavily infected scallops. Scallop with left valve removed (A) displaying heavily infected tissues including the adductor muscle (a) in which large round colonies (A,a insert, solid arrow, and sloughed off colony, open arrow) are typical. Algal colonies within the gill filament (B, solid arrow). Note how the colony is being pushed out. Small thin colonies within the gill filaments (C). Algal cells from a colony extruding through a break in the epithelium of the gill filament (D, white arrow). Round colony within the gill filament (E) showing thick boundary between the algae and the animal tissue.

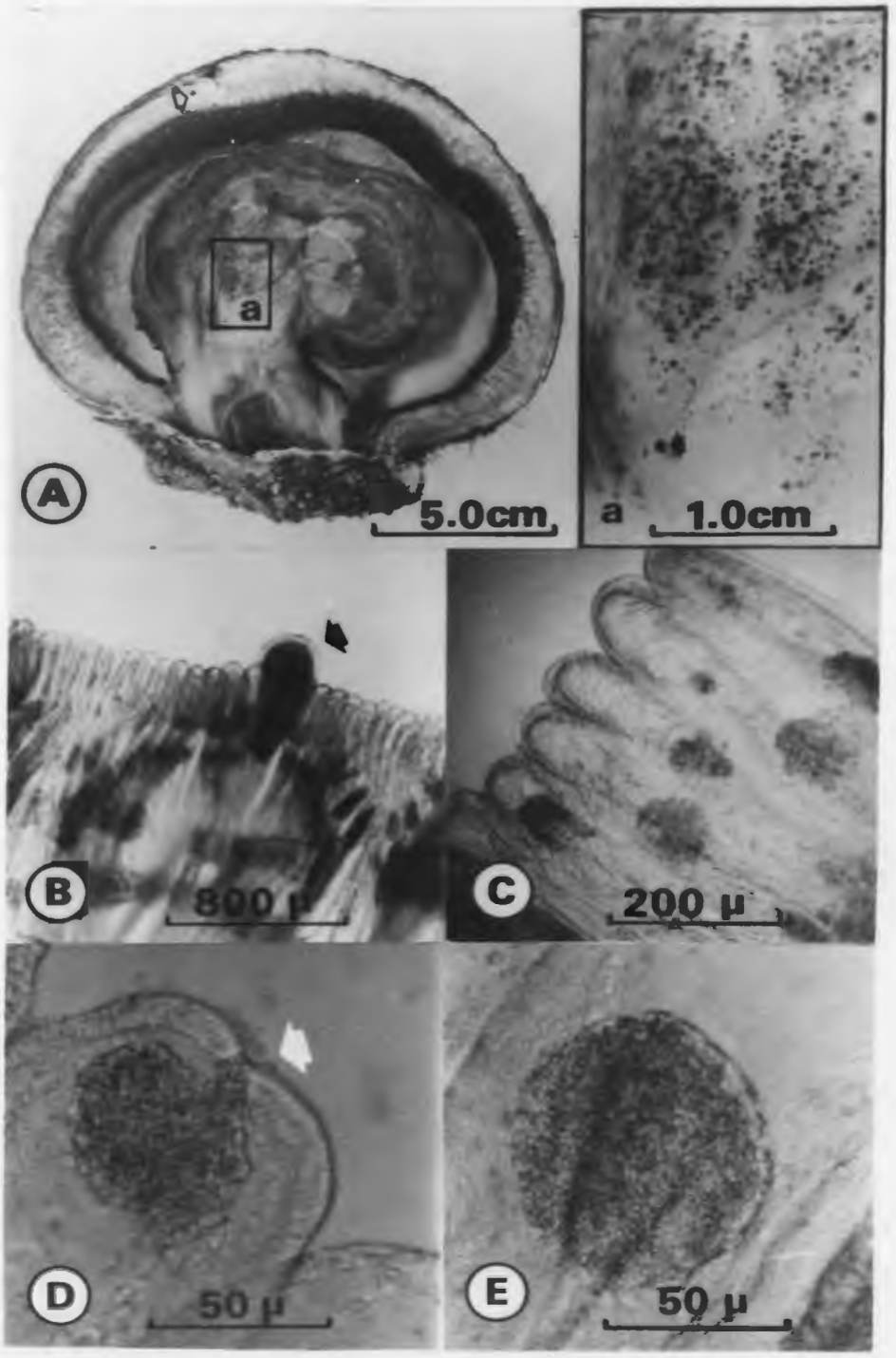

FIGURE 6. Algal colonies within the tissues of a heavily infected scallop. See explanation on page 29.

The areas of algal settlement in the infected scallops examined during this study and the frequency of algal settlement in certain anatomical areas is presented in Table 1.

TABLE 1: Analysis of frequency of algal infection of various organs of 96 scallops.

Anatomical area	Number of scallops having colonies of cells*.
Right (lower) mantle	
(a) Anterior	72
(b) Mid-mantle	81
(c) Posterior	79
Left (upper) mantle	
(a) Anterior	84
(b) Mid-mantle	96
(c) Posterior	87
Anus	3
Adductor Muscle	12
Gill filaments	3
Shell	10
Amoebocytes in stomach	2*
Leucocytes in blood	2*

* Single algal cells observed.

Preliminary examination of a large number of infected scallops indicated that there was a definite distribution pattern of the algal colonies. As indicated by Naidu (1971), the areas most heavily infected were the mantles (left and right), particularly the mantle edges. The apparent pattern of increased colony number and size in the mantle edges (Naidu, 1971), is substantiated here, but it is noted that in some very heavily infected scallops large and numerous colonies may be present deep within the adductor muscles. Superficial examinations indicated that the colonies were more numerous and larger within the mid-line mantle regions, especially in the left or upper mantles.

These preliminary observations on the apparent pattern of colony distribution warranted a closer quantitative assessment of the phenomenon. Three scallops (one slightly infected, one light-moderately infected, and one moderately infected) were studied intensively, and the density of colonies determined over the mantle length as prescribed in MATERIALS AND METHODS. The counts for each of the 10% segments of the entire mantle length is presented in Figure 7 for these three scallops.

Figure 7 shows that colony density is highest in the mid-line region of each mantle (left = dorsal, right - ventral), but is noticeably higher in the dorsal mantles. There is a decrease in colony density from the mid-line regions towards the posterior and anterior in the dorsal and ventral mantles in the three scallops requested.

To assess the algal colony distribution pattern within the mantles as applied to the population of infected scallops, a sample of 10

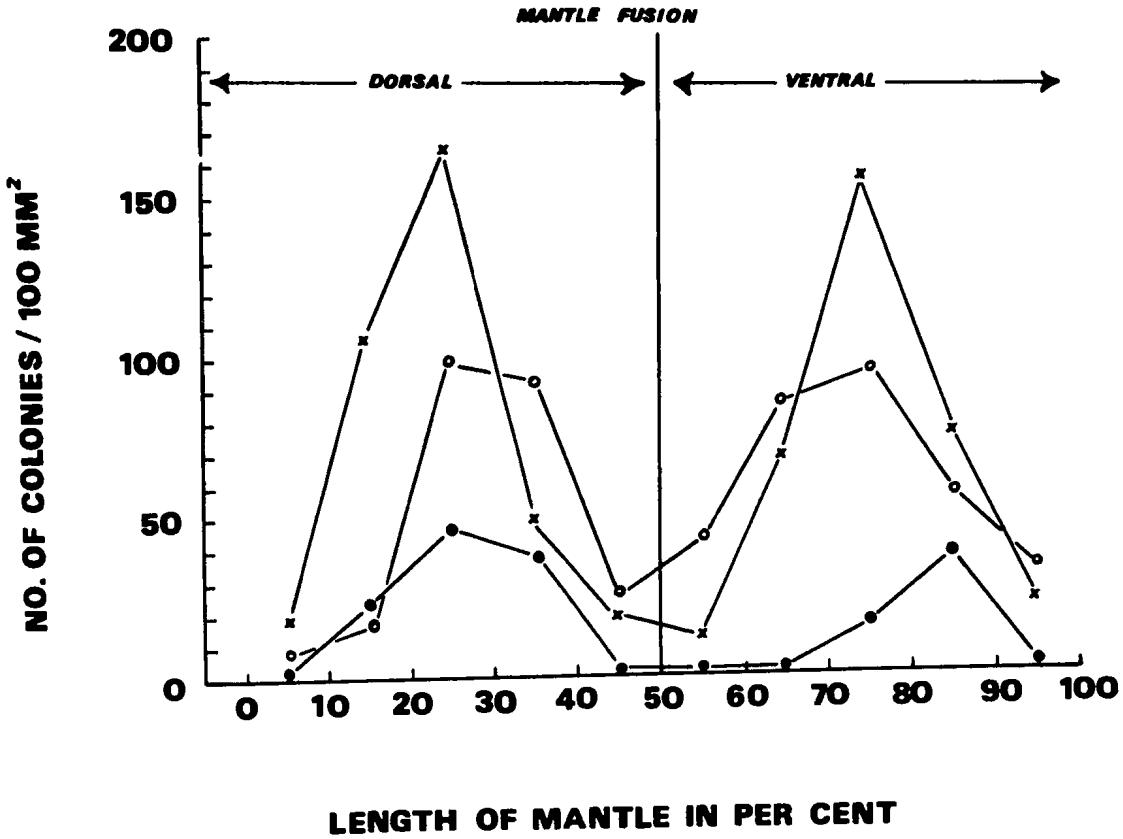

FIGURE 7. Distribution of algal colonies along the mantle represented as the number of colonies per 100 mm^2 plotted against length of mantle in percent for a moderately infected scallop (-x-), a light-moderately infected scallop (-o-) and a lightly infected scallop (-o-).

additional animals was selected at random and studied as prescribed in MATERIALS AND METHODS. The mean number of colonies per 100 mm^2 for each of the 10% mantle segment samples is represented in Figure 8. In this population sample there is a much higher concentration of colonies within the mid-line region of the mantles especially in the dorsal (left) mantle.

Further studies on the actual position of the colonies within the mantle segments revealed that the highest concentration occurs in a very narrow, delicate strip of tissue called the shell fold (Figure 9A, sf), which lies in direct contact with the shell surface. Figure 10 represents the percentage of colonies counted in each anatomical area (Figure 9A, sf, mm, v) plotted against the total percentage length of mantle.

The graph indicates that from 67 to 97% of the colonies in the mantle segments are localized in the shell fold area and the highest percentage values in the shell fold tissue correspond to the mid-line regions of the dorsal (left) and ventral (right) mantles.

Naidu (1971) concluded that the size of the algal colonies decreases from the mantle edge towards the interior of the scallop. The three anatomical areas, namely the shell fold, mantle margin and the mantle velum were selected for comparison and investigated as prescribed in MATERIALS AND METHODS. The number and size of the algal colonies were determined for each of the three areas and represented as size frequency histograms in Figure 11. As may be seen from Figure 11, the shell fold contains the larger colonies as well as the

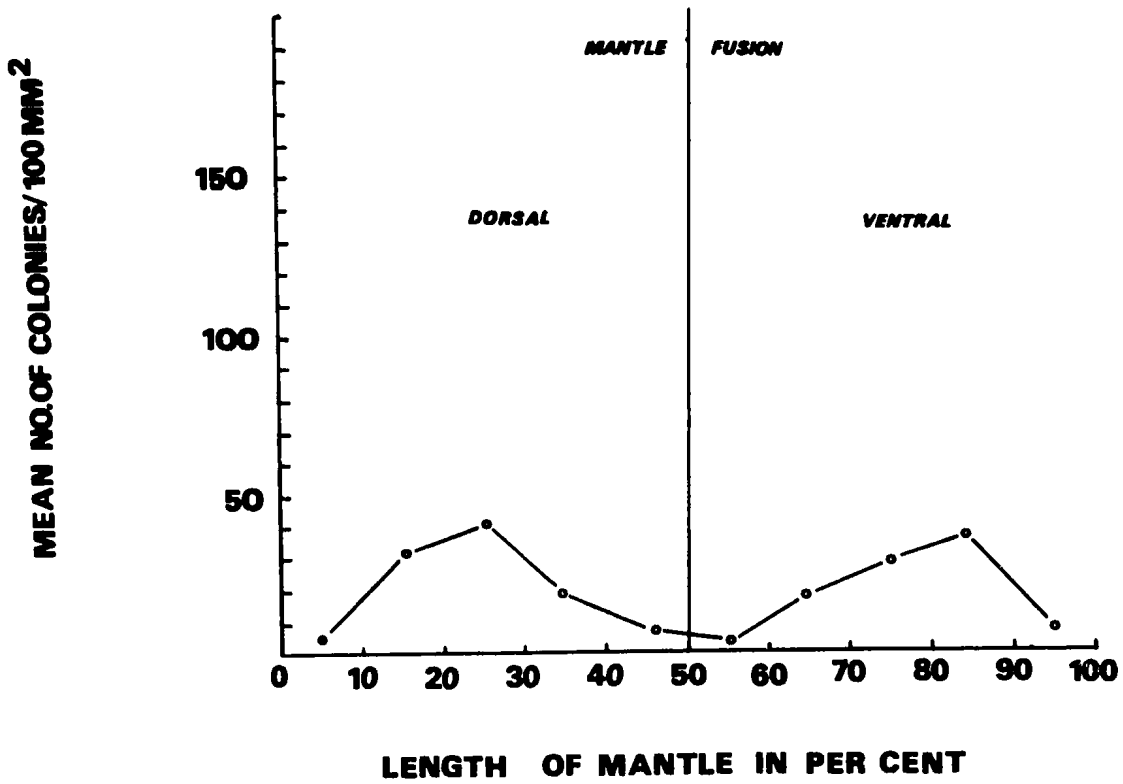

FIGURE 8. Distribution of algal colonies within the mantle represented as the mean number of colonies per 100 mm^2 plotted against mantle length in percent for a sample of 10 scallops of varying degrees of infection.

FIGURE 9

Histological sections through the mantles of infected scallops. Designation of tissues studied in distribution analysis; Shell fold area (A, Sf); Mantle margin (A,MM); Velum (A,V). Heavily infected scallop (B) showing concentration of the algal colonies (ac) within the shell fold area (Sf) and Velum (V). Algal colonies under epithelium layers (arrows). Note proximity to the blood sinus (C,bs).

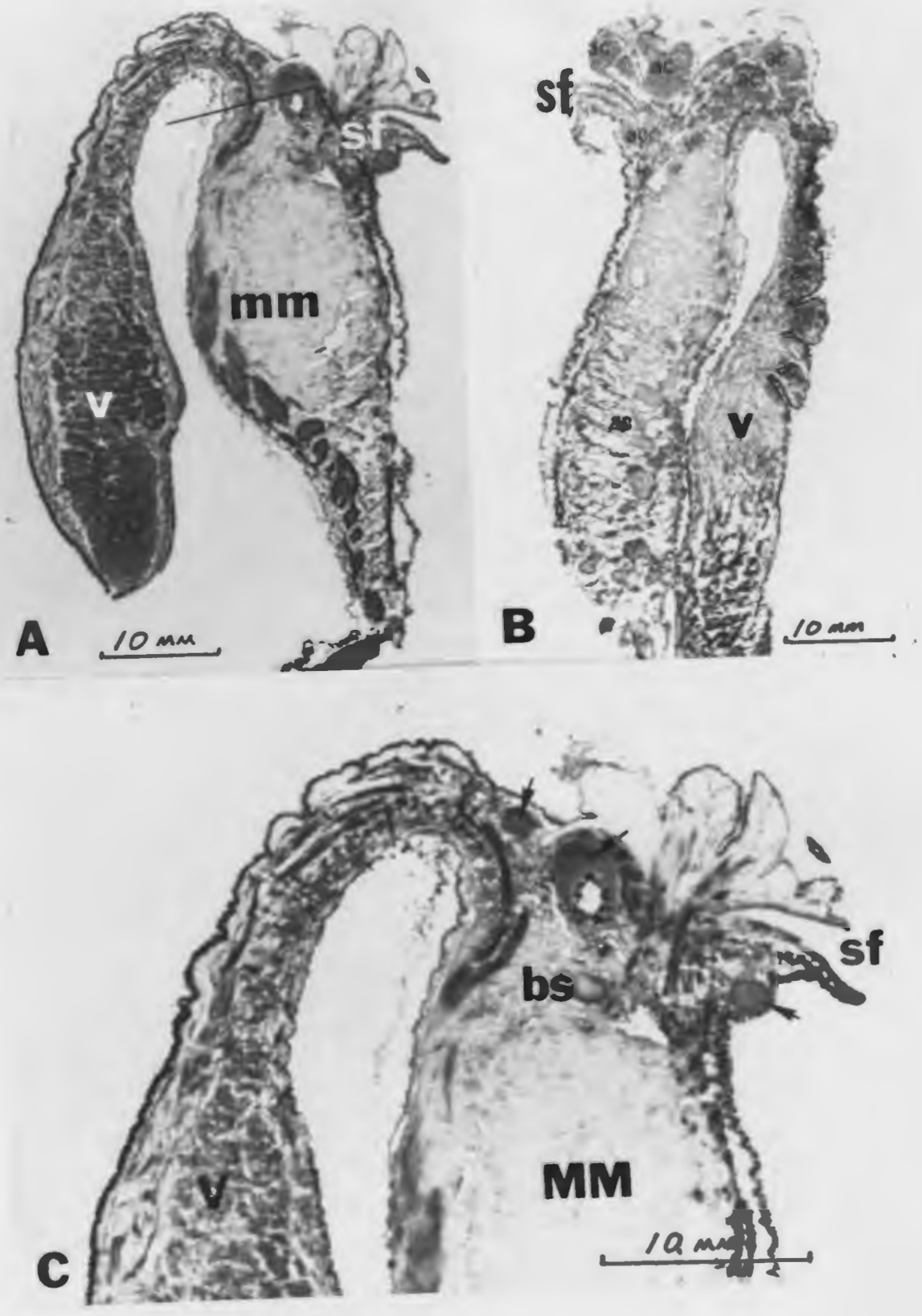

FIGURE 9. Longitudinal sections through the mantle tissue of infected scallops. See explanation on page 36.

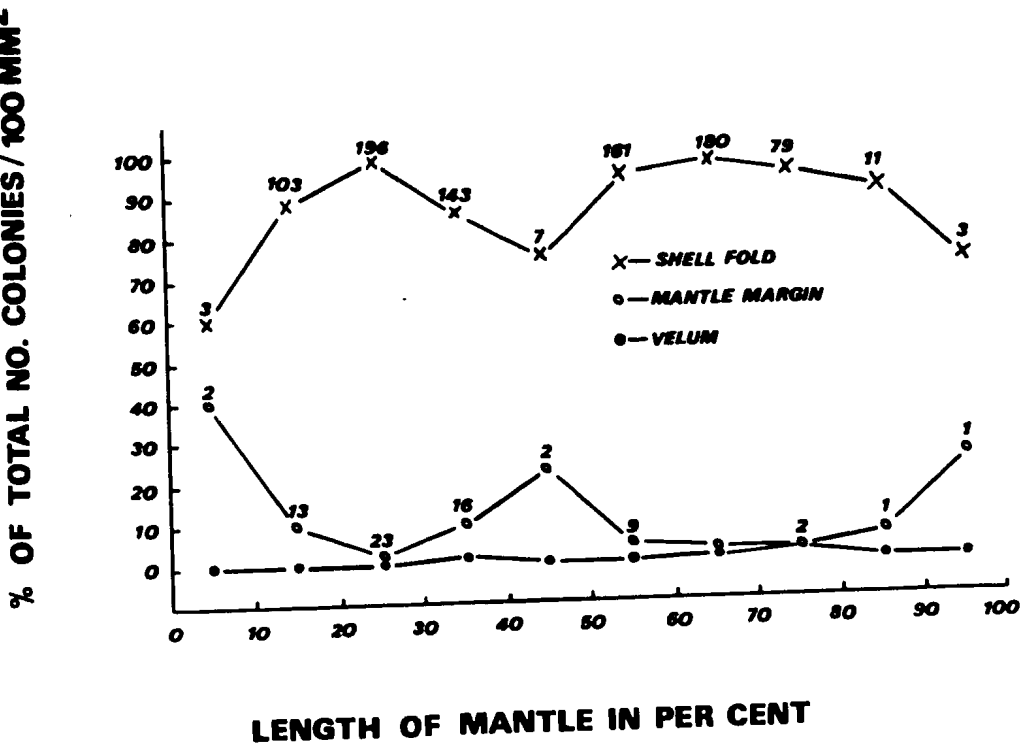

FIGURE 10. Analysis of the number of algal colonies, represented as percent of the total number of colonies, within the three mantle tissues compared; Shell fold (-x-); Mantle margin (-o-); Velum (-o-). Number above each point corresponds to the actual number of colonies recorded.

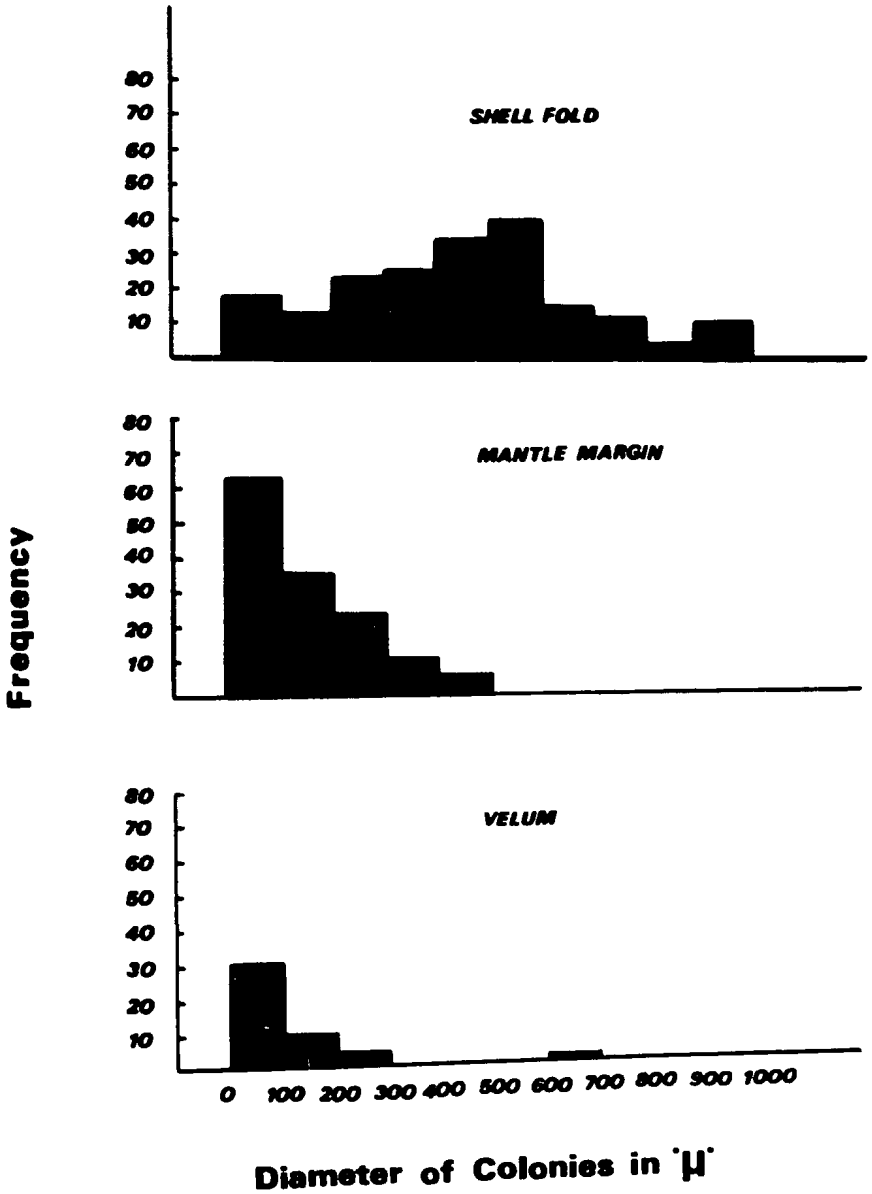

FIGURE 11. Size frequency distributions for the colonies measured in the three mantle areas compared, namely: Shell fold; Mantle margin; Velum.

highest number of colonies within the mantles. Colonies decrease in size and number from the mantle edge towards the interior of the animal, in this case the mantle margin.

In all other infected animals studied subjectively it was found that the general pattern of distribution as demonstrated above held true.

Morphologically the algal colonies appear to be uniformly spherical when observed superficially. Closer examination showed, however, that they may be spherical, elliptical or completely irregularly shaped. The general shape of the colonies is dependent on their location within the scallop's tissues. The greatest variation in shape occurs in the mantle regions, while in the adductor muscle they appear to be uniformly spherical (Figure 6Aa, insert). In the gill filaments the colonies are either round or oval (Figure 6 B and C). Closer examination of these colonies with the optical microscope reveals that unlike the adductor muscle or mantle colonies, these are in fact round or oval discs. In the other areas the colonies assume a more pronounced spherical morphology.

In lightly infected scallops the colonies appear to be separate bodies embedded in the tissues. In heavily infected scallops this characteristic may be lost in areas where colonies are highly concentrated since one colony may lie so close to is neighbour as to give the effect of one continuous algal mass (Figure 6A).

Colonies appear to be very smooth-edged (Figure 6Aa, insert, and Figure 6E) when observed with the naked eye or under low power with the microscope. Under higher magnifications many of the colonies are

seen to be very irregular with numerous algal cells jutting into the surrounding animal tissue. Colonies have been observed in which animal tissue extends inward towards the centre of the algal mass.

Color of the colonies is dependent on size and shape. Small irregular colonies appear greenish yellow while larger compact colonies are deep green. Areas of the mantle tissue containing numerous compacted colonies appear very dark green when observed superfically (Figure 4). Colonies found on the shell appear green in color, but often have a yellow tint imparted by a layer of calcareous material covering the colony (Figure 12, arrow).

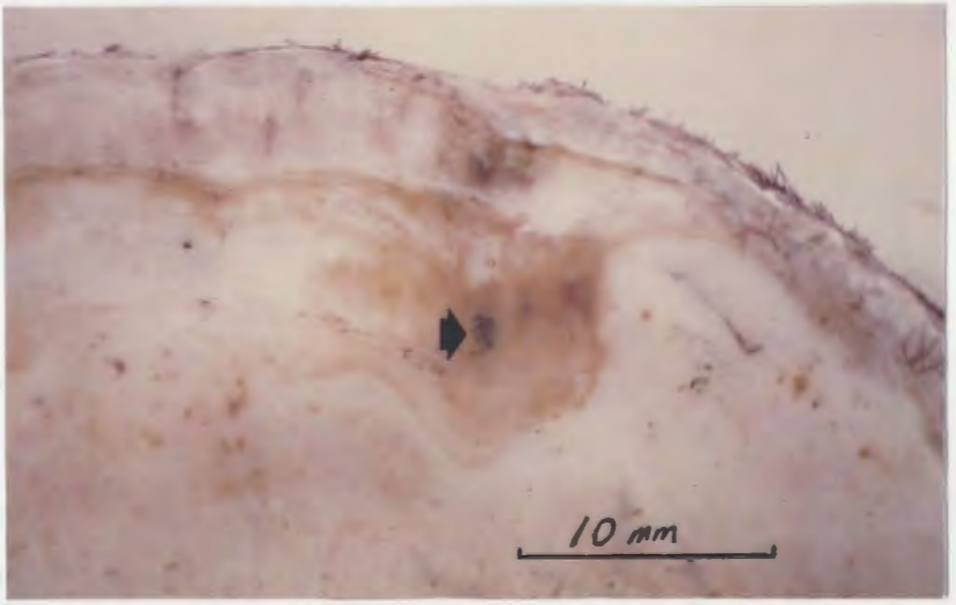

FIGURE 12 Algal colony on inner face of shell. Note calcareous material covering the 'sloughed off' colony.

The algal colonies may occur at different depths in the animal tissue. In the mantle tissue and the gill filaments colonies may be found very close to the epithelium giving the impression of elevated bodies similar to a human skin tumor (Figure 5, arrow). Colonies like these can be picked off the tissue very easily with a capillary pipette, even though there is a layer of epithelium covering the algal mass (Figure 9C, solid arrows). In the thicker mantle edge tissue the colonies occur deeper (Figure 9B, solid arrow), several mm from the surface. In the adductor muscle the colonies may be distributed through the organ at different depths. Colonies on the gonad and the anus are restricted to the surface layers.

(2) Morphology of Endozoic Alga

Naidu and South (1970) presented a brief description of the endozoic alga based on preliminary work of the algal-animal relationship. In the present study a more exhaustive examination of the alga as it occurs in the animal was carried out based on light and phase contrast microscopy.

A generalized drawing of the endozoic alga is presented in Figure 13. The majority of cells observed in a fresh smear from an infected scallop range in size from 1.0 to 11.0 μ, and usually contain a single band shaped chloroplast (Figure 13a) which rarely occupies more than 3/4 of the cell. The cell wall (Figure 13b) is thick (approximately 0.5 μ) and rigid with little ornamentation observed

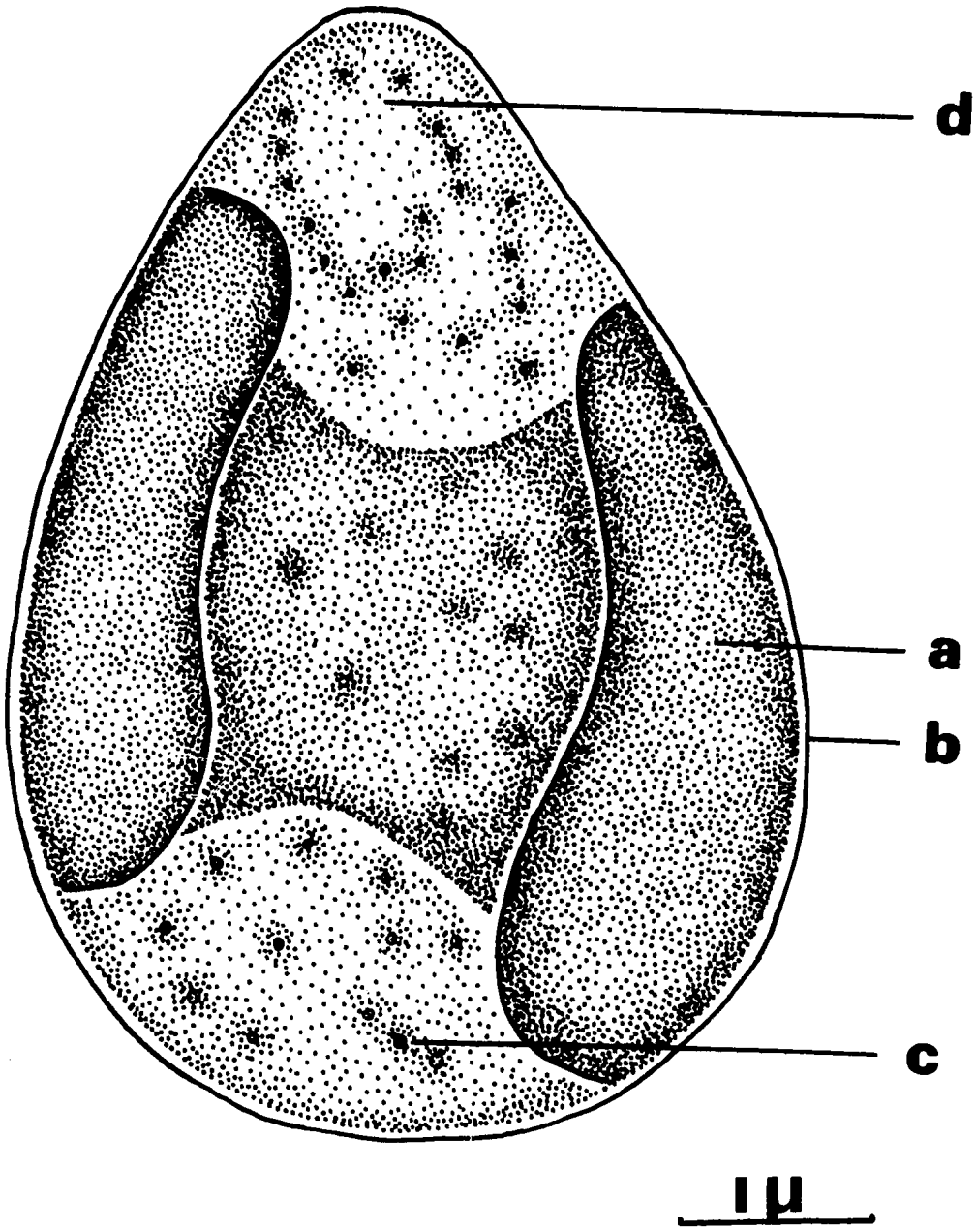

FIGURE 13. Generalized illustration of the endozoic alga *in vivo* showing the well defined parietal band-shaped chloroplast (a), thick cell wall (b), refractile cytoplasmic inclusions (c) and tapered anterior tip (d).

with the light microscope. The cells have a definite polarity with a characteristic thick posterior end and a tapered anterior end often terminating in a hyaline tip (Figure 13d).

Refractile cytoplasmic inclusions can be observed clearly under the light microscope. A definite arrangement of inclusions frequently occurs and vacuoles may be present (Figure 13c, Figure 14, arrow and Figure 15B, arrow).

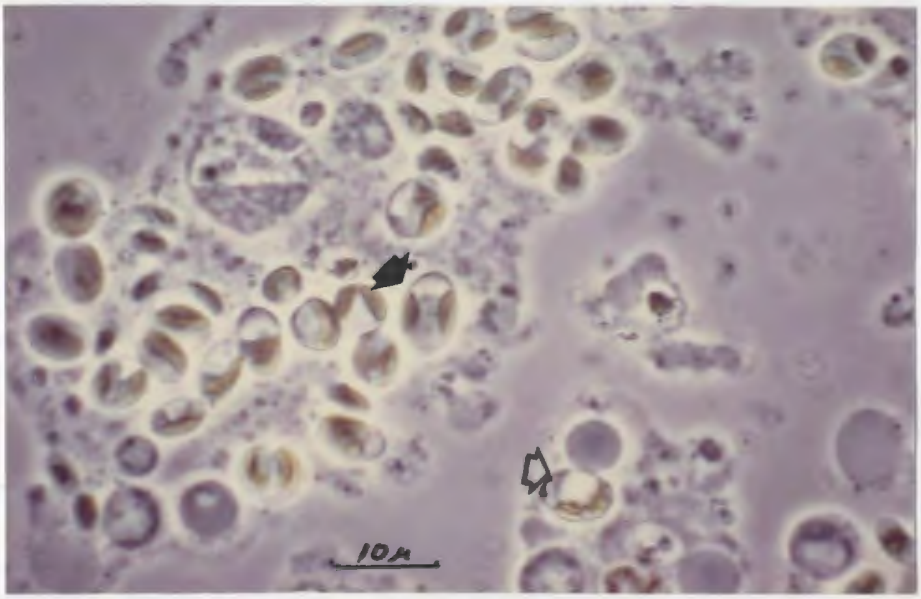

FIGURE 14 Algal cells observed in a smear prepared from a fresh infected scallop. Note the well defined parietal chloroplasts (solid arrow), the refractile bodies within the cytoplasm and the vacuoles (open arrow).

FIGURE 15

Algal cells from an infected scallop showing the variation in morphology (A) in which cells contain refractile cytoplasmic inclusions (A, solid arrow) and well defined chloroplast (A, open arrow). Vacuoles are observed as shown in B, solid arrow. Note the well defined chloroplast and polarity of the cell in C. Scale bar = 10 μ

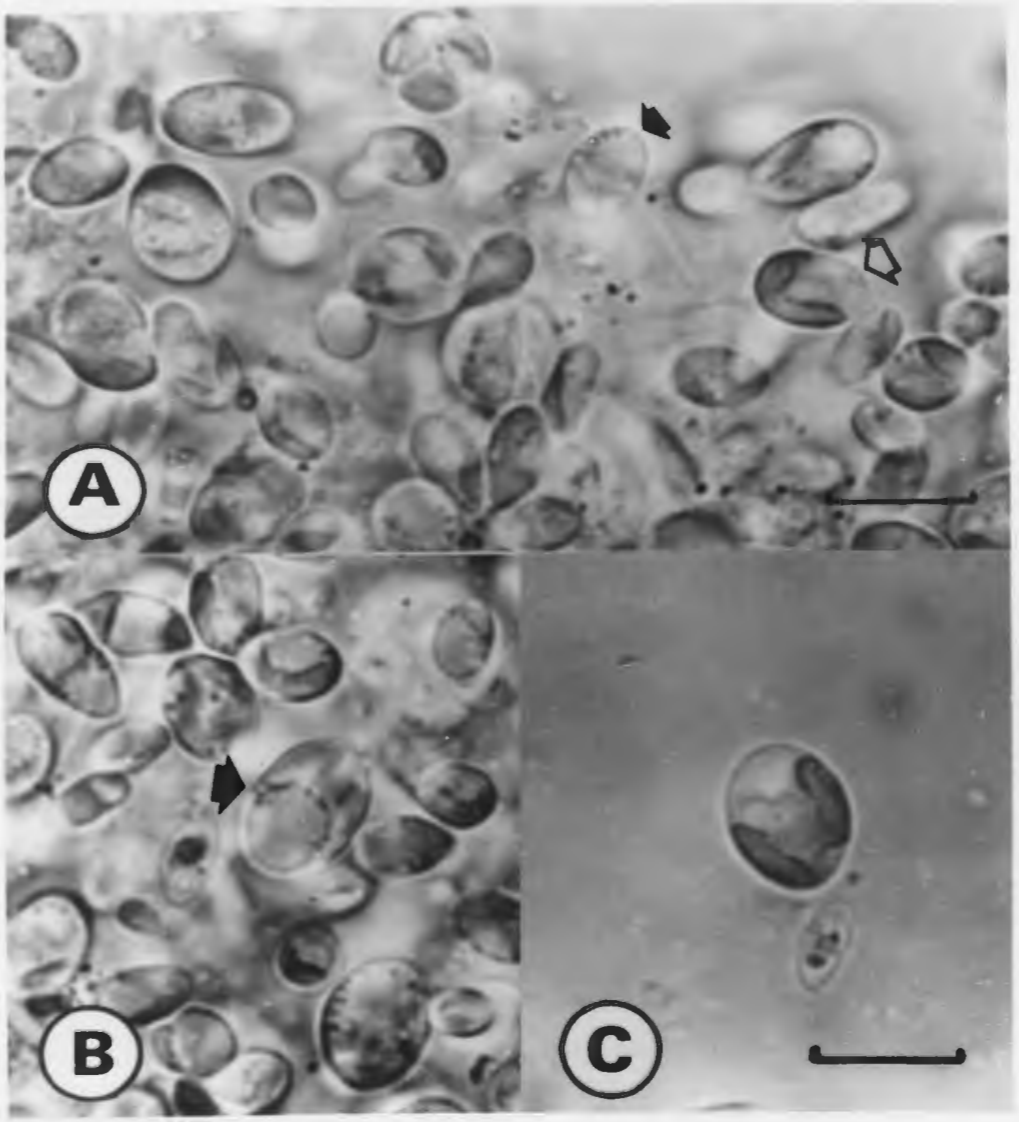

FIGURE 15. Algal cells from infected scallop. See explanation on page 45.

Cell types representative of a fresh smear from an infected scallop are shown in Figure 16. The cells may be rod-, oval-, or sausage-shaped, round or sickle-shaped; many may have smooth uniform ends or rather accentuated hyaline tips. Some cells may contain two, and very rarely three band chloroplasts while others may contain one very well defined cup-shaped chloroplast.

A frequency distribution of the length of cells measured from a lightly infected scallop is presented in Figure 17. Two noticeable peaks appear for cells 3.0 to 4.0 μ in length and cells 7.0 to 8.0 μ in length. The measurements were made on cells which were not dividing. The average length of cells as determined from 40-50 measurements per smear for 10 smears from each of 10 scallops chosen as a sample of the population was found to be 5.5 μ. The average width was found to be 3.0 μ on the same sample.

Cytological tests performed on the algae were of little help in determining the composition of the cell wall, as well as that of the storage products. The I_2-KI-H_2SO_4 test for cellulose proved negative as did the chloro-zinc - iodide test. The Meyer's Starch test proved negative and was thought to be so because of the toughness of the cell wall which prevented the chemicals from penetrating the cell.

To investigate the toughness of the cell wall, 75% H_2SO_4 was added drop by drop onto the algal smear and observed under the microscope. The cells remained intact for as long as 10 min. before the walls ruptured, spilling out their cytoplasm.

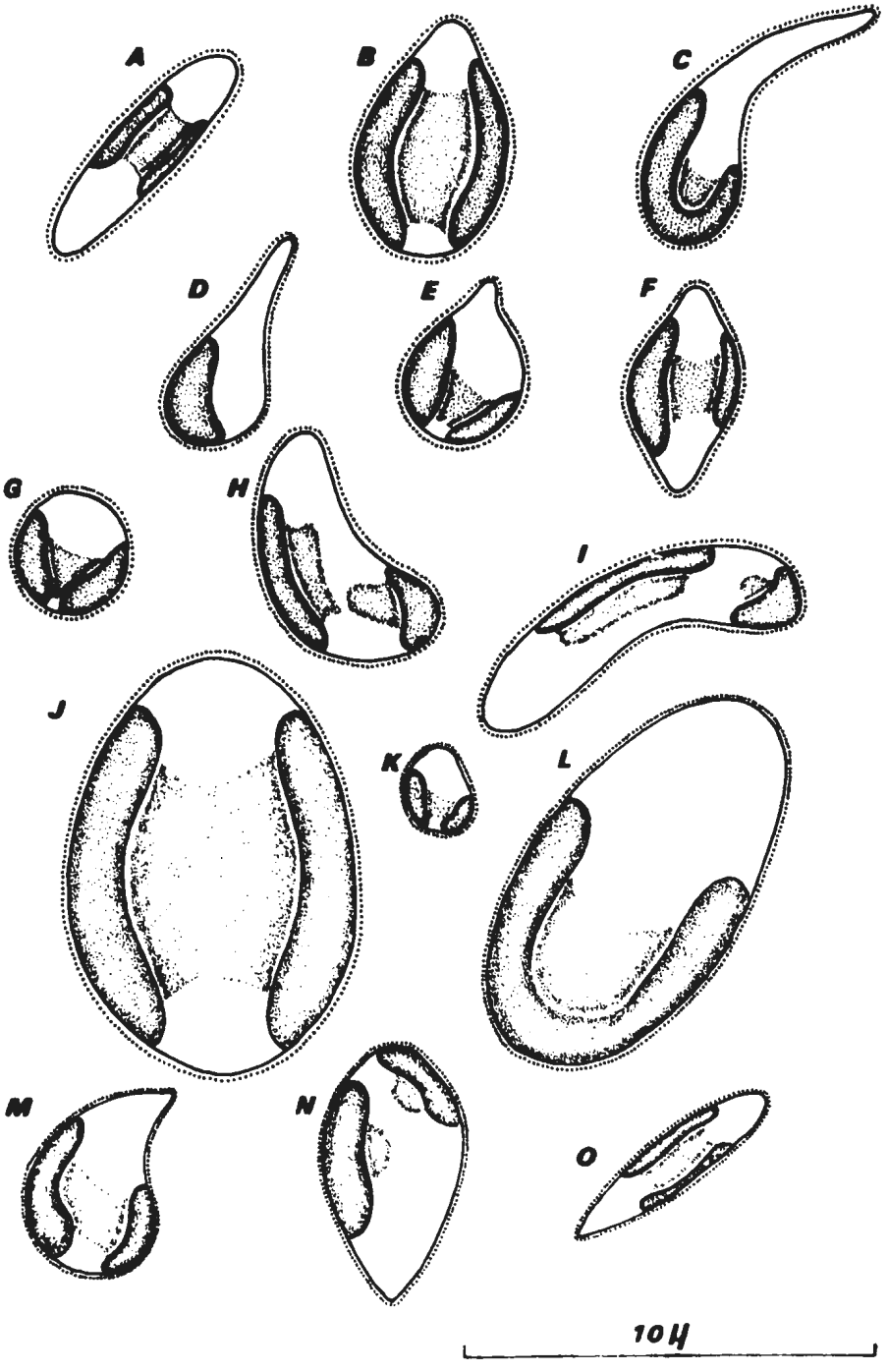

FIGURE 16. Selection of the morphologically variable cell types found *in vivo*.

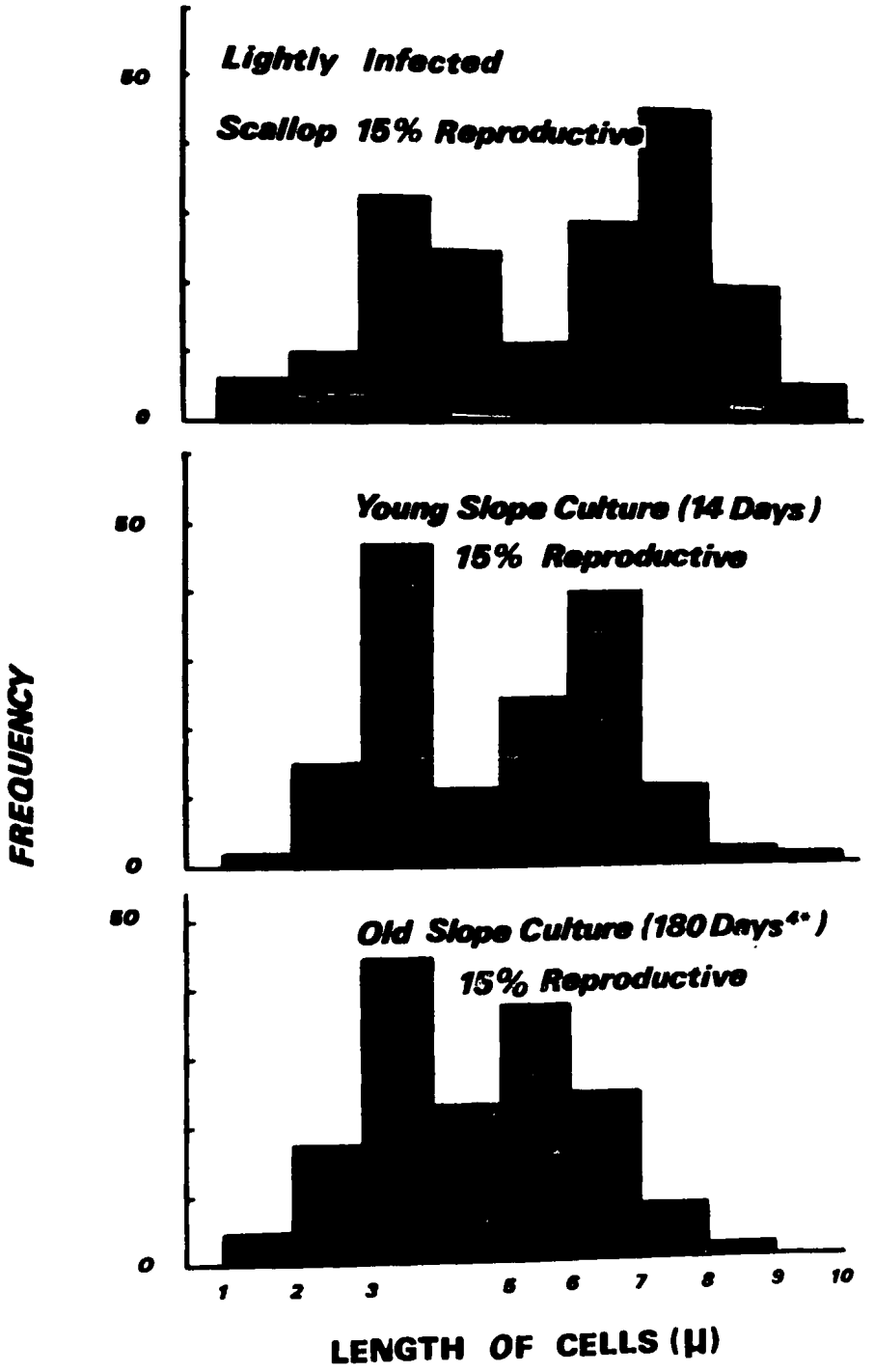

FIGURE 17. Size frequency distributions for algal cells from a lightly infected scallop, young slope culture 14 days after initiation and an old slope culture 180 days old which was subcultured 4 times.

Material fixed in 3:1 alcohol: acetic acid for 12 hours was stained by aceto-carmine and aceto-orcein methods* to investigate the nuclear materials of the algal cells. The aceto-orcein method worked well and it was found that the algal cells contain a single small centrally located nucleus. No nucleolus could be resolved in the preparations.

(2) Reproduction *in vivo*

Reproduction *in vivo* occurs entirely by means of aplanospores. Two-, 4-, and 8-celled aplanosporangia were observed in the smears prepared from live scallops. Figure 18 presents an interpretation of the sequence of events in the formation of 2-, 4-, and 8-celled aplanosporangia as well as 16-celled aplanosporangia observed later in culture. In the formation of a 2-celled aplanosporangium the cell divides along a line which is oblique to the longitudinal axis of the mother-cell. In Figure 18A, it will be noted that the division plate has formed in this manner. Figure 18B illustrates the complete cell division thus forming the 2-celled aplanosporangium.

The formation of the 4-celled aplanosporangium can be seen in Figure 18C, in which the four lobes of the chloroplast are evident from a polar view. Figure 18D, shows the four aplanospores held compactly within the capsule from polar (solid arrow) and side (open arrow) views. Figure 18E illustrates the complete division of the four aplanospores and there is an apparent re-orientation of the cells within the mother-cell membrane. Figure 18F reveals the

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* Appendix A, Fixatives and cytological stains.

FIGURE 18

Formation and release of aplanospores *in vivo* (A - J) and *in vitro* (K and L). Formation of the division plate (A) followed by complete division and formation of 2-celled aplanosporangium (B). Polar view of initial division to form 4-celled aplanosporangium (C). Complete division from polar view (D, solid arrow) and from a side view (D, open arrow). Note that the 4 spores are held tightly within the sporangium. Re-orientation of the 4 spores (E) in which they appear to be held loosely within the capsule. Rupture and release of spores from sporangium (F). Initial division (G) and formation of 8-celled aplanosporangium (H and I). Rupture and release of spores from 8-celled aplanosporangium (J). Division to form 16-celled aplanosporangium (K) and complete formation of 16-celled aplanosporangium as observed in culture (L).

A. Phase contrast
B. Bright field
C. Bright field
D. Bright field
E. Phase interference
F. Phase contrast
G. Bright field
H. Bright field
I. Bright field
J. Bright field
K. Bright field
L. Bright field

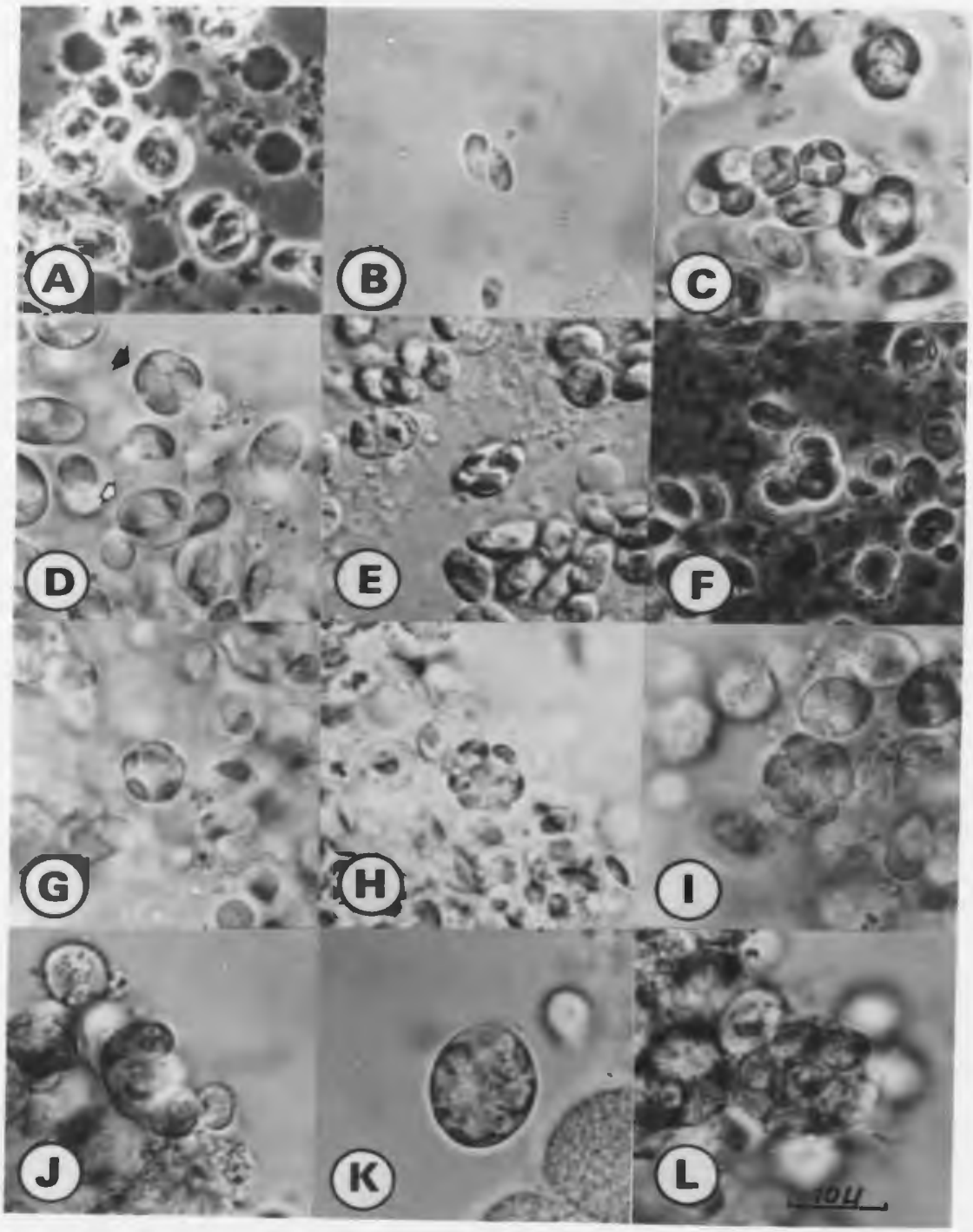


FIGURE 18. Formation and release of aplanospores *in vivo* (A - J) and *in vitro* (K and L). For explanation see page 51 and text.

rupture of the 4-celled aplanosporangium wall and the subsequent release of the aplanospores. Figure 18G illustrates the initial formation of the 8-celled aplanosporangium in which the segmented chloroplast is clearly visible, followed by the complete division of the cell (Figure 18H), rupture of the aplanosporangium wall (Figure 18I) and eventual release of the aplanospores (Figure 18J). Formation of the 16-celled aplanosporangium is presented in Figure 18K and L as was observed in culture.

Microscopic examination of living algal cells *in vivo* indicated that division in the formation of 2- and 4-celled aplanosporangia is oblique to the long axis of the cell. Formation of 2-celled aplanosporangia appears to be a very simple process. However, formation and release from 4-celled aplanosporangia is more complicated and warrants elucidation. Division is clearly seen to be oblique to the long axis of the cell but after complete division there appears to be a re-orientation of the spores within the mother cell capsule. Figure 19 presents a graphical sequence of this formation and re-orientation based on light and phase contrast observations.

Formation of the oblique division plate is represented in Figure 19A from a side view and division of chloroplast and cytoplasm from a polar view (Figure 19B). Complete division and formation of the aplanospores is evident in Figure 19C and it will be noted that the spores remain tightly together, still retaining their oblique orientation. After formation, the spores separate and "re-orient" themselves within the spore capsule. Repeated observations on this

phenomenon suggest that this is accomplished by one or more of the aplanospores rotating away from the initial plane of division (Figure 19D and E) until complete separation is achieved (Figure 19F). The aplanospores are now held loosely within the mother cell capsule and may continue to "move" about. It is speculated that growth and expansion of the spores after division may account for this process and also the arrangement of the spores may facilitate easier release from the mother cell capsule.

Determination of percentage reproductivity each month for 1 year using 10 scallops of varying degrees of infection showed only a slight variation. The range was found to be from 15% to 17% with the majority of values around 15%. There is consequently little variation in reproductivity with either season or host animal.

Aplanosporangia range in size from 5.0 to 11.0 μ. A size frequency distribution of the aplanosporangia is given in Figure 20. The plot is representative of measurements performed on aplanosporangia containing 2, 4, and 8 aplanospores, found in 10 random microscope fields for a total of 10 smears from 10 different scallops of varying degress of infection. It will be noted that there is a definite peak at 7.0 to 8.0 μ.

Frequency distributions for 2-celled, 4-celled and 8-celled aplanosporangia respectively are presented in Figure 21. There is considerable overlap in size ranges for the three types of aplanosporangia. Two-celled aplanosporangia range from 5.0 to 9.0 μ, 4-celled from 6.0 to 10.0 μ and 8-celled aplanosporangia from 7.0 to 11.0 μ. Two-celled aplanosporangia predominate with 4-celled and 8-celled examples occurring less frequently (Figure 22).

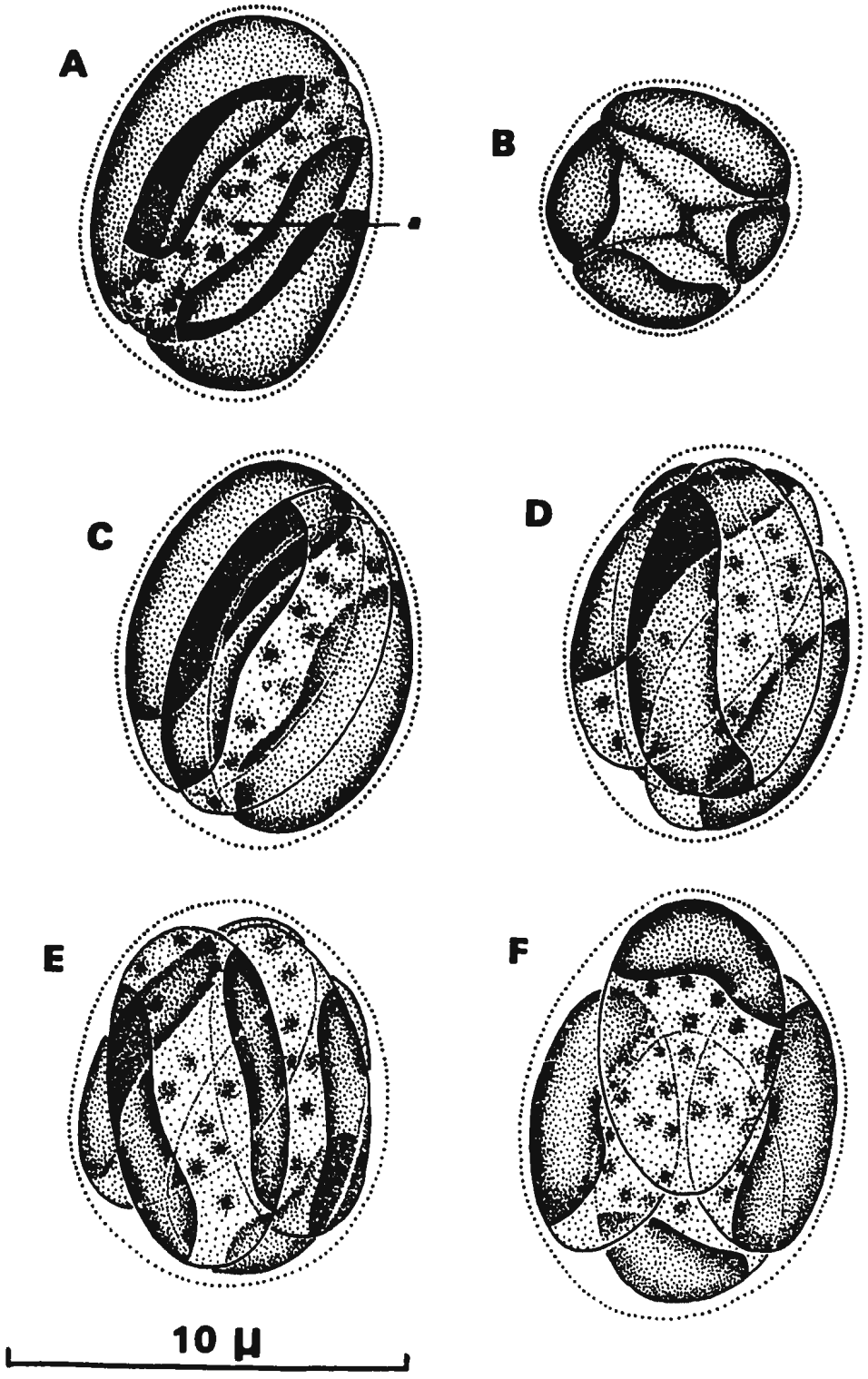


FIGURE 19. Formation of 4-celled aplanosporangium and reorientation of spores within the capsule. For explanation see text.

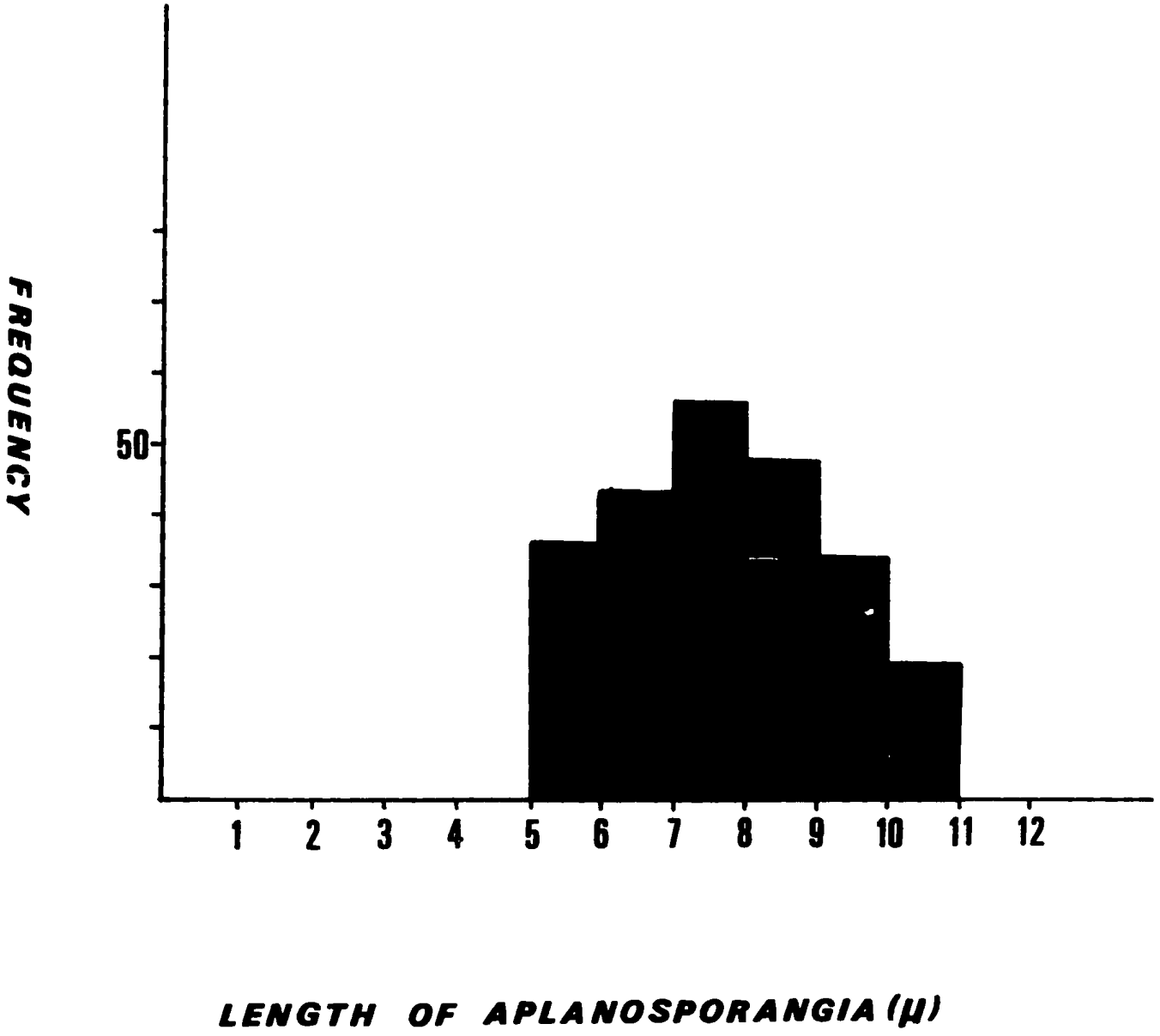


FIGURE 20. Size frequency distribution for aplanosporangia *in vivo*.

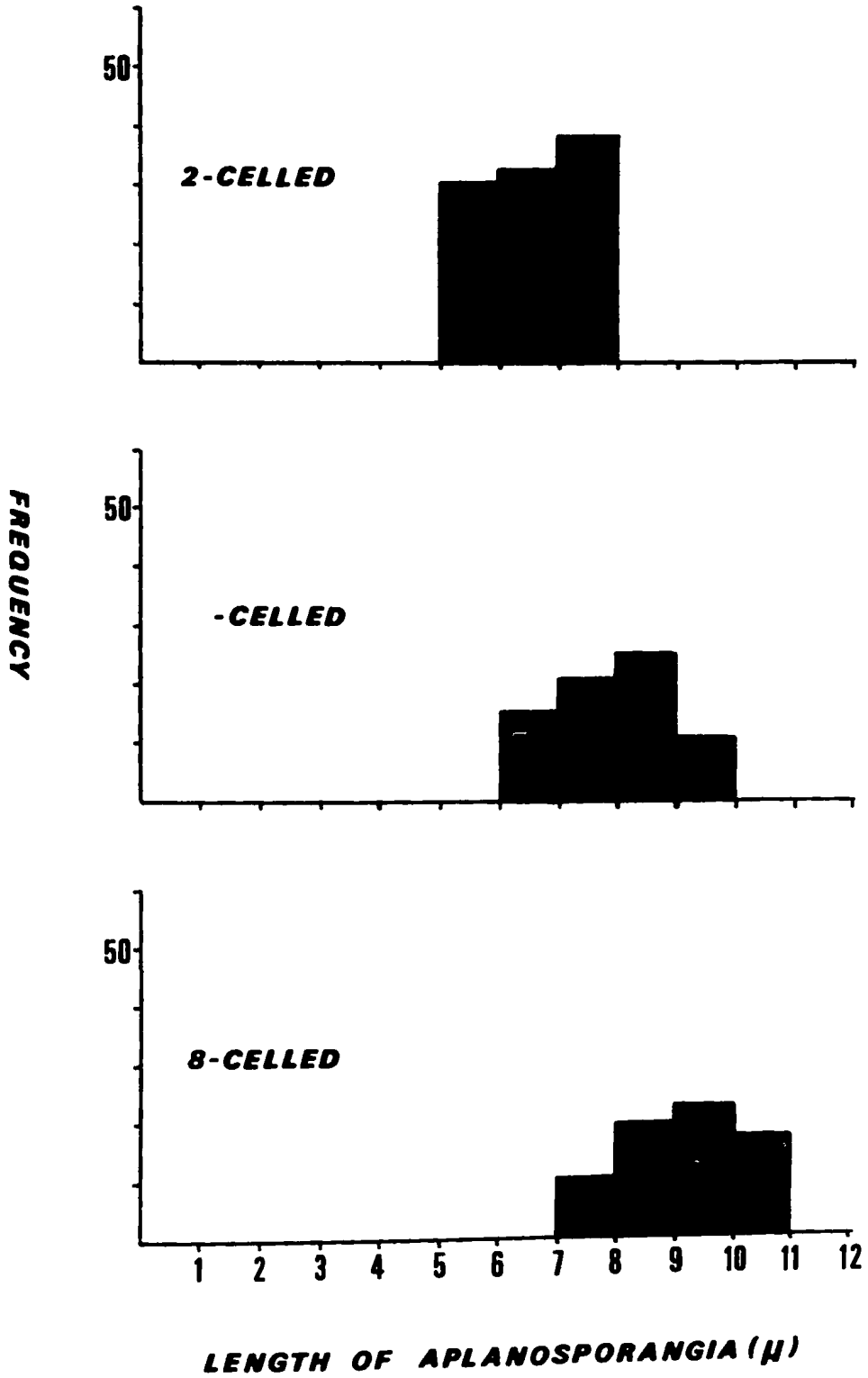


FIGURE 21. Size frequency distributions for 2-celled, 4-celled and 8-celled aplanosporangia *in vivo*.

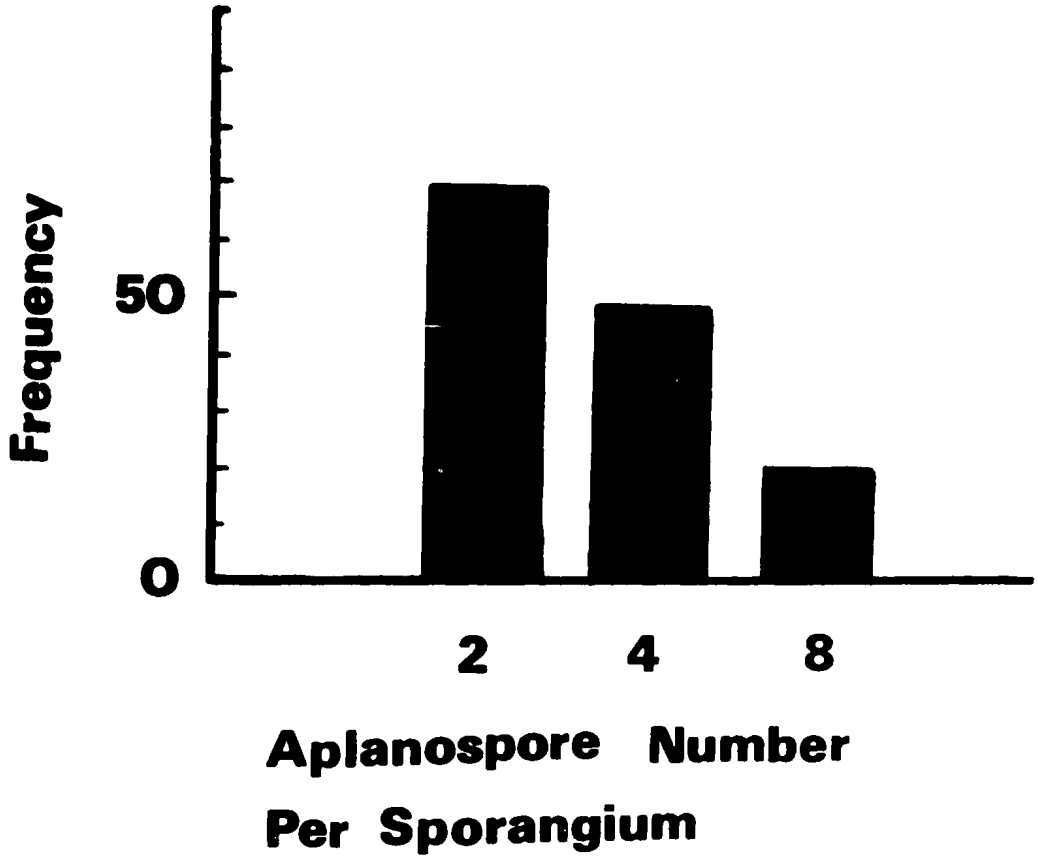


FIGURE 22. Frequency of 2-celled, 4-celled and 8-celled aplanosporangia *in vivo*.

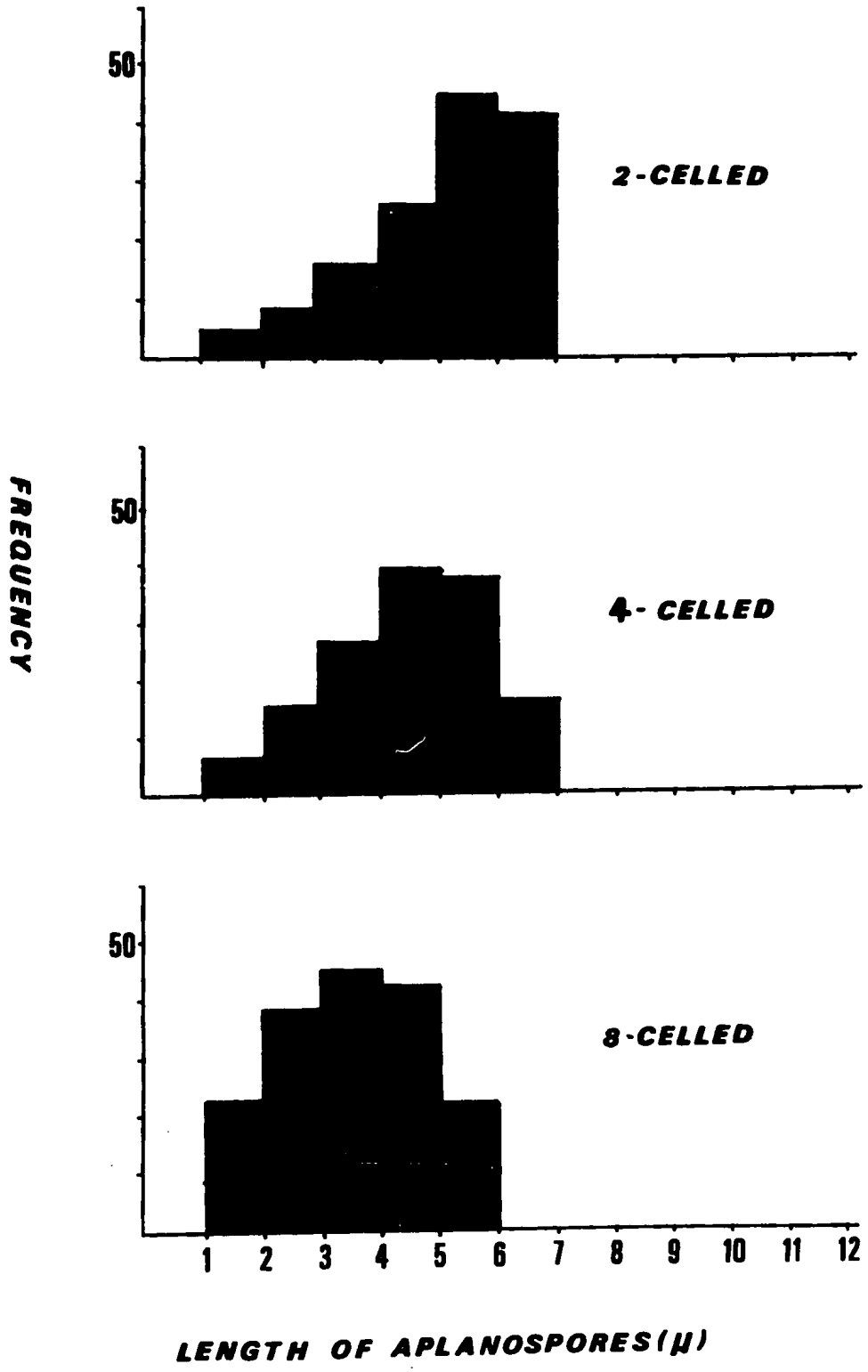


FIGURE 23. Size frequency distributions of aplanospores from 2-celled, 4-celled and 8-celled aplanosporangia *in vivo*.

Size distribution for the aplanospores present in the 2-, 4-, and 8-celled aplanosporangia is presented in Figure 23. Spores range in length from 1.0 to 7.0 μ. A peak occurs at 5.0 - 7.0 μ for spores from 2-celled aplanosporangia, 4.0 to 6.0 μ for spores from 4-celled aplanosporangia, while 8-celled aplanosporangia have spores with a size distribution peak at 2.0 to 5.0 μ.

Morphology and Reproduction of Endozoic Algae *in vitro*

(1) Culture characteristics and morphology of algae *in vitro*:

Actively growing liquid cultures appear to be very deep green in color when concentrations of algal cells are great. When a liquid culture of the endozoic algae is initiated the cells deposited in the liquid medium settle to the bottom of the vessel. Growth at first is uniform along the bottom of the vessel providing it is not disturbed. With further elapse of time the algal cells show a definite phototropic response growing up the side of the culture vessel furthermost from the light source. Growth may continue up the side of the vessel to the air-liquid interphase where a "ring" of the green cells may form.

Algal cells grown on an agar substrate during the first few weeks of growth display a lime green color, but as the numbers increase the color becomes brilliant deep green. The algal colonies established on agar are smooth and often very shiny. In clonal cultures clones were uniformly circular on the surface of the agar, but during aging it was noted that the clones often became irregularly

shaped and often penetrated the surface of the agar. In older agar cultures it was noted that colonies grew quite well several mm beneath the surface of the agar.

In stab cultures the cells remained alive and grew actively under the surface of the agar. In many cases the clones spread and eventually formed colonies on the surface of the agar above the original inoculation site.

On agar slopes algal growth covered most of the slope surface after several weeks of incubation.

In biphasic cultures (Chihara liquid medium covering Chihara agar substrate), growth was first evident on the surface of the agar, however, within a few weeks algal cells could be seen on the side of the vessel furthermost from the light demonstrating the phototrophic response.

Inoculation of 5-20 cells was an unsatisfactory method of establishing liquid subcultures; no visible growth could be observed after several months. When larger numbers of cells were used, actively growing subcultures could be initiated and growth was evident within a few weeks.

Bacteria- free cultures were not achieved in this study. Close examination of the algal cells showed that in many instances rod-shaped bacteria were attached to the cell walls of the algae (Figure 24). Repeated washings of the cells proved of little consequence to the firmly attached bacteria.

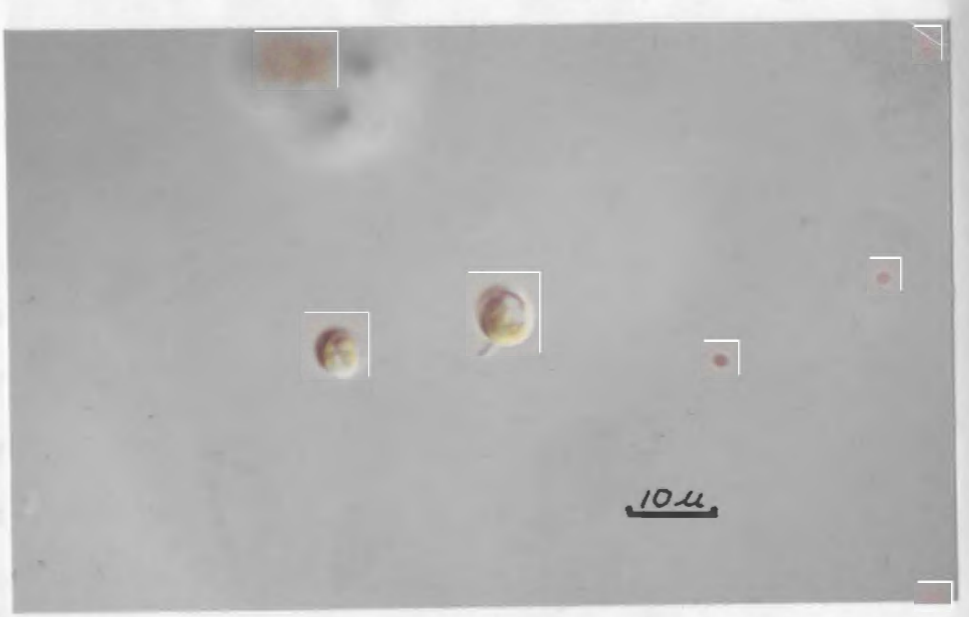


FIGURE 24 Rod-shaped bacterium firmly attached to the algal cell wall.

Morphological characteristics of the algae *in vitro* vary greatly with changes in culture media, age of cultures and the number of times the algae have been subcultured.

Young liquid and agar cultures freshly initiated from infected animals differ only slightly from algae *in vivo*. The cells show a great variation in size and shape; however, in many cells there is a greater accentuation of hyaline tips and an increase in refractile cytoplasmic inclusions than in cells observed *in vivo*. The characteristic parietal chloroplast is well defined, usually single and band or cup-shaped, although two and very rarely three chloroplasts have been observed in some cells.

Cells from young cultures range in length from 1.0 to 10.0 μ with an average length of 4.5 μ and from 1.0 to 6.0 μ in width with an average width of 4.0 μ.

With increase in age of the isolated algae there was an apparent loss of morphological variability. Older agar cultures displayed less variability than liquid cultures of comparable ages. The older liquid cultures (6-10 weeks after inoculation) displayed cell types very similar to the young freshly initiated cultures, whereas agar cultures contained cells which were generally smaller and more uniformly spherical.

Cells from older liquid cultures ranged in length from 1.0 to 10.0 μ with an average length of 5.5 μ, and 1.0 to 5.0 μ in width with an average width of 3.5 μ. Cells displayed a great variety of shapes but there were fewer cells displaying well defined

hyaline tips. Chloroplasts were usually parietal bands with few cup-shaped examples, and usually occupied up to 3/4 of the cell. Vacuolated cells were common, and coarse refractile cytoplasmic inclusions were evident in most of the cells.

Old agar cultures of comparable ages were characteristic in having cells which ranged from 1.0 to 9.0 μ in diameter (with few cells larger than 7.0 μ) usually round, containing a single band chloroplast, a thick cell wall, and were relatively free of vacuoles or refractile bodies.

Liquid and agar cultures which were subcultured on numerous occasions were characteristic in having cells which were small, round and free of refractile bodies. In these cultures reproduction was accomplished almost exclusively by the formation of two-celled aplanospores, unlike freshly initiated cultures which displayed 2-celled, 4-celled, 8-celled and on occasions 16-celled aplanosporangia.

Cells in cultures which were left for long periods of time without replenishment of nutrients showed a general degeneration. The chloroplast became very minute, often only a fragment at one side of the cytoplasm. Refractile bodies were very evident with numerous vacuoles and large well defined round bodies which were interpreted as oil globules (Figure 25). The cells were spherical and displayed a very thick cell wall (Figure 26). These cultures, however, were used to initiate subcultures which grew actively. The subcultured algal cells displayed a well defined band chloroplast and were free of vacuoles, refractile granules and oil globules.

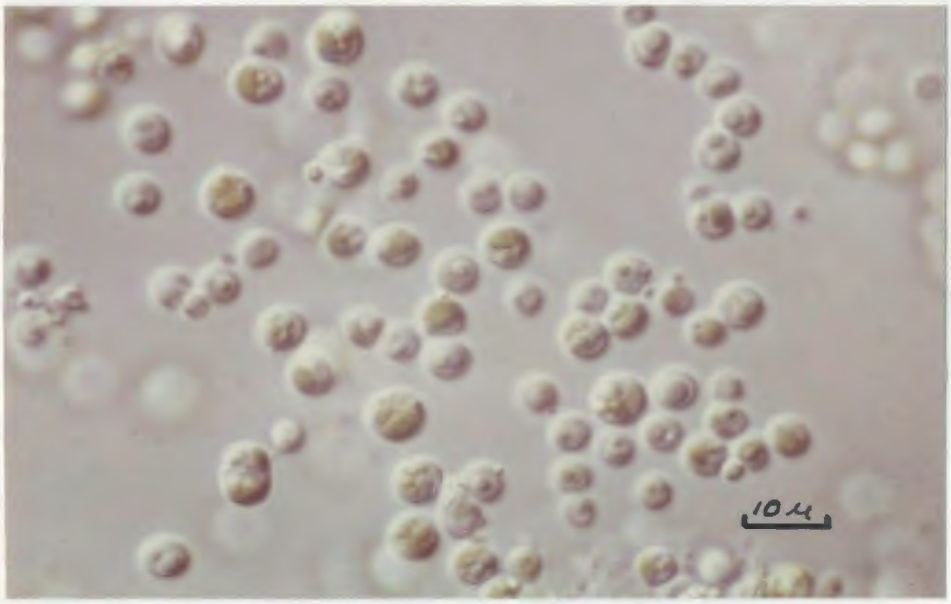


FIGURE 25 Cells from old agar plate culture displaying oil globules and numerous refractile cytoplasmic inclusions.

FIGURE 26 Cell casts from algal cells in old culture illustrating the characteristic thick cell wall (arrow).

To illustrate the changes in cell size in various cultures as compared with cells *in vivo* , cell size distributions were plotted. Figure 17 presents size frequency distributions of algal cells from: a lightly infected scallop; a young slope culture (14 days); and an old culture (180 days, subcultured 4 times). Size distributions for old (175 days) liquid and young (14 days) liquid cultures are presented in Figure 27.

Peaks were observed at 3.0 to 4.0 μ and 6.0 to 7.0 μ, 3.0 to 4.0 μ and 5.0 to 6.0 μ for young slope cultures and old slope cultures respectively (Figure 17). Old liquid and young liquid cultures displayed peaks at 3.0 to 4.0 μ and 7.0 to 8.0 μ; and 2.0 to 3.0 μ and 7.0 to 8.0 μ respectively (Figure 27).

Viability studies were conducted on cultures under various conditions. Viability was assessed by the ability of cultures to propagate new subcultures in Chihara medium.

It was found that liquid and agar (plate and slope) cultures which were kept in the dark at room temperature or 10°C for periods up to 8 months could give rise to actively growing subcultures, which displayed healthy spherical cells free of refractile bodies or oil globules.

Algal cultures were found to withstand temperatures as low as 2°C for periods up to 2 weeks. After 2 weeks, however the cells failed to reproduce and hence subculturing was impossible. The cells appeared normal in that the chloroplast was large and well defined. However after 2 weeks at this low temperature the cells quickly deteriorated and the cells bleached when exposed to the light.

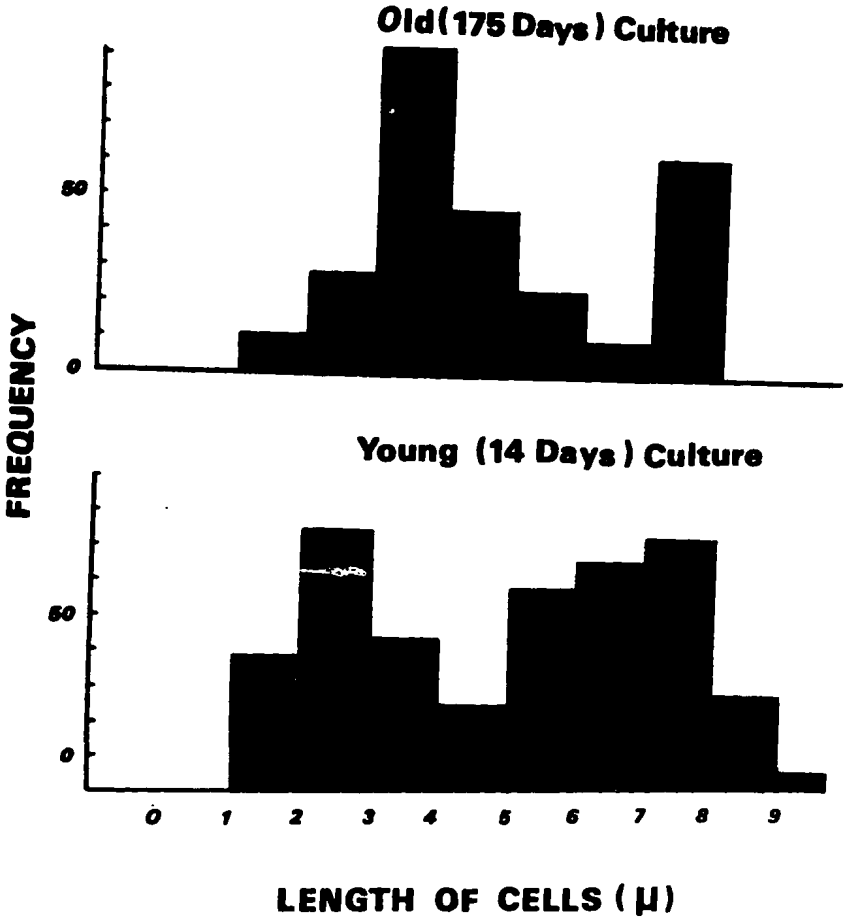


Figure 27. Cell size frequency distributions for an old (175 day) culture and a young (14 day) culture.

The high temperature tolerance limit of the algae was determined by exposing the cultures to 20^{o}, 25^{o}, 30^{o}, and 35^{o} for prolonged periods of time. Normal growth and reproduction as well as successful subcultures were accomplished in cultures exposed to 20^{o} and 25^{o} but rapid deterioration was found with cultures placed in baths at 30^{o} and $35^{o}C$. It was found that the algae could tolerate these temperatures for no longer than two days.

Algal cells placed in 27 and $28^{o}C$ water baths survived up to a week and were able to propagate new subcultures. After 7 days in the baths, however, the cells quickly died, and were bleached out when exposed to light.

Cells exposed to bright direct sunlight quickly died and were bleached out if the exposure time was longer than 4 hours. Cultures exposed to sunlight for periods less than 3-4 hours survived and could give rise to actively growing subcultures even if the cultures received this light shock on consecutive days.

Temperature shock seemed to have little effect on the survival of the alga. Cultures maintained at $15^{o}C$ and exposed to $0^{o}C$ for periods up to 36 hours were able to give rise to new subcultures which grew actively at $15^{o}C$. Cultures maintained at $2^{o}C$ for several days and then exposed to temperatures of $20^{o}C$ and $25^{o}C$ survived and gave rise to new subcultures.

A brief subjective study was undertaken to determine optimal culture conditions for the endozoic algae. All subcultures were initiated from one actively growing agar slope culture 5 weeks after inoculation. The culture chosen was relatively 'clean'

i.e., very little bacterial growth could be observed. Subcultures on agar Chihara subtrates placed in an illumination of 3500 lux and a temperature of 15°C were found to grow best. A summary of the conditions and the success of the subcultures based on "visible" growth within two weeks is presented in Table 2.

(2) Reproduction

Reproduction *in vitro* is accomplished by the formation of 2-, 4-, and 8-celled aplanosporangia with less frequently 16-celled aplanosporangia. Division was not observed to be oblique since few oblong cells were observed in stable cultures. Figure 28 illustrates various views of *in vitro* cells undergoing aplanospore formation. On occasions capsules containing only 3 spores were observed, however, as pointed out by Fott and Novakova (1969) irregular division is often observed in cultures of unicellular green algae. As *in vivo* the aplanospores after complete division move about with a degree of freedom within the mother cell wall, and subsequent release is by rupture of the capsule wall.

All attempts to induce motility met with failure. As a consequence, reproduction appears to be entirely asexual and by aplanospores.

Rapid rates of division were characteristic of young or new subcultures, in which 2-celled aplanosporangia were of highest frequency. Frequency distributions of aplanospores per sporangium for representative agar cultures are presented in Figure 29.

TABLE 2: Culture conditions and success of subcultures as based on period of time before 'visible' growth was observed, and on average time for 10 subcultures exposed to each condition.

Medium	Type	Illumination	Temperature	Time
Chihara	Liquid	3500 lux	15°C	13 days
Chihara	Liquid	3500 lux	10°C	--
Chihara	Liquid	1500 lux	15°C	14
Chihara	Liquid	1500 lux	10°C	--
Chihara	Agar	3500 lux	15°C	8
Chihara	Agar	3500 lux	10°C	11
Chihara	Agar	1500 lux	15°C	12
Chihara	Agar	1500 lux	10°C	14
Erdschreiber	Liquid	3500 lux	15°C	--
Erdschreiber	Liquid	3500 lux	10°C	--
Erdschreiber	Liquid	1500 lux	15°C	--
Erdschreiber	Liquid	1500 lux	10°C	--
Erdschreiber	Agar	3500 lux	15°C	14
Erdschreiber	Agar	3500 lux	10°C	--
Erdschreiber	Agar	1500 lux	15°C	--
Erdschreiber	Agar	1500 lux	10°C	--
Symbiodinium	Liquid	3500 lux	15°C	13
Symbiodinium	Liquid	3500 lux	10°C	--
Symbiodinium	Liquid	1500 lux	15°C	--
Symbiodinium	Liquid	1500 lux	10°C	--
Symbiodinium	Agar	3500 lux	15°C	12
Symbiodinium	Agar	3500 lux	10°C	14
Symbiodinium	Agar	1500 lux	15°C	14
Symbiodinium	Agar	1500 lux	10°C	--

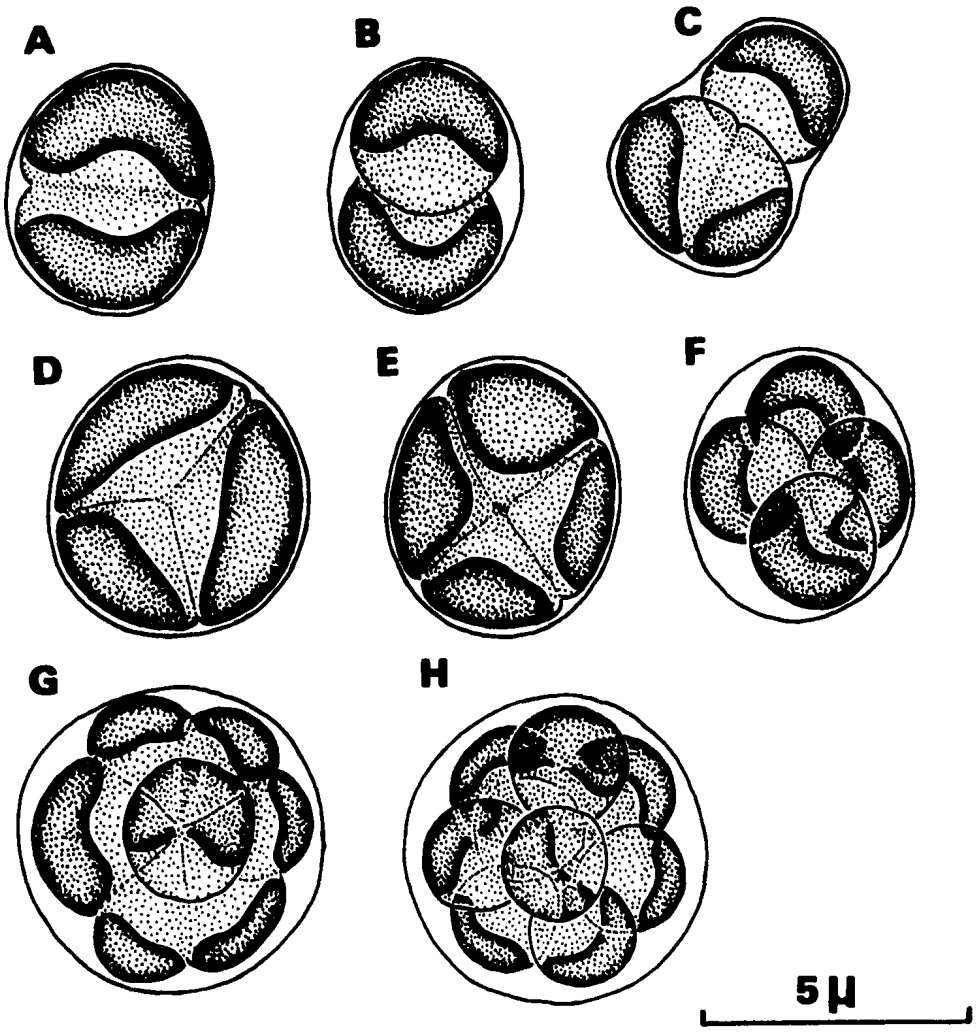


FIGURE 28. Aplanosporangia formation as observed *in vitro*.

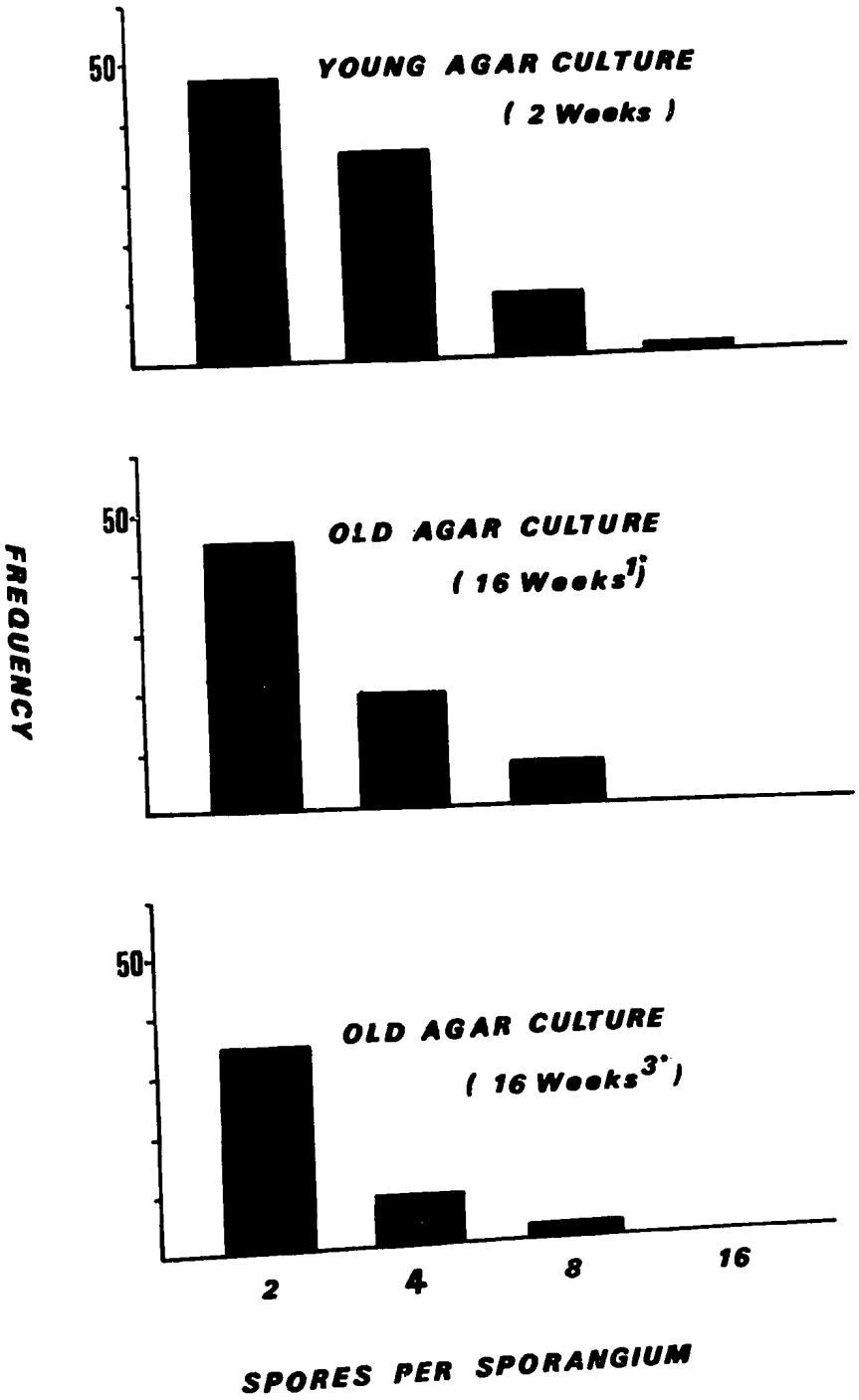


FIGURE 29. Frequency of 2-celled, 4-celled, 8-celled and 16-celled aplanosporangia in cultures of different ages. (*... number of times subcultured)

Pigmentation

(1) Paper chromatography

Figure 30 shows a typical one dimensional paper chromatogram for the endozoic algae run and developed as prescribed in MATERIALS AND METHODS. Neoxanthin (Figure 30-5) chlorophyll *b* (Figure 30-4) and chlorophyll *a* (Figure 30-3) were clearly resolved but β-carotene (Figure 30-2) and α-carotene (Figure 30-1) were not completely separated.

The color description, Rf values and identity for the five pigment fractions are presented in Table 3.

(2) Spectrophotometry

Absorption spectra were used in specific identifications of the pigment fractions. An absorption spectrum for the total pigment extract as well as for the individual fractions were prepared as described in MATERIALS AND METHODS. Total extract (Figure 31) showed absorption maxima at 665 and 430 mμ indicating a strong presence of chlorophyll *a*. It was suspected that the strong presence of chlorophyll *a* masked the other pigment fractions.

Fraction 1, α-carotene (Figure 32) showed absorption maxima at 420, 445, and 473 mμ. Fraction 2, interpreted as β-carotene showed absorption maxima at 420, 450, and 473 mμ (Figure 33). Maxima at 409, 430 and 664 mμ were recorded for the third fraction, chlorophyll *a* (Figure 34) and maxima at 424, 453, and 644 mμ were recorded for the fourth fraction, chlorophyll *b* (Figure 35). Neoxanthin, the fifth fraction showed absorption maxima at 415, 439, and 465 mμ (Figure 36).

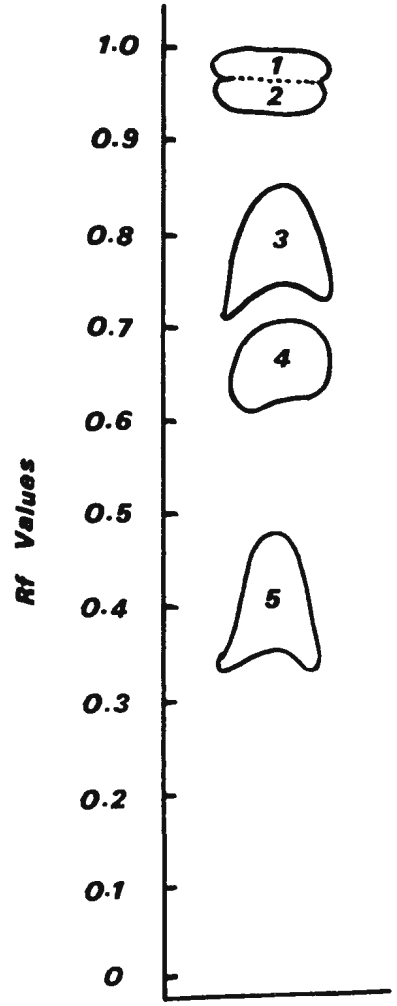


FIGURE 30. Typical one dimensional paper chromatogram indicating the relative positions of the pigment fractions; α-carotene (1); β-carotene (2); chlorophyll *a* (3); chlorophyll *b* (4); neoxanthin (5).

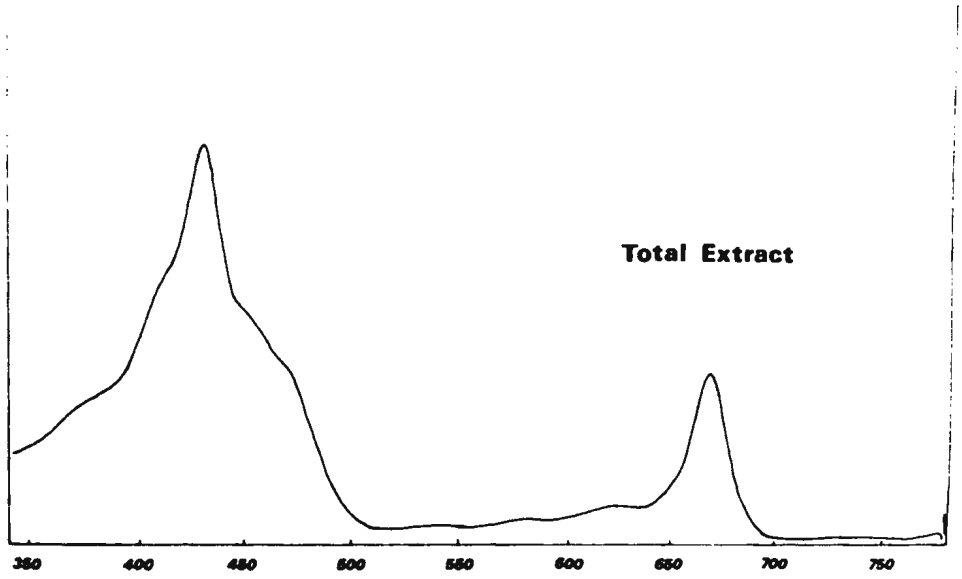


FIGURE 31. Spectrophotometric absorption curve for total pigment extract indicating a strong presence of chlorophyll *a*.

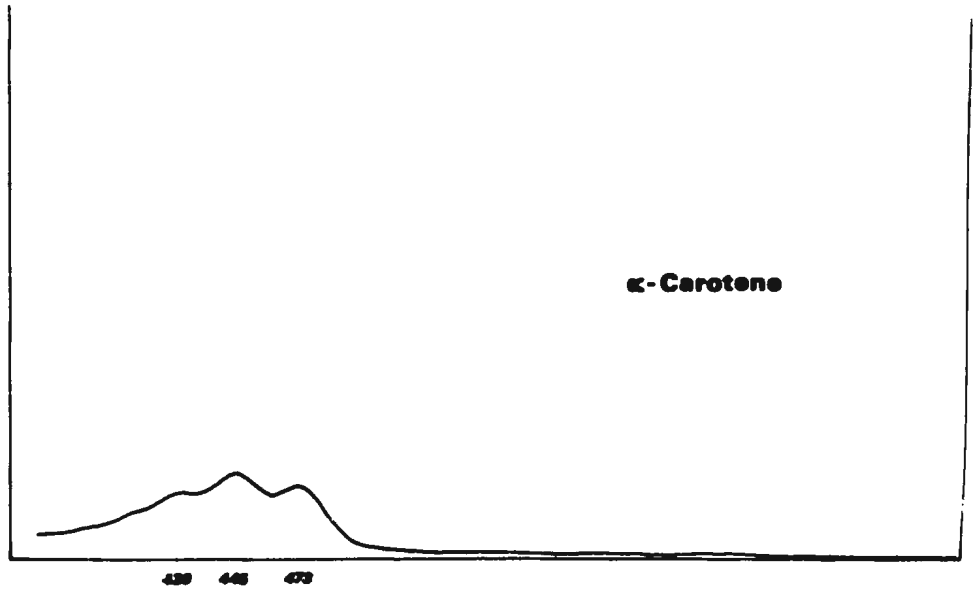


FIGURE 32. Absorption curve for 1st pigment fraction identified as α-carotene showing maxima at 420, 445, 473 mμ.

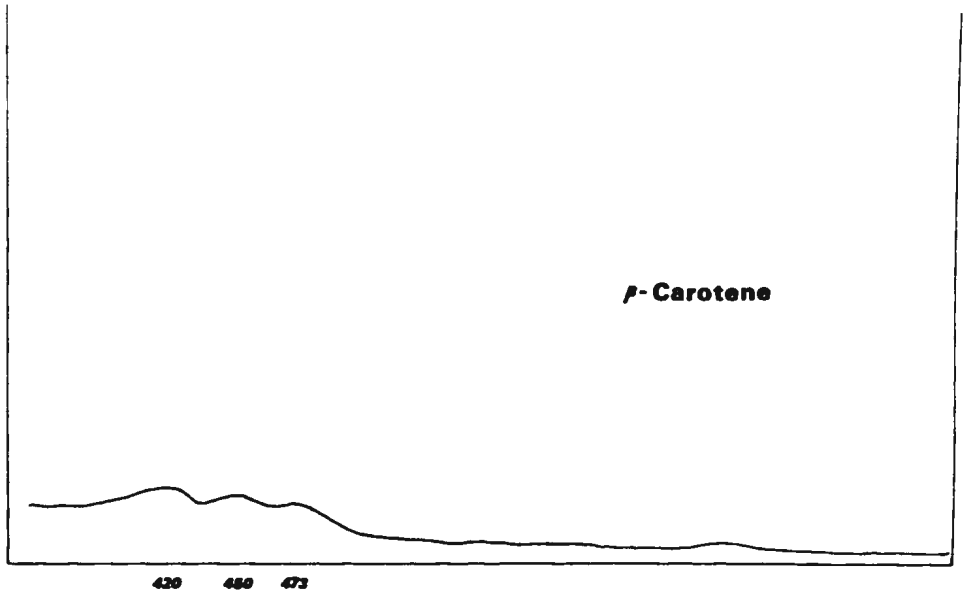


FIGURE 33. Absorption curve for 2nd pigment fraction identified as β-carotene showing maxima at 420, 450, 473 mμ.

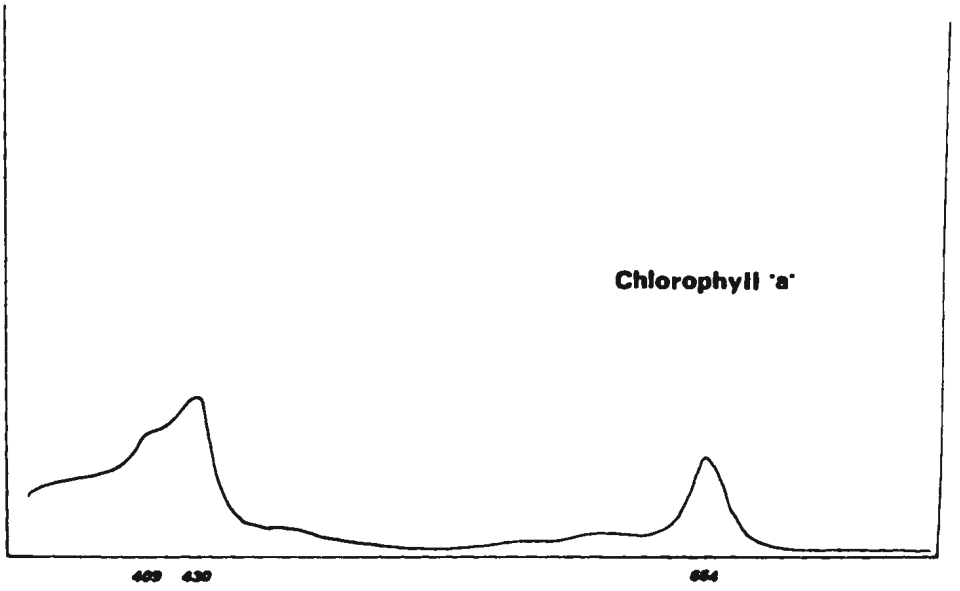


FIGURE 34. Absorption curve for 3rd pigment fraction identified as chlorophyll a showing maxima at 409, 430, 664 mμ.

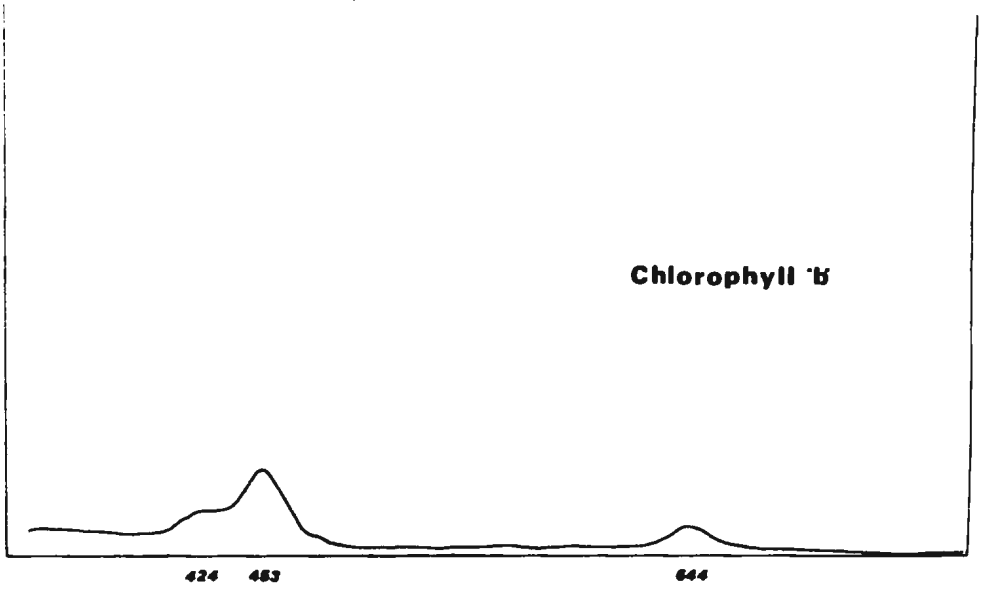


FIGURE 35. Absorption curve for 4th pigment fraction identified as chlorophyll *b* showing maxima at 424, 453, 644 mμ.

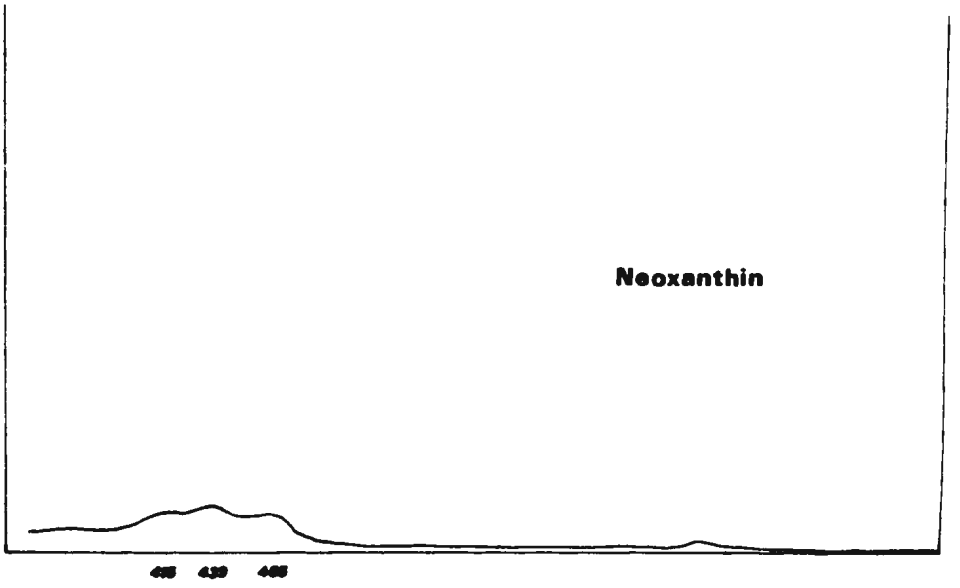


FIGURE 36. Absorption curve for 5th pigment fraction identified as neoxanthin showing maxima at 415, 439, 465 mμ.

A summary of the description, and absorption maxima for each pigment fraction is presented in Table 4.

TABLE 3: Rf values, color description and tentative identification of pigment fractions.

FRACTION	Rf VALUE	COLOR	IDENTIFICATION
1	0.97	yellow	α-carotene
2	0.95	pale orange	β-carotene
3	0.78	blue-green	chlorophyll *a*
4	0.65	yellow-green	chlorophyll *b*
5	0.41	bright yellow	neoxanthin

TABLE 4: The absorption maxima and identification of each pigment fraction.

FRACTION	ABSORPTION MAXIMA mμ	IDENTIFICATION
1	420, 445, 473	α-carotene
2	420, 450, 473	β-carotene
3	409, 430, 664	chlorophyll *a*
4	424, 453, 644	chlorophull *b*
5	415, 439, 465	neoxanthin

Animal Experimentation

(1) Phagocytosis

Natural phagocytosis

In routine examinations of the algal-animal smears it was noted that numerous animal cells contained the endozoic algae. It was concluded that this phenomenon was a cellular response (phagocytosis) of the animal to the infecting algae.

Animal cells of various shapes, sizes and cytoplasmic characteristics were observed containing the algal cells (Figure 37) within the mantle tissues, haemolymph and stomach contents of infected scallops. Some types of animal cells were never observed containing the algal cells (Figure 37F and G).

Large round, oval or irregular phagocytes (Figure 37A,B,C,D,E, and Figure 39) were observed in the infected tissues of the scallop. These phagocytes are characterized by having plasticity of shape, varying degrees of granulation and in many cases well defined vacuoles (Figure 37B and Figure 40, solid arrow). A single large nucleus oval in shape (Figure 37A, nu) occupies one side of the cytoplasm as revealed in Geimsa stained smears.

The shape of the phagocyte appeared to be dependent on the phagocytosed particles. Cells free of engulfed algal cells were usually round or oval whereas phagocytes containing large numbers of algal cells were irregularly shaped (Figure 39A,B,C, and D). The smaller phagocytes, round or oval in shape, were observed carrying few algal cells (Figure 37C and D) whereas the larger cells contained large numbers of algae (Figure 39B and D). As many as 35-40 algal cells were counted in some phagocytes.

FIGURE 37

Animal cells observed which actively phagocytosed the endozoic algae (A-E and H-J) and animal cell types never observed containing the endozoic algae (Fand G). Cells varied in shape and size depending on the number of algae contained. gr, granules; nu, nucleus; va, vacuole; fl, flagella; al, alga; ps, pseudopodia.

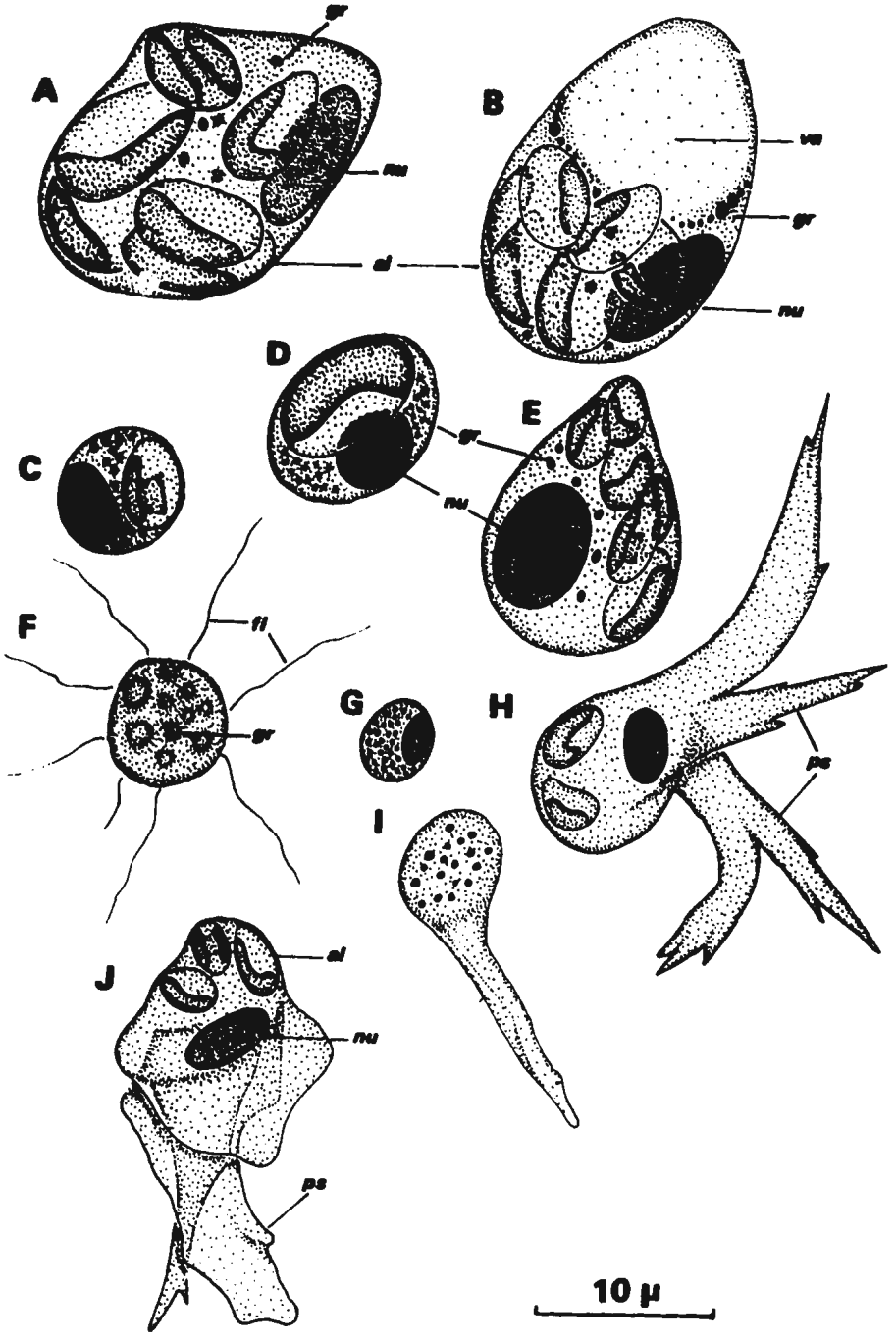


FIGURE 37. Animal cells observed which actively phagocytosed the endozoic algae (A - E and H - J) and animal cell types never observed containing the algae (F and G). For explanation see text and page 83.

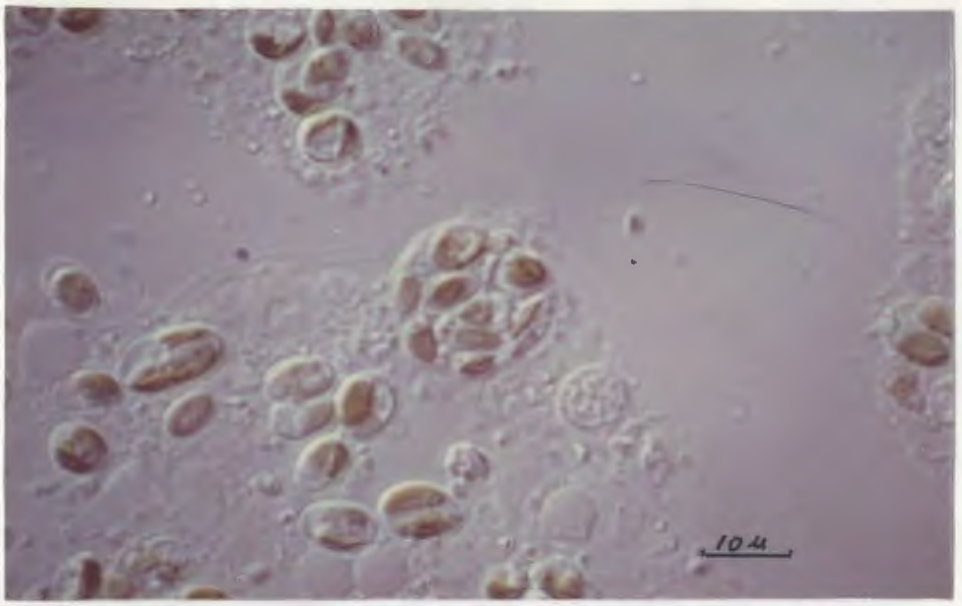


FIGURE 38 Large phagocyte observed in infected mantle tissue displaying generally round shape and containing numerous algal cells.

Phagocytes observed in the infected tissues of the scallop were relatively free of cytoplasmic extensions. Phagocytes observed in the haemolymph, however, were characterized by having large spiked-edged (Figure 37H and I) or rounded-edged (Figure 37J) pseudopodia. This characteristic was also very common in phagocytes observed in the feces and stomach contents of heavily infected scallops.

Haemolymph phagocytes were usually agranular, carried only a few algal cells (1-4) and displayed a great variety of cytoplasmic extensions (pseudopodia). The cells contain a single oval nucleus which occupies usually a central position in the cytoplasm. On only a few occasions granules (Figure 37I) were observed within the cells but this was later thought to be non-algal phagocytosed material.

There appeared to be no specific pattern of phagocytic activity

in relation to degree of infection, site of infection or seasonal variation. Phagocytic activity of the animal cells ranged from 12% to 35% in the smears studied but an inadequate sampling technique hampered proper elucidation of this phenomenon.

In algal colonies picked from the epidermal layers of the mantle it was noted that large numbers of phagocytes containing algal cells surrounded the intact colonies. On occasions phagocytes containing the algae were observed within the centre of the algal colonies as revealed by Cryostat sections through the round colonies.

Examination of blood and stomach contents as well as feces from two heavily infected scallops revealed phagocytic leucocytes of the type in Figure 37H. These cells as pointed out above contained only a few algal cells. The algae were recognized by their shape, size and chloroplast. In heavily infected scallops which had algal colonies resting on the exposed inner surface of the shell, phagocytes of the type in Figure 37A were observed within and surrounding the sloughed off colonies.

Phagocytes containing the algae were found resting on or loosely held within the epidermal layer of the mantle edges. Clean microscope slides pressed gently against the infected mantle edge and later observed under high power revealed numerous algae-carrying phagocytes.

Generally healthy looking single algal cells were observed in phagocytes, but on a few occasions degenerated cells (Figure 40, solid arrow) and dividing cells (Figure 40, open arrow) were observed.

FIGURE 39

Natural and experimental or induced phagocytosis. Large phagocyte (A) in which it will be noted that it takes the general shape of the contents. Note the plasticity of the cell membrane. Large numbers of algae are contained in some phagocytes (B, C and D). The spherical cultured alga is clearly visible within the blood cells (leucocytes), (E open arrow) which are characterized by pseudopodia (E solid arrow). The phagocyte generally take the shape of the ink particles (F).

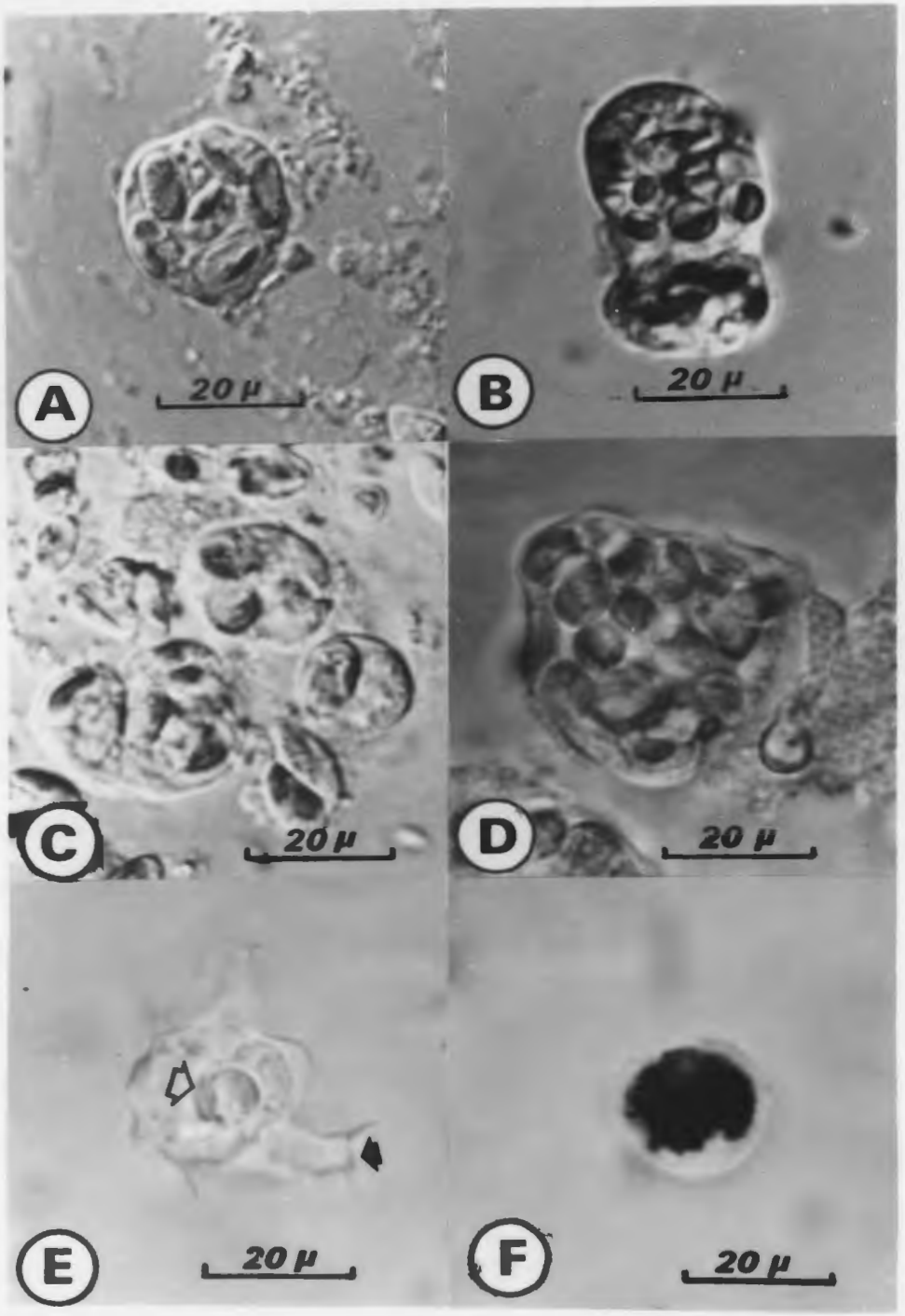
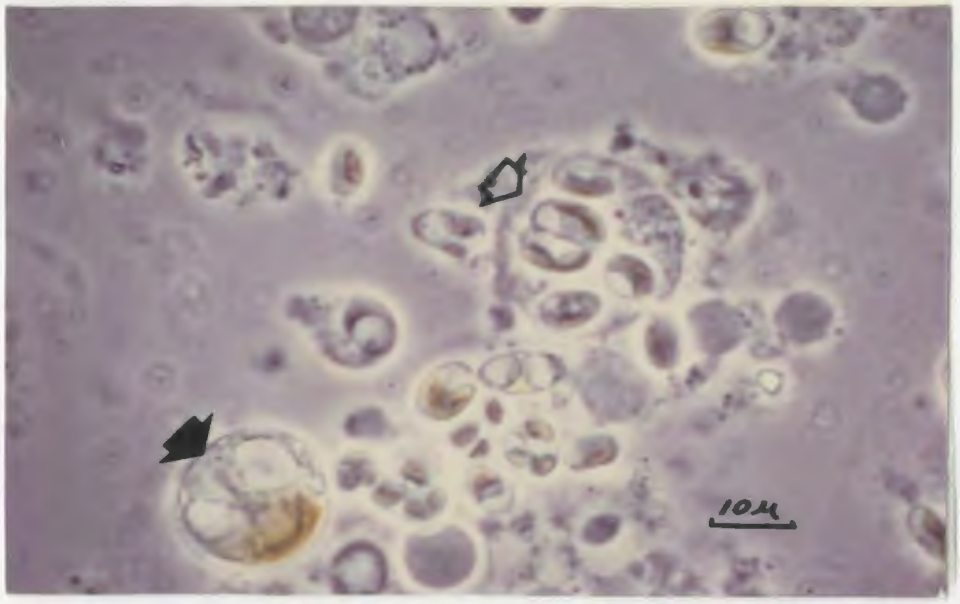


FIGURE 39. Natural phagocytosis (A - D) and induced phagocytosis (E and F). For explanation see text and page 87.

FIGURE 40 Phagocytes containing degenerated algae and large vacuoles (solid arrow) and dividing algae (open arrow).



Experimental phagocytosis:

Experiments were carried out to illustrate the phagocytic activity of the leucocytes within the haemolymph and the amoebocytes within the tissues of uninfected scallops.

Cultured endozoic algae and India ink were injected into the ventricles of visibly uninfected scallops and studied as prescribed in MATERIALS AND METHODS. Cultured algae were observed in numerous leucocytes (Figure 39E) which displayed their characteristic pseudopodia. India ink could be clearly resolved within the leucocytes and in many cases the phagocyte assumed the general shape of the ink particle (Figure 39F).

Algal cells injected into the adductor muscle and mantle tissues were engulfed by large generally circular phagocytes (Figure 41).

Unlike the haemolymph cells these phagocytes could engulf large numbers of the cultured algae.

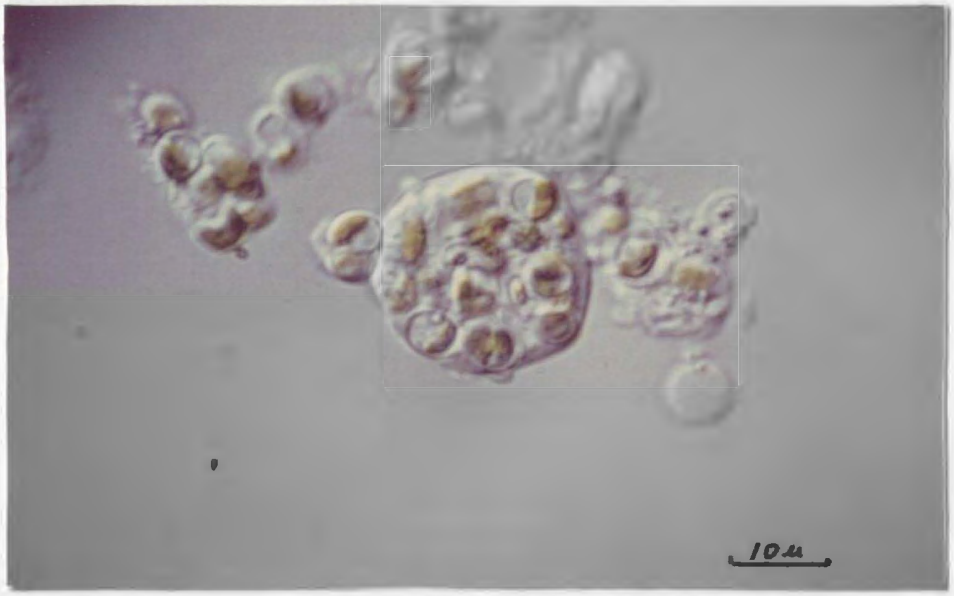


FIGURE 41 Large phagocyte from mantle tissue containing cultured algae.

(2) Artificial infection of scallops

Of the methods employed in attempting to infect scallops with the endozoic algae grown in culture as described in MATERIALS AND METHODS one was found to successfully initiate colonies within the mantle tissues. Cultured endozoic algae injected into the left mid-mantle edge successfully established colonies in two scallops and were clearly visible within 8 weeks. The colonies displayed the normal discrete round shape and appeared as 'natural' endozoic algal colonies. Sections through these artificially infected areas revealed encapsulation of the algae by fibrous tissue as described by Naidu (1971).

Systematic observations on the algae injected into the mantle tissues of uninfected scallops indicated that after a period of time

the cultured algae gradually changed their morphological appearance. Cultured algae which were uniformly spherical, small and showed no hyaline tips before injection were noted to take on the characteristics of naturally occurring endozoic algae. Two weeks after injection many of the cells observed were typical of the cultured algae, however, some contained hyaline tips. Within 8 weeks after injection the algal cells appeared to be identical to the endozoic algae from naturally infected scallops. A graphic sequence of events is presented in Figure 42.

Active phagocytosis as well as encapsulation of the injected algae occurred within the mantle tissue. Many of the animals which were injected but failed to produce 'visible' colonies nevertheless, upon examination, indicated that many of the cultured cells were present within the phagocytes. No apparent morphological changes of algal cells were observed within the phagocytes.

(3) Incidence of infection and age composition of scallops collected:

The incidence of infection for the samples (based on the percentage of scallops containing algal colonies) is presented in Table 5. It was found that the highest incidence occurred in January 1970 from the St. Joseph's area, in which 32.7% of the scallops collected contained the algae. The overall incidence of infection for the 6 samples was 18.4%.

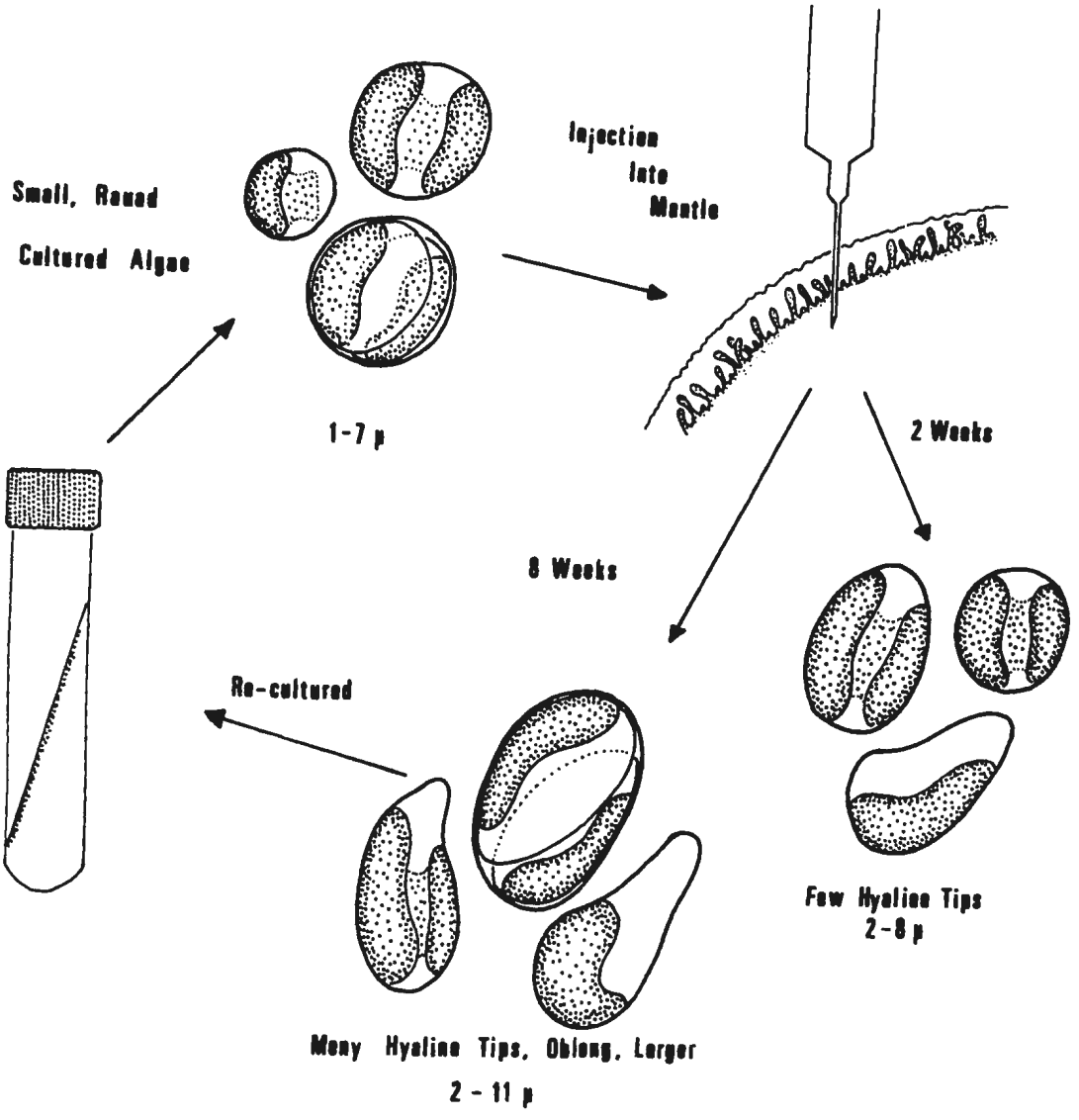


FIGURE 42. Sequence in which small uniformly spherical cultured algae are injected into the mantle tissue of an uninfected scallop. Few hyaline tips are observed after 2 weeks, however after 8 weeks the algae appear identical to the naturally occurring endozoic algae. Cells are larger, division clearly oblique and many hyaline tips are observed.

TABLE 5: Collection data and incidence (% infection) of endozoic algae.

DATE	LOCATION (Area)	TECHNIQUE	DEPTH	NO. COLL.	NO. INF.	INCIDENCE (%)
Nov. 7/68	1	SCUBA	40 ft.	25	1	4.0
Feb. 20/69	1	SCUBA	40 ft.	30	5	6.0
June 21/69	1	SCUBA	30-40 ft.	207	21	10.2
July 10/69	2	DRAG	30-40 ft.	68	12	17.6
Oct. 20/69	1	SCUBA	40 ft.	83	21	25.2
Jan. 18/70	1	SCUBA	30-40 ft.	110	36	37.2
TOTALS				523	96	18.4

Table 6 presents the percentage infection in relation to the age of the scallops collected. Scallops from 5 to 16 years old were collected. Highest incidence of infection occurred in scallops 14 years old in which 32.0% were found to contain the algae.

TABLE 6: Age composition and incidence of infection for the scallops collected.

Age in years	Number infected	Number Collected	Incidence of Infection (%)
5	0	3	0
6	1	18	5.6
7	1	31	3.2
8	3	30	10.0
9	14	58	24.2
10	27	156	17.3
11	22	108	20.2
12	12	52	23.1
13	7	28	25.0
14	8	25	32.0
15	1	9	11.1
16	0	5	0
TOTALS	96	523	18.4

DISCUSSION

Parasitic algal infections

Few cases of algal infections in animals have been reported in the literature. Mortensen and Rosenvinge (1910) first reported on an algal infection in the sea star *Ophiura texturata*. They identified the alga *Coccomyxa ophiurae* within the epidermis of the host and commented on possible calcium dissolution by this pathogen resulting in the eventual death of the echinoderm. Later these authors (1933) documented a second parasitic association involving the alga *Coccomyxa astericola* and the echinoderm *Hippasteria phrygiana* (Parel) in which they suspected the same detrimental effect on the host animal.

Davies, Spencer and Wakelin (1964) isolated the colorless alga, *Prototheca segbwema* from cutaneous lesions of a man in Sierre Leone and also demonstrated the pathogens within the tissues of the human host. These authors later (1967) commented on the spread of the alga to the regional lymph nodes and considered the alga to be a potentially dangerous pathogen since it was resistant to commercial antibiotics and antifungal agents.

Hoffman, Prescott and Thompson (1965) reported on cysts 0.29 to 1.16 mm in diameter containing large numbers of algae of the genus *Chlorella*, within the orbit of the eye of a bluegill, *Lepomis macrochirus*. Because of the tissue response to the algae by the animal these algae were considered parasitic.

Klintworth, Fetter and Nielsen (1968) also encountered an algal

parasite (*Prototheca wickerhamii*) within the tissues of a woman from Salisbury, North Carolina. Large numbers of algae were observed in lesions on the distal portion of the leg. The infection was considered to be secondary as the patient was prone to a wide variety of bacterial and fungal infections.

R.E. Lee (pers. comm.) encountered the alga *Prototheca* in localized lesions on the face of a South African man. The infection spread to cover oneside of the face and attempts to contain the algal growth have failed to date.

Dr. Richard Norris (pers. comm.) has encountered unicellular green algae within the epidermis of several starfish species in the Friday Harbor area. It was found that the algal parasite eventually causes the death of the host animals.

The parasitic algal infections in animals reported to date all involve unicellular green algae (Chlorophyceae). In only one case, i.e., *Prototheca*, have the algae apparently taken on a role of complete dependence on the host animal. In other cases (including the present alga under study) the algae have maintained well defined chloroplasts and presumably the ability to photosynthesize. The ability to exist in intimate association with the host yet retain the ability to photosynthetize indicates that these algae are facultative parasites or may indicate a recent evolutionary trend towards true obligatory parasitism as is apparently the case with *Prototheca* in man.

Algal-Scallop Association:

Naidu (1971) reporting on the infection of the giant scallop

with an endozoic alga concluded that the alga was parasitic because of the adverse effects it has on the molluscs. Body weight, adductor muscle weight and adductor muscle scar area were found to be significantly lower in infected scallops. Encapsulation of the algae by the animal tissue was interpreted as a defense response by the animal to the 'intruder' and was considered to be additional evidence for designating this alga as parasitic.

Naidu (*op. cit.*) commented on the possible mode of infection, colonization of the host and transmission of the algae to other scallops. In the present study feeding experiments, distribution patterns of the algal colonies and the observations on the cellular responses to the algae, lead to some doubts as to the validity of these theories.

Naidu (*op. cit.*) concluded that the algae gain entry into the scallops through the mantle epithelium; however, Tripp (1969) points out that "the viscid mucous layer covering exposed molluscan tissue acts as a physical (and perhaps chemical) deterrent to tissue penetration; the mucous net traps potential parasites and the entangled material is ejected." The hypothesis presented by Naidu suggests that the alga has the ability to bore its way into the scallop tissue, however no evidence has been found to substantiate this.

Feeding experiments carried out in this study indicate that a more likely mode of infection is via the digestive system. Stauber (1961) suggests that "some (parasites) might enter a host like the oyster passively, swept along in the incurrent water to gain the digestive tract.....where ingestion by a phagocyte might carry

them into deeper tissues." It has been shown that the scallops will readily filter out large quantities of cultured algae and ingest them. It has also been shown that amoebocytes within the alimentary canal will and do ingest the algae. Yonge (1926) indicated that it is possible for wandering amoebocytes to pass through the wall of the oyster stomach, ingest partially digested material and intact diatoms and then pass back through the gut wall into the circulatory system.

Observations indicate that digestion of the endozoic algae is not readily accomplished by the scallop phagocytes. Few engulfed algal cells were observed lysed or denatured inside the phagocytes. This suggests that the alga in some way (possibly because of its thick cell wall) resists digestion by the phagocyte. Mackin (1962) found that the phagocytes of the American oyster were unable to digest the spores of *Nematopsis*. Pan (1956) observed yeast like organisms within the amoebocytes of *Australorbis glabratus* and it was shown by Michelson (1961) that acid-fast organisms within the phagocytes of planorbid snails not only resisted digestion but also multiplied within the amoebocytes. On numerous occasions large numbers of the endozoic algae were observed inside scallop phagocytes. On a few occasions dividing cells were observed within the phagocytes. Conclusive evidence for the growth and reproduction within the phagocyte has not been presented in this study; however, it is speculated that this process may well be taking place.

Stauber (1961) has pointed out the importance of the number of infecting organisms gaining entry into the host, with regards to the eventual establishment of the parasite. If a few algal cells

were to enter the host, chances of successful settlement would be slim and once more it appears that entry via the mantle would be more difficult to accomplish unless great numbers were to 'invade' together. Invasion via the digestive system seems more reasonable in this respect since the filtering apparatus of the scallops tends to concentrate algae from the water column together with other food materials for passage to the gut.

Based on experimental observations and reports from the literature it is suggested that the algae are filtered from the water column, passed on to the gut where they may be engulfed by phagocytes which may then pass back through the gut wall to the circulatory system, and then transported to areas where settlement and establishment is favorable.

Observations on the colonies within the mantle tissue indicate that there is a definite distribution pattern. With reference to Figures 7 and 8 it will be noted that colonies are more abundant in the mid-mantle shell fold areas especially in the dorsal mantle. A similar distribution pattern has been reported for *Nematopsis* cysts within the mantles of oysters. Sprague and Orr (1956) discovered that *Nematopsis* cysts occurred predominantely in a band two mm wide and parallel to the mantle margin. Feng (1958) carried out extensive cyst counts along the entire mantle lengths of oysters with varying degress of *Nematopsis* infection. He demonstrated a definite non-random distribution in which cysts were more numerous in the mid-mantle regions with a decrease in concentration towards the anterior and posterior in both upper and lower mantles.

This non-random distribution was hypothesised to be closely related to the circulatory system in the mantle tissues. Discovery of the accessory heart in the oyster by Hopkins (1934, 1936) revealed that there was much surging of blood back and forth in the circumpallial arteries. It was shown that the circumpallial arteries receive both arterial and venous blood and as was shown by Stauber (1957) meet at a point corresponding to the mid-mantles both dorsal and ventral. Highest cyst counts were recorded in these areas and it was speculated by Feng (1958) that the *Nematopsis* cysts accumulate where blood flow is slowest.

Injections of food dyes into the ventricles and auricles of the scallop to illustrate the circulatory system in the mantle tissue proved unsuccessful. The close proximity of the circumpallial blood vessels with the shell fold tissue where algal colony concentration is greatest indicates, however, a distribution possibly related to circulation.

The circulatory system may play a very important part in the distribution of the algal colonies within the mantle tissue; however, the physiological requirements of the alga itself must not be dismissed as a possible explanation of the alga's affinity to the shell fold tissue. Naidu (1971) pointed out that the availability of light within the scallop may be important and that the mantle edge areas receive the most light. The fact that the higher concentration of algal colonies as well as the larger colonies occur in the shell fold indicates a possible light dependent association. The shell fold area itself is a very rich area since active

metabolism occurs during shell deposition. The high correlation between shell deformity and incidence of infection may not be as suggested by Naidu (1971) that the scallop has become enfeebled but rather because of an acceleration of shell deposition in areas of shell breakage. Wagge (1955) showed an accelerated metabolic process in the mantle tissue of *Helix* when shell breakage occurred. Large numbers of phagocytes carrying calcium and protein were mobilized to the area of shell damage and a delicate layer of shell material was deposited.

In heavily infected scallops "sloughed off" colonies were observed on the exposed shell. Also microscope slides tapped gently against the infected tissue revealed numerous phagocytes containing the endozoic algae. It is speculated that the scallop, in trying to combat the infection, is discarding or eliminating the algal cells, and in some cases whole colonies, through the epithelium to the outside (Figure 6B, arrow, and D). Studies carried out by Stauber (1950), Tripp (1958, 1960, 1961 and 1963) and Feng (1958, 1969) in which a wide variety of foreign particles were injected into the hearts of oysters indicate a general sequence of cellular responses to these particles. Particles were readily engulfed by the phagocytes; the particle-laden phagocytes became widely distributed throughout the oyster tissue; if the particles were digestible the phagocyte quickly digested them; if the particles were indigestible by the phagocyte, migration soon took place through the epithelial layers and the particles discharged to the outside.

Naidu (1971) illustrated by histological and histochemical examinations of the infected mantle tissues that the algae are encapsulated. Generally it is reported that particles too large for phagocytosis are encircled by amoebocytes which flatten out to form fibrous tissue around the material and thus 'contain' it. It has been shown, however, that not only large particles are encapsulated but also indigestible particles such as acid-fast bacilli and India ink when injected into oysters are encapsulated (Metalnikov and Chorine, 1930, Cameron 1934). In areas where foreign particles though small enough to be phagocytosed aggregate in large masses, encapsulation was found to occur as illustrated by Tripp (1958) with avian red cells. Encapsulation of the organisms may result in the death of the intruders; however, it has been reported that acid-fast bacilli not only remain viable (Cameron 1934) but also increase in numbers within the capsule (Michelson 1961).

In the current study colonies of varying sizes have been observed in which numerous cells are found to be reproducing. It is suggested that the algal cells inside the capsule continue to grow and reproduce thus expanding the size of the colonies. It is natural to assume that the larger colonies are older or because of some undetermined physiological requirement grow at a much faster rate than the other colonies. Since large and small colonies are found in close proximity with one another it is more reasonable to accept the former hypothesis. This being the case it may be possible to determine the areas in which algal establishment has first occurred. Observations on the distribution and size of the algal colonies (Figure 11) indicate that the larger colonies

are found in the mid-mantle shell fold area.

Naidu (1971) suggested that spread of the infection may occur from the initial point of settlement to other areas of the scallop by means of respiratory and feeding currents. Colonies of varying sizes lying in close proximity to one another indicate that smaller colonies may be initiated by larger older colonies but a process other than water or feeding currents may be involved.

Microscopic examinations of the colonies revealed phagocytes containing the endozoic algae deep within the encapsulated algal mass. Phagocytes containing the algae were also observed outside the capsule. This suggests that it is possible for the phagocytes to pass through the fibrous capsule wall and engulf the algal cells, and possibly pass back to the outside tissue. It is speculated that the actual spread of the algal infection in a localized area may be accomplished by the phagocytes themselves, and that feeding or respiratory currents are not involved.

Spread of the infection in a localized area may also be accomplished by the rupture of the capsule itself, thus sending large quantities of algal cells into the surrounding tissue to be gradually encapsulated to form new colonies.

Naidu (1971) indicated that the association terminated with the death of the scallop and commences anew in each scallop generation. From observations on the apparent discharge or elimination of the algal cells and colonies into the water column it seems reasonable that the 'liberated' algae are able to re-infect new scallops. Fecal samples from infected scallops revealed algal cells which (as established

from successful culture initiation) remain viable and may be able to re-infect uninfected scallops. It was found that dead infected scallops when left to decay in aquaria did not result in the death of the algae. The algae were 'freed' from the scallop tissue and remained alive and actually reproduced. Successful cultures were initiated from these algal cells which demonstrated the viability of the cells. From these observations it is hypothesized that a heavily infected scallop upon death could potentially set enough algae free into the water column to infect uninfected scallops within the surrounding areas.

It is also suggested that the "shucking" practices of the scallop fishermen in which the shell and the mantle tissues (the areas most likely to be infected with the algae) are returned to the sea, usually in the same areas where the scallops are harvested, may in fact accelerate the incidence of infection as well as spread the infection throughout unaffected areas.

The seasonal variation in the incidence of infection in the scallop has been little studied but it has been suggested by Naidu (1971) that there is perhaps a massive re-infection of scallops in early spring, when the alga is presumably abundant, with fewer new infections as the summer proceeds. Feng (1958) indicated that heavily infected oysters (with *Nematopsis* spores) could eliminate the spores from the mantle tissue. Feng (1965) illustrated that temperature may play a very important role in discharging potential parasites. In oysters an increase in temperature results in an increase in heart rate and thus an increase in phagocytic

activity and an increase in elimination or discharge of indigestible particles through the epithelium. It is possible that a similar process occurs in the scallop in which few new infections occur in summer (when the water is warmer) since there is an increase in cellular activity and hence a greater resistence to infection.

Little information on the incidence of infection in relation to age of the scallops has been presented in this study. From the observations, however, it appears that there is an increase in incidence of infection with age as was indicated by Naidu (1971) for the Port au Port Bay scallop population. Naidu (1971) found that there was a pronounced increase in percentage infection in scallops 8 years old. In the current study scallops 14 years old showed the highest incidence of infection. However, this apparent discrepency is held in reservation since fewer scallops were examined in this study.

Conflicting views on invertebrate susceptibility in relation to aging have been presented in the literature. Duxbury *et al* (1961) in their work on *Anopheles quadrimaculatus* found that more and longer larvae of *Dirofilaria uniformis* were obtained from specimens infected at 12 to 13 days old than specimens infected at 4 to 5 days old. Wijers (1958) on the other hand illustrated that there was a decline in infection rate of *Trypanosoma gambiense* in the host *Glossina palpalis* with age of the tsetse fly.

Webbe (1965) pointed out that the age of the host may be indirectly involved in the incidence of infection. If a host is constantly exposed to a potential parasite the probability of

infection increase with age.

The Algal Symbiont:

Few detailed descriptions of the algal partner involved in symbiotic associations with animals have been presented in the literature. With some notable exceptions (Ax and Apelt, 1965; Parke and Manton, 1967; Apelt, 1969; Kevin, Hall, McLaughlin and Zahl, 1969; Taylor, 1969a, 1969b, 1969c, 1971a, and 1971b), little progress has been made in the field of symbiotic algal taxonomy. In newly reported symbiotic associations the algal partner is usually placed in one of three broad catagories, viz. "zoochlorellae", "zooanthellae" or "cyanallae" depending whether they are green, yellow or blue green. However, "no precise taxonomic connotation is now attached to these terms" (Droop, 1963).

Problems in symbiotic algal taxonomy may arise from incomplete descriptions of the algal partners, failure to "discover" the free living algae within the environment, confused taxonomic criteria for many of the free living algal genera believed to have species involved in symbiosis with animals and the apparent morphological variability of many coccoid algae themselves when grown in culture.

As may be apparent these problems are interrelated. Determination of precise taxonomical criteria for the algae in various associations as well as in culture is essential. Taylor (1969a) points out that incomplete descriptions for symbiotic dinoflagellates is "mainly due to the uniform appearance of zooxanthellae *in situ* under the optical microscope." In such instances the author points out the necessity

of cytochemical and ultrastructural studies to determine "stable and easily recognized taxonomic characters" which may be applied to the alga *in vitro* as well as *in vivo*. Experiments of Provasoli, Yamasu and Manton (1968) in which symbiosis in *Convoluta roscoffensis* was resynthesised *in vitro* indicate a very useful tool for the identification of the symbionts. However, Taylor (1971a) suggests that such experimentation is useful only if pertinent "taxonomic data are available to facilitate rapid identification of the symbionts *in situ*." Because few symbiotic algae have been completely described such experimentation on the resynthesis of symbiosis with various algae would prove to be unfeasible.

The experiments of Provasoli *et al.* (1968) illustrate the importance of detailed descriptions of the algae in culture. In many instances the morphology of the algae in culture differs very markedly from the algae observed *in vivo*. The authors point out that once the symbiotic relationship has been established in a worm "most of the usual criteria needed for recognition of species and genera by light microscopists cease to be available" and often the only details which remain unchanged are at the ultrastructural level. Detailed ultrastructure description of the organelles such as the pyrenoids is essential for establishing the similarities with cultured species.

In many cases the culture conditions of the unicellular coccoid algae may play a very important part in the morphology as observed under the optical microscope. Fott and Novakova (1969)

pointed out that much of the confusion in the taxonomy of *Chlorella* is because many desciptions have been made from various culture media and conditions and a particular taxon in one condition may appear morphologically different under other culture conditions and thus be described as a different species. The authors point out the importance of defining the culture media as well as comparing suspected different species when they are under exact conditions. Schlichting and Bruton (1971) illustrated that pleomorphism often occurs in algae under culture conditions. Using Bold's Basal Medium they illustrated that Mg, N and microelements were essential for the maintenance of taxonomically important characteristics for *Ankistrodesmus falcatus* and *Tetraedron bitridens*. The exclusion of N caused oil accumulation in *A. falcatus* and cell wall thickening in *T. bitridens*. If microelements were excluded, autospore formation was inhibited in *A. falcatus* and aplanospores varied in shape and size in relation to the medium in which they were maintained. Andreeva (1970) also demonstrated that the taxonomic characters of *Chlorella* species are dependent on the concentration of medium components. The author illustrated that the size of the cells may also vary with the age of the cultures.

Algal Morphology *in vivo* and *in vitro*

As pointed out earlier in the RESULTS the endozoic algae when viewed *in vivo* appear morphologically different from the *in vitro* forms. During the initial isolation of the alga it was noted that

certain morphological changes take place in culture. Subsequent subculturing showed that morphologically variable cell types, typical of the *in vivo* populations were replaced by a population of cells which were generally smaller and uniformly spherical. These observations re-inforced the premise that in newly reported symbiotic associations in which description of the algal partner is based on optical microscopy it is necessary to present both *in vivo* and *in vitro* observations and equally important to specify the culture conditions to which the algae are exposed.

Stabilization of morphological characteristics is accomplished in older agar cultures and subcultures. It is speculated that the rate of division and reproduction in the cultures may greatly influence the acquisition of the "cultural characteristics". Increase in the reproductivity in agar cultures and subcultures gives rise to large numbers of smaller cells. Taylor (1969a) speculated that zooxanthellae in conditions which favoured rapid division (eg. in *Tridacna*) would be represented by a smaller cell size population than in habitats where cell division was suppressed, as might be the case in coelenterates. Liquid cultures were characteristic in having slower growth rates than the agar cultures. It is suggested that acquisition of the "cultural characteristics" was retarded in these cultures since it required a longer time for a complete turnover in the cell population.

No differences between the *in vivo* and *in vitro* forms in relation to the cell wall thickness were observed. Generally the cell wall is described as thick and well defined. In many symbiotic algal

associations there is a decrease in the cell wall thickness or complete loss of the cell wall *in vivo* as is the case for the symbionts of *Convoluta roscoffensis* (Oschman and Gray, 1965; Oschman, 1966; Parke and Manton, 1967). The authors speculate on the adaption of the algal symbiont towards a more intimate association with the host organism. In the case of *Platymonas convolutae* in *C. roscoffensis* loss of the cell wall results in an irregular shape of the algae in which the plastid lobes are pushed out to come in contact with the animal cells (Provasoli *et al.*, 1968). Such a close arrangement may facilitate a more efficient transfer of carbohydrate materials from the symbiont to the host cells as has been suggested by Smith *et al.* (1969).

In other associations there may be a gradual thickening of the cell wall in the symbiotic form. Taylor (1968) found that the zooxanthellae associated with *Anemonia sulcata* acted in this way. The author suggests that this thickening in the zooxanthellae periplast may in fact prevent the transport of metabolites and may also act as a protective measure by the algae to the animal tissues. In the association between the dinoflagellate *Amphidium klebsii* and the turbellarian *Amphiscalops langerhansi* Taylor (1971b) reported that the algal symbionts *in vivo* are identical to those isolated in culture. Unlike *Platymonas convolutae* these symbionts still retain their flagella *in vivo*. The cell wall in the symbiont is identical to that of the cultured species. The author hypothesised that failure to change morphologically within the host may be because

this alga does not go trhough an intracellular phase as is the case in *P. convolutae* (Oschman, 1966), but instead passed directly from the gut to the intercellular spaces. Taylor (1971b) speculated that failure to undergo morphological adaptation may be indicative of a recently formed symbiosis.

In the association currently under discussion it is apparent that some morphological changes have taken place in the algae *in vivo* (compared to algae *in vitro*), cell shape and size being the major ones. The nature of the cell wall although apparently unchanged in culture may nevertheless be one of the reasons the alga is "successful" within the scallop tissue. As pointed out earlier, the cell wall being so rigid may resist digestion by the phagocytes. Changes in the cell wall structure of the alga *in vitro* may well take place but are not obvious with light microscopy. Ultrastructual studies on the cell wall will no doubt clarify the situation.

Taylor (1971b) pointed out that direct contact with the host cells or an "intracellular phase" may play a major role in morphological changes. In the scallop such an intracellular phase may in fact take place. As illustrated earlier the scallop phagocytes are able to pick up the algae in the alimentary canal and transport them throughout the scallop tissues. Feeding experiments, however, failed to illustrate changes in morphology of the algae within the phagocytes. It is suspected, however, that time of exposure to the intracellular environment may be a major factor in determining such changes.

In the re-infection experiments it was shown that the scallop tissue in some way causes a change in the morphology of the algal cells. As illustrated in the RESULTS round small cultured algae when injected into the mantle tissue gradually changed in appearance to become identical to "natural" endozoic algae. This experimentation leads to the conclusion that an adaptation process may be occurring and that it requires constant exposure to the living scallop tissues in order to occur. Manipulations of the algal cultures (e.g. addition of scallop tissue extract) did not alter the morphology of the alga; the cells remained small and uniformly spherical.

The re-infection experiments served to establish that the small uniformly spherical algae observed in the cultures were the original endozoic algae. This artificial infection though not as refined as the experiments of Provasoli *et al.* (1968) illustrate a practical method (in this current study) by which cultural material can be compared with the naturally occurring algae. It must be stressed, however, that ultrastructural studies must be carried out to establish more stable taxonomic criteria before further re-infection experiments are designed. Cell size and shape though applicable to the simple experiments outlined above, would not satisfy the prerequisites for subsequent studies on other potential symbionts and competition studies, as those carried out by Provasoli *et al.* (1968) and Taylor (1971b).

Reproduction and Life Cycle

Reproduction appears to be asexual and accomplished solely by

the formation of aplanospores. The formation and subsequent release of the aplanospores have been used as taxonomic criteria in many unicellular green algae (Fritsch, 1935 and Fott and Novakova 1969).

Based on *in vivo* and *in vitro* observations a diagrammatical life cycle is proposed as in Figure 43. Cell size distributions and size of aplanosporangia suggest that mature algal cells can give rise to the 2- 4- 8- and 16-celled aplanosporangia. Formation of 2-and 4-celled aplanosporangia is typically accomplished by oblique division of the mature algal cell in which growth of the spores inside the sporangium may increase the size of the capsule. Release of these spores may then give rise to mature cells which repeat the process. The size of the 8-celled and 16-celled aplanosporangia undergoing division (Figure 43G,H) indicate that growth of the "mature" cell must occur (Figure 43F) which may give rise to 8-celled and 16-celled aplanosporangia, these after release giving rise to mature cells which can repeat the process.

Pigmentation:

Although initial pigment extraction from the endozoic algae was difficult to accomplish, ascending paper chromatograms were easily prepared on which five major pigment fractions were resolved. Identification of the fractions was accomplished by comparing the Rf values, color description and absorption maxima for these fractions with published data for marine algae (Jeffrey, 1961; Jeffrey, 1968; Jeffrey and Haxo, 1968). Rf values alone cannot be used in identification since Sestak (1958) points out that Rf values obtained in paper chromatography depend on the composition of the developing solvent mixture, the quality of the paper, the quantity of the extract

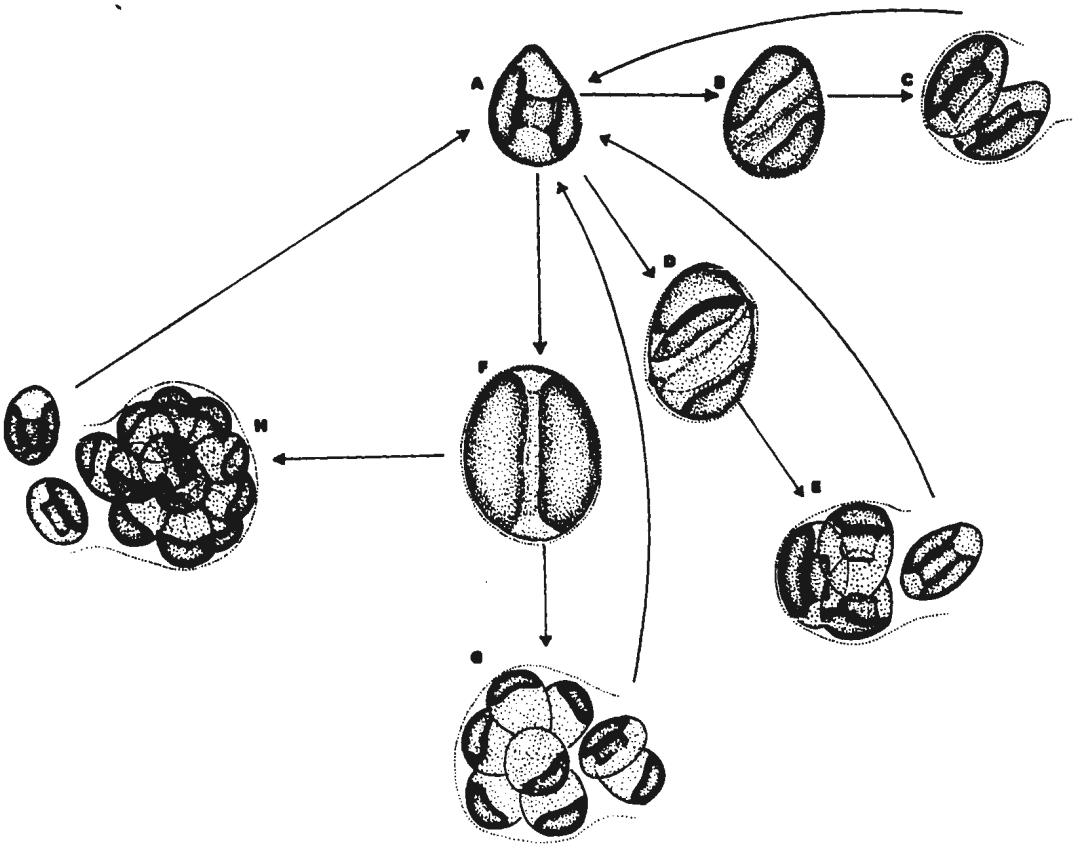


FIGURE 43. Diagrammatical life cycle illustrating formation of 2-celled, 4-celled, 8-celled and 16-celled aplanosporangia from the mature endozoic alga. Growth of the mature cell must occur prior to formation of 8-celled and 16-celled aplanosporangia. See explanation in text.

applied to the paper and the time of development. Rf values serve, however, to present the relative positions of the fractions on the chromatograms. The Rf values obtained for the pigment fractions from the endozoic alga are presented in Table 3.

The pigments were characterized after elution by their absorption maxima. Since the maxima are dependent on the solvent used, all pigment fractions were compared with the published maxima in which a similar solvent was used, in this case ether.

Table 7 presents the absorption maxima for each fraction with examples from the literature. Good agreement with the published data was found for all the fractions.

Based on the Rf values, color characteristics and spectrophotometric absorption maxima, the endozoic alga shows pigmentation characteristic of the algae from the class Chlorophyceae.

Taxonomic Position of the Endozoic Alga:

Positive identification of this alga will have to await further culture and ultrastructural studies *in vivo* and *in vitro*. Based on observations in this study it is, however, possible to place the alga in the Class Chlorophyceae and the Order Chlorococcales. Designation of the alga to a Family is attempted, however, there appear to be two possible families which satisfy the morphological and reproductive characteristics of the endozoic alga, namely, Oocystaceae and Coccomyxaceae.

TABLE 7 Absorption maxima of pigments from the endozoic algae and pigments from other algae separated by paper chromatography using same solvent.

Fraction	Organism	Absorption Maxima (mμ)	Solvent	Identification
1 (yellow)	Endozoic alga	420,445,473	diethyl ether	α-carotene
	Codium *	417,445,475	diethyl ether	
	Dunaliella tertiolecta	429,450,478	diethyl ether	α- and β-carotene
2 (pale orange)	Endozoic alga	420,450,473	diethyl ether	β-carotene
	Published**	430,450,480		
3 (blue green)	Endozoic alga	409,430,664	diethyl ether	chlorophyll *a*
	*Codium**	409,428,663		
	Published**	---,429,661		
4 (yellow green)	Endozoic alga	424,453,664	diethyl ether	chlorophyll *b*
	*Codium**	---,453,643		
5 (bright yellow)	Endozoic alga	415,439,465	diethyl ether	neoxanthin
	*Codium**	416,440,468		
	Dunaliella tertiolecta	414,437,466		

* Jeffrey (1968)
**Jeffrey and Haxo (1968)

The pigmentation of the endozoic alga best satisfies the plastid pigment characteristics of the Chlorophyceae. As pointed out earlier the alga contains large quantities of chlorophyll *a* and chlorophyll *b* which are the only chlorophylls found in the Chlorophyceae (Strain, 1958). Only three carotenes have been reported in the Chlorophyceae viz. α-carotene, β-carotene and γ-carotene. In most members of the Chlorophyceae β-carotene predominates with lesser amounts of α- and γ-carotenes. In the endozoic algae both α-carotene and β-carotene were found in measurable quantities. Xanthophylls of the chlorophycean algae are represented by lutein, violaxanthin and astaxanthin (Nakayama, 1962), however only one xanthophyll, neoxanthin, was resolved in the endozoic algae.

In vivo and *in vitro* morphology and reproduction indicates that this alga is a member of the order Chlorococcales. Since no motile reproductive phases were observed in culture or *in vivo* it is further designated to the azoosporic line of the Chlorococcales. Spherical or ellipsoidal cells with or without a thick cell wall, containing a single parietal band or cup-shaped chloroplast with or without a pyrenoid are typical characteristics of the order Chlorococcales. Reproduction by the formation of 2-, 4-, 8-, and 16-celled aplanosporangia is also further evidence for designating this alga to the order Chlorococcales (Bourrelly,1966).

Placing the alga in a specific Family within the Chlorococcales is difficult to accomplish because of the great morphological variability *in vivo* and the differences exhibited by the cells *in vitro*. *In vivo* the characteristics of the alga best satisfy

the criteria for the Family Coccomyxaceae. Absence of pyrenoid, band shaped chloroplast, refractile cytoplasmic inclusions, hyaline tips and oblique cell division as well as morphological variability are all characteristics of *Coccomyxa* spp. (Jaag,1933). Bourrelly (1966) however, points out that members of the Coccomyxaceae display simple vegetative division and in *Coccomyxa* spp. this division is oblique to the long axis of the cell. In the Coccomyxaceae reproduction is not accomplished exclusively by vegetative division but also by the formation of aplanospores. In the current study confusion arises in interpreting the process by which two daughter cells are produced; whether it is vegetative division or asexual aplanospore formation. Conclusive evidence for stating that this division is vegetative has not been found in this study but it must be pointed out that in many *Coccomyxa* spp. aplanospore formation (by oblique division) appears identical to simple vegetative division (Jaag, 1933).

In culture, oblique division is only observed in young freshly initiated cultures or cultures containing oblong cells. In culture the algae display characteristics typical of the Family Oocystaceae in which reproduction is by the formation of 2-,4-,8-, and 16-celled aplanosporangia (Bourrelly, 1966). The spherical cells with band or cup-shaped chloroplast, absence of pyrenoid and thick cell wall are typical of many *Chlorella* spp. (Fott, 1969).

This apparent discrepency between *in vivo* and *in vitro* morphology and cell division necessitates the presentation of alternatives in the possible taxonomic positioning of the endozoic alga. It

would not suffice to classify the alga solely on its cultural characteristics since it has not been established that the Chihara medium employed in this study satisfies all the requirements of the alga. The morphological characteristics displayed by this alga may in part be influenced by the culture medium. Alternatively it would be unwise to classify the alga solely on its characteristics *in vivo* as it has been pointed out that morphological adaptation is common on many algae involved in symbiosis. On the basis of this, classification can only be made with reservations, namely, *in vivo* the alga morphologically satisfies the characteristics of *Coccomyxa* sp. or a related genus, however, *in vitro* it satisfies the morphological characteristics of *Chlorella* sp. or some related genus.

SUMMARY

(1) Description of the alga *in vivo*

Morphology: unicellular, variable in shape and size; rod-, oval-, sausage-shaped, round or sickle-shaped; definite polarity with thick posterior end and tapered anterior end often terminating in a hyaline tip; chloroplast distinct, usually single, parietal band-shaped, sometimes cup-shaped, occupying up to 3/4 of the cell periphery; cell wall distinct and thick with no ornamentation.

Cytology: Negative test for cellulose and starch; single centrally located nucleus; refractile cytoplasmic inclusions; vacuoles may be present in some cells.

Reproduction: Asexual by formation of 2-, 4-, and 8-celled aplanosporangia; spores released by rupture of the mother cell wall; 2-celled aplanosporangia predominate with less frequency of 4-celled and 8-celled aplanosporangia; division in the formation of 2- and 4-celled aplanosporangia distinctly oblique to the long axis of the cell; re-orientation of the spores in 4-celled aplanosporangia occurs; formation of 8-celled aplanosporangia by radial division.

Dimensions: Vegetative cells range in length from 1.0 to 11.0 μ; average length 5.5 μ; average width 3.0 μ. Spores 1.0 to 9.0 μ long; aplanosporangia 5.0 to 11.0 μ long.

Habitat: In discrete packets or colonies within and upon the

tissues of the giant scallop *Placopecten magellanicus*; found singly within blood cells of the giant scallop.

(2) Description of the alga *in vitro*

Morphology: Cell shape and size varies with culture conditions; in freshly initiated cultures cells are similar to the *in vivo* forms; cells in older more stable cultures are uniformly spherical; chloroplast parietal band or cup-shaped occupying up to 3/4 of the cell periphery; cell wall distinct and thick with no ornamentation.

Cytology: Negative starch and cellulose tests; single centrally located nucleus; refractile bodies and vacuoles may be present in some cells; oil bodies prevalent in older cultures.

Reproduction: Asexual by the formation of 2-, 4-, 8-, and 16-celled aplanosporangia; 2-celled predominate with less frequency of 4-, 8-, and 16-celled aplanosporangia; division not conspicuously oblique in spherical cells but oblique in oblong cells found in young cultures.

Dimensions: Young cultures:- vegetative cells 1.0 to 10.0 μ long; 1.0 to 6.0 μ in width; average length 5.5 μ; average width 3.5 μ; old agar cultures:- 1.0 to 9.0 μ in diameter with few cells larger than 7.0 μ.

Culture conditions: Chihara liquid and agar cultures maintained at 15°C and light intensity of 3500 lux with an 18-6 hr. light-dark periodicity.

(3) The endozoic alga has been demonstrated to belong to the algal Class Chlorophyceae and the Order Chlorococcales. Designation to a family and genus status cannot be made possitively since discrepencies arise *in vivo* and *in vitro*. Possible identification is given as *Coccomyxa* sp. or some related genus in the Family Coccomyxaceae or *Chlorella* sp. or some related genus in the Family Oocystaceae.

(4) Re-infection experiments demonstrated the change in morphology from *in vitro* to the *in vivo* habitat. A morphological adaptation process is suspected, however, further culture studies and electron microscopy as well as demonstration of a "free-living" form in the water column is required to fully elucidate this process.

(5) Besides the tissues reported by Naidu (1971) to be infected by the endozoic algae, colonies were found in the gill filaments illustrating that settlement may occur in tissues where ciliary action takes place. Colonies were also found on the anus and single cells were found in blood cells in the circulatory system as well as in phagocytes within the infected tissues.

(6) Mode of infection is more likely to be via the digestive system than via mantle penetration. It is suggested that the algal cells are filtered out of the water column, passed on to the gut where they are engulfed by phagocytes which transport them into deeper tissues. To verify this hypothesis audioradiography studies should be carried out in which uninfected scallops are fed radioactive labeled algae and the progress followed over prolonged periods of time.

(7) Phagocytosed algae appear to resist digestion. It is speculated that this is accomplished because of the thick cell wall of the algae. Electronmicroscopy and cytochemical studies must be carried out to investigate the composition of the algal cell wall. Studies on the enzymes produced by the scallop phagocyte may also help explain the failure to digest the the algal cells.

(8) Algal colonies within the mantle tissue show a definite distribution pattern. Possibility of a blood circulation related process is suggested, however the physiological requirements of the alga cannot be dismissed. Detailed studies on the circulation system of *Placopecten magellanicus* as well as nutritional studies of the alga *in vitro* and biochemical composition of the shell fold region is required to fully elucidate this phenomenon.

(9) Observations indicate that algal cells and whole colonies can be discharged from the mantle tissue into the water column. It is thus suspected that new infections may be propagated by already infected scallops.

(10) Large quantities of actively reproducing algae are "set free" from the scallop tissues after death and subsequent decay. Release of these algae may cause new infections of uninfected scallops in the immediate area around the morbid animal. It is also suggested that the "shucking" practices of the scallop fisherman over the scallop beds may cause an increase in the rate of infection and possibly spread the infection to uninfected areas.

(11) The existence of infected scallops and uninfected scallops within the same immediate area indicates a possible insusceptibility by some animals to the infection. Age of the scallop as well as physical state have been suggested as reasons for this phenomenon (Naidu, 1971). Immunological techniques should be applied in subsequent studies on susceptibility and insusceptibility of the scallops to the algal infection.

(12) Tissue and cellular responses typical of parasitic associations have been demonstrated here and by Naidu (1971). Even though the presence of this alga in large quantities causes considerable stress to the animal further studies are required to determine if this organism will cause the death of the host animal. Pathogenicity studies are required to investigate the effects of this alga on similar invertebrates.

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APPENDICES

Appendix A. Fixatives and cytological stains.

Alcohol:Acetic acid

Absolute alcohol	100 pts
Galacial acetic acid	33 pts

Newcomer Fixative (1954, Science ii8: 161)

Isopropyl alcohol	6 pts
Propionic acid	3 pts
Ether (pet)	1 pt
Acetone	1 pt
Dioxane	1 pt

Bleaching Solution

Hydrogen peroxide	1 pt
80% Ethyl alcohol	3 pt

Prepared for immediate use.

FAA. Formalin, Alcohol, Acetic Acid

Formalin	8 pts
Ethyl alcohol (absolute)	1 pt
Acetic acid	1 pt

90% ethyl alcohol

10% Formalin

Appendix A. Cont'd.

Meyer's Starch Test

Chloral hydrate..... 5.0 gm.
Distilled water..... 2.0 gm.
Add few crystals of I_2 in finely ground state.

IKI-H_2SO_4 test for cellulose

Material soaked in a solution of 0.3% I_2 and 1.3% KI in water, followed by a few drops of 65% H_2SO_4. The cellulose will turn blue.

Chlor-zinc-iodide test for Cellulose

20.0 gm $ZnCl_2$ dissolved in 8.5 ml. H_2O
Add to this drop by drop until a saturated solution is accomplished: 3.0 gm KI, 1.5 gm I_2 and 60 ml. H_2O. Material is soaked in I_2-KI solution for a few moments and then the solution is replaced with the chlor-zinc-iodide solution. Cellulose turns blue.

Aceto-carmine nuclear stain

45 ml. glacial acetic acid
55 ml. distilled water
Add 0.5 gm of carmine, boil gently for 5 mins in a reflux condenser. Shake well and filter when cool. A drop or two of 45% acetic acid saturated with iron acetate can be added.

Acetic-orcein nuclear stain

45 ml glacial acetic acid
55 ml distilled water
1.0 gm orcein.
Boil gently, cool and filter when used.

Appendix B. Culture Media.

Chihara Medium (Courtesy of Dr. M. Chihara)

Oceanic Seawater	100 ml.
$NaNO_3$	0.2 gm.
$NaH_2PO_4.12H_2O$	0.025 gm.
Trace elements*	2 ml.

* Na_2EDTA..... 3.0 gm. $FeCl_3$..... 0.08 gm.
$MnCl_2$..... 0.12 gm. $ZnCl_2$..... 0.015 gm.
$CoCl_2$..... 0.003 gm. $CuCl_2$..... 0.0012 gm.
$NaMo.H_2O$..... 0.050 gm. H_3BO_3..... 0.6000 gm.
Made in stock solution of 1000 m. autoclaved distilled water.

Erdschreiber's Solution (Foyn, B. 1934., Arch. Protistenk. 83: 1-56)

Filtered seawater	1000 ml.
soil water extract*	50 ml.
$NaNO_3$	100 mg.
Na_2HPO_4	20 mg.

* Soil water extract: Place about 1/2 to 1 inch of a sandy loamy soil in a 2 litre flask and fill with distilled water. Steam for 1 hour on 3 consecutive days. Allow to settle for a few days then pour off the clear liquid into a sterile flask for use.

Symbiodinium nicroadriaticum medium (McLaughlin and Zahl, 1966*)

NaCl	2.5 gm
HCl	0.07 gm.
$MgSO_4.7H_2O$	0.9 gm.
Ca (as Cl)	0.03 gm.
$NaNO_3$	0.005 gm.
KH_2PO_4	0.001 gm.
PII Metal mix*	1.0 ml.
Vitamin B_{12}	0.1 µ gm.
$Na_2SiO_3.9H_2O$	0.003. gm.
Vitamin mix 8-A**	0.05 ml.
Nitrilotriacetic acid	0.003 gm.
Tris (buffer pH 7.8-8.0	0.05 gm.

* See references.

Appendix B cont'd.

* In stock of 1000 ml, 1.0 ml. contains.....

Na_2EDTA	1.0 mg.
Fe	1.0 mg.
H_3BO_3	0.2 mg.
Mn	0.04 mg.
Zn	0.005 mg.
Co	0.0001 mg.

(Adjusted to pH of 7.5 with conc. NaOH)

** In stock of 1000 ml, 1.0 ml. contains.....

Thiamine HCL	0.2 mg.
Biotin	0.5 μ g
B_{12}	0.05 μ g
Folic Acid	0.0025 mg.
Folinic Acid	0.2μ g
p-aminobenzoic	0.01 mg.
Nicotinic acid	0.10 mg.
Thymine	0.80 mg.
Choline	0.50 mg.
Inositol	1.00 mg.
Putrescine 2HCl	0.04 mg.
Orotic acid	0.02 mg.
Riboflavin	0.005 mg.
Pyridoxamine 2CHl	0.02 mg.
Pyridoxine.2HCl	0.05 mg.
Ca pantothenate	0.10 mg.

U. R. II Medium (Hanic)

Filtered seawater	1000 ml.
KNO_3	72.2 mg.
KH_2PO_4	8.8 mg.
Fe EDTA	0.5 mg.
Glycyl glycine	0.5 mg.
Biotine	6 μ g
Thiamine	10 μ g
B_{12}	0.02 μ g

Appendix C Preparation of Scallop Extract.

Scallop Extract

10 gm. wet scallop tissue (Mantle tissue)
200 ml. Distilled water
Homogenized in Waring Blendor for 5 min.
Autoclaved for 15 min. at 15. p.s.i.
Diluted with distilled water for the
desired concentration of scallop extract and
added to the liquid or agar cultures.